Molecular modelling and site-directed mutagenesis of the inositol 1,3,4,5-tetrakisphosphate-binding pleckstrin homology domain from the Ras GTPase-activating protein GAP1^{IP4BP}

Gyles COZIER, Richard SESSIONS, Joanna R. BOTTOMLEY, Jon S. REYNOLDS and Peter J. CULLEN¹

Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K.

GAP1^{IP4BP} is a Ras GTPase-activating protein (GAP) that *in vitro* is regulated by the cytosolic second messenger inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P₄]. We have studied Ins(1,3,4,5)P₄ binding to GAP1^{IP4BP}, and shown that the inositol phosphate specificity and binding affinity are similar to Ins(1,3,4,5)P₄ binding to Bruton's tyrosine kinase (Btk), evidence which suggests a similar mechanism for Ins(1,3,4,5)P₄ binding. The crystal structure of the Btk pleckstrin homology (PH) domain in complex with Ins(1,3,4,5)P₄ has shown that the binding site is located in a partially buried pocket between the $\beta 1/\beta 2$ - and $\beta 3/\beta 4$ -loops. Many of the residues involved in the binding are conserved in GAP1^{IP4BP}. Therefore we generated a model of the

PH domain of GAP1^{IP4BP} in complex with Ins(1,3,4,5)P₄ based on the Btk–Ins(1,3,4,5)P₄ complex crystal structure. This model had the typical PH domain fold, with the proposed binding site modelling well on the Btk structure. The model has been verified by site-directed mutagenesis of various residues in and around the proposed binding site. These mutations have markedly reduced affinity for Ins(1,3,4,5)P₄, indicating a specific and tight fit for the substrate. The model can also be used to explain the specificity of inositol phosphate binding.

Key words: GAP, GTPase-activating protein, PIP₃, PH domain, Ras.

INTRODUCTION

Pleckstrin homology (PH) domains are independent protein modules of approximately 120 amino acids that were initially identified as regions of weak sequence similarity repeated in pleckstrin [1,2]. Subsequently more than 100 different PH domain-containing proteins have been identified, many of which are involved in intracellular signalling. These include protein kinases [Bruton's tyrosine kinase (Btk), β -adrenergic receptor kinase (β -ARK), protein kinase B and phosphoinositide-dependent kinase-1], all phospholipase C (PLC) isoforms, the insulin receptor substrates (IRS-1 and -2), the phosphoinositide 3-kinase p110 γ subunit, ADP-ribosylation factor (ARF) guanine nucleotide exchange factors, general receptor for phosphoinositides-1 (GRP1), cytohesin-1 and ARF nucleotide-binding site opener (ARNO) and the dynamin GTPase [3–5].

Recently our appreciation of PH domains has dramatically increased following the observation that certain PH domains contain specific binding sites for various phosphoinositides. For example, the PH domains of both PLC- δ_1 and β -spectrin specifically bind PtdIns(4,5)P₂ and its soluble inositol head group Ins(1,4,5)P₃, albeit with distinct affinities (K_d of PLC- δ_1 for Ins(1,4,5)P₃ is 210 nM; K_d of β -spectrin for Ins(1,4,5)P₃ is 40 μ M [6–9]). In the case of the PH domain from PLC- δ_1 , its ability to bind PtdIns(4,5)P₂ results in the targeting of this protein to the plasma membrane [10]. Interestingly, since PLC- δ_1 hydrolyses PtdIns(4,5)P₂ to generate Ins(1,4,5)P₃, the *in vivo* membrane localization of PLC- δ_1 appears to be a reversible process governed by whether the PH domain is bound to PtdIns(4,5)P₂ or Ins(1,4,5)P₃ [11,12].

In contrast, the PH domain from Btk specifically binds PtdIns(3,4,5)P₃ and its corresponding soluble inositol head group, Ins(1,3,4,5)P₄ [13,14]. *In vivo* this PtdIns(3,4,5)P₃-binding results in the PH domain-dependent recruitment of Btk from the cytosol to the plasma-membrane following agonist stimulation [15]. A similar plasma-membrane recruitment has also been described for the PtdIns(3,4,5)P₃/Ins(1,3,4,5)P₄-binding PH domains from the ARF-exchange factors GRP1, cytohesin-1 and ARNO, and the Ras GTPase-activating protein GAP1^m ([16,17] and references therein). It is unclear, however, whether in a manner analogous to that of PLC- δ_1 , the plasma-membrane association of these proteins is regulated by the interaction of their PH domains with either plasma-membrane PtdIns(3,4,5)P₃ or cytosolic Ins(1,3,4,5)P₄.

GAP1^m is a member of the GAP1 family of Ras GTPase-activating proteins (GAPs), which also contains GAP1^{IP4BP} and Ras GTPase-activating-like ('RASAL') (reviewed in [18] and see 19). As with Btk, both GAP1^{IP4BP} and GAP1^m contain C-terminal Ins(1,3,4,5)P₄-binding PH domains [20–22]; however, in contrast with GAP1^m, the available data suggest that the function of GAP1^{IP4BP} may be regulated not by PtdIns(3,4,5)P₃ but by Ins(1,3,4,5)P₄. For example, GAP1^{IP4BP} is predominantly a plasma-membrane-localized protein [21,23,24] which, from *in vitro* plasma-membrane-based liposomes assays, appears unable to function as a Ras GAP unless Ins(1,3,4,5)P₄ is present [25]. Importantly, the ability of Ins(1,3,4,5)P₄ to regulate the Ras GAP activity of GAP1^{IP4BP} is not affected by the presence of PtdIns(3,4,5)P₃ [25]. Furthermore, there is compelling evidence that GAP1^{IP4BP} is involved in what is now a well-established phenomenon whereby Ins(1,3,4,5)P₄ influences Ca²⁺ mobilization

Abbreviations used: ARF, ADP-ribosylation factor; ARNO, ARF nucleotide-binding site opener; β -ARK, β -adrenergic receptor kinase; Btk, Bruton's tyrosine kinase; GAP, GTPase-activating protein; GRP1, general receptor for phosphoinositides-1; GST, glutathione S-transferase; PH, pleckstrin homology; PLC, phospholipase C; SOS1, son-of-sevenless 1; 3-D, three-dimensional.

¹ To whom correspondence should be addressed (e-mail Pete.Cullen@bristol.ac.uk).

induced by Ins(1,4,5)P₃ in permeabilized L-1210 cells [26,27]. Together these data suggest that GAP1^{IP4BP} may function *in vivo* as an unique receptor for Ins(1,3,4,5)P₄ rather than PtdIns(3,4,5)P₃.

The three-dimensional (3-D) structures of PH domains from seven different proteins have so far been determined, namely those from pleckstrin [28], β -spectrin [9,29,30], dynamin [31–34], PLC- δ_1 [7], son-of-sevenless 1 (SOS1) [35,36], Btk [37,38] and β -ARK [39]. Despite sharing sequence identity which averages only 10–20%, all of these structures share the same overall topology, i.e. seven β -strands arranged in two almost orthogonal β -sheets ($\beta 1$ – $\beta 4$ and $\beta 5$ – $\beta 7$) capped on one side by a C-terminal α -helix. Each of these structures contain clusters of lysine and arginine residues that form a positively-charged surface, which interacts with the negatively-charged phosphate groups of their respective phosphoinositide ligands. The precise manner of phosphoinositide binding has only been inferred from crystal structures of Ins(1,4,5)P₃ bound to the PH domains of β -spectrin [9] and PLC- δ_1 [7], and of Ins(1,3,4,5)P₄ bound to the PH domain of Btk [38]. Intriguingly whereas the Ins(1,4,5)P₃-binding site in β -spectrin comprises the $\beta 1/\beta 2$ - and $\beta 5/\beta 6$ -loops, the PLC- δ_1 PH domain binds Ins(1,4,5)P₃ through residues from the $\beta 1/\beta 2$ - and $\beta 3/\beta 4$ -loops. From studies using NMR the ligand binding described for PLC- δ_1 appears to also be present in the PH domains from dynamin [40,41] and SOS1 [35], and has also been loosely characterized on the N-terminal pleckstrin and β -ARK PH domains [42,43]. A 3-D structure of the ligand-free R28C mutant of the Btk PH domain has been described, and mutational analysis has suggested an analogous phosphoinositide-binding site to that of PLC- δ_1 [37,41]. This has recently been confirmed by the structure of the wild-type Btk PH domain being solved in complex with Ins(1,3,4,5)P₄ [38]. Interestingly, although the Ins(1,3,4,5)P₄ is bound in an equivalent binding pocket to Ins(1,4,5)P₃ in PLC- δ_1 , it is in a different orientation, being rotated 180° about the P1–P4 axis [38].

In the present study we have compared, in detail, the binding of Ins(1,3,4,5)P₄ to the PH domains of Btk and GAP1^{IP4BP}. This has established that these proteins bind Ins(1,3,4,5)P₄ with similar affinities and importantly almost identical specificities, evidence which suggests a similar mechanism of Ins(1,3,4,5)P₄-binding. Therefore we have used the 3-D structure of Btk in complex with Ins(1,3,4,5)P₄ to obtain a molecular model describing the Ins(1,3,4,5)P₄-binding site within the GAP1^{IP4BP} PH domain. This model, which has been verified through the analysis of a number of site-directed mutants, explains the molecular nature of the high-affinity isomer-specific binding of Ins(1,3,4,5)P₄ to GAP1^{IP4BP}.

EXPERIMENTAL

Expression and purification of GAP1^{IP4BP} and GAP1^{IP4BP} mutants

Both wild-type and mutagenic glutathione S-transferase (GST)–GAP1^{IP4BP} fusion proteins were isolated as follows. An overnight 20 ml culture was used to inoculate four 2.5 l conical flasks, each containing 500 ml of Luria–Bertani broth, 50 μ g/ml ampicillin and 25 μ g/ml kanamycin. The cells were cultured at 25 °C until D_{600} reached 0.6. Protein expression was then induced by the addition of 0.1 mM isopropyl β -D-thiogalactoside, followed by a further 4 h incubation 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 1 mM EDTA, 1 mM EGTA, 1 mM β -mercaptoethanol and 0.1 mM PMSF, and sonicated for 3 periods of 15 s, with 3 min on ice between each sonication. Triton X-100 [1% (v/v), final

concentration] was added and the suspension was left stirring for 45 min on ice prior to the addition of lysozyme (2 mg/ml, final concentration). This was incubated for a further 15 min, before cell debris was removed by centrifugation (36000 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 overnight. The beads were pelleted by centrifugation and washed sequentially with 3 $\times$ 20 ml of PBS containing 0.1% (v/v) Triton X-100, followed by 75 mM Tris/HCl (pH 8.0) containing 0.1% (v/v) Triton X-100 and 500 mM NaCl. The beads were finally washed with 4 $\times$ 20 ml of PBS. The GST fusion protein was liberated by eluting with 3 $\times$ 1 ml of 75 mM Tris/HCl (pH 8.0), 1 M NaCl, 0.5% (w/v) CHAPS and 20 mM glutathione at room temperature over the course of 1 h. The eluants were pooled and concentrated using a Centricon apparatus (Amicon, Conneticut, MA, U.S.A.).

Site-directed mutagenesis of the GAP1^{IP4BP} PH domain

This was performed as previously described [21,22] using the Transformer Kit[™] (Clontech Laboratories, Basingstoke, Hants., U.K.) and pCIneo-GAP1^{IP4BP} as the template. Primers used were as follows: K585R, 5'-GAAGGGTTCATGATCAGGAGGG-CCCAAGGACGG-3'; A587F, 5'-TTCATGATCAAGAGGT-TCCAAGGACGGAAGCGC-3'; N597D, 5'-TTTGGGATGA-AGGATTTTAAGAAGAGATGG-3'; F598Q, 5'-TTTGGGA-TGAAGAATCAGAAGAAGAGATGGTTTC-3'; R601K, 5'-GATGAAGAATTTTAAGAAGAAGTGGTTTCGTTTGAC-CAAC-3'; R601C, 5'-GATGAAGAATTTTAAGAAGTGGT-GGTTTCGTTTGACCAAC-3'; K614E, 5'-GAATTTACCTA-CCACGAAAGCAAAGGGGACCAG-3'; W668F, 5'-GTGG-AGGCCAAGGACTTTATCGACATTCTCACC-3'. In each case the underlined codon describes the particular mutation.

[³²P]Ins(1,3,4,5)P₄-binding assays

The binding of radiolabelled Ins(1,3,4,5)P₄ was performed as previously described [21]. An individual binding assay contained 100 mM KCl, 20 mM NaCl, 1 mM EDTA, 30000 d.p.m. of [³²P]Ins(1,3,4,5)P₄ (prepared as in [21]), 0.5 μ g of GST fusion protein and various concentrations of competing unlabelled inositol phosphates in 10 mM Hepes/NaOH (pH 7.0) buffer, to a final volume of 0.5 ml. Equilibrium binding was achieved by a 15 min incubation at 4 °C, after which the receptor complex was precipitated by the addition of 100 μ l of 5 mg/ml γ -globulin and 1 ml of 25% (w/v) poly(ethylene glycol) in Hepes/NaOH buffer (pH 7.0) containing 100 mM KCl, 20 mM NaCl and 1 mM EDTA and incubated at 4 °C for 10 min. The precipitated proteins were pelleted by centrifugation (21000 g for 10 min) and washed with ice-cold water prior to counting. Non-specific binding was defined as the binding remaining in the presence of 10 μ M unlabelled Ins(1,3,4,5)P₄.

Molecular modelling

All analysis and modelling of the PH domains was performed using the program packages Quanta/CHARMm and InsightII. The alignment of PLC- δ_1 and Btk was structure-based, with the GAP1^{IP4BP} PH domain being aligned by sequence, initially using Omega and then refined by hand where necessary. Any insertions or deletions in the aligned sequences were, where possible, introduced in the loop regions, rather than in the β -sheet or α -helical regions. The backbone co-ordinates of aligned residues, and the side-chain co-ordinates of the identical amino acids, in

Btk were then mapped to the equivalent amino acids in the model. Where insertions or deletions occurred in the sequence that was being modelled, a fragment database [43] was used to search for short sequences that overlapped with the structure either side of the unknown region and which contained the same number of residues. The best-fit fragment was then used in the model structure. After defining the backbone co-ordinates the validity of the emerging structure was assessed using the Protein Health module in Quanta. The irregularities identified were either re-modelled manually or by re-using the fragment database. The non-identical amino acids were modelled in their most likely rotameric form and major side-chain clashes were removed manually.

To form the binding site, the Ins(1,3,4,5)P₄ was positioned in the same position and orientation as observed in the Btk–Ins(1,3,4,5)P₄ complex. Conserved residues in the binding site were fixed so as to retain the interactions with Ins(1,3,4,5)P₄, and other side chains were manually positioned to maximize interactions and minimize steric hindrance. The InsightII Discover program was then used to perform an energy minimization using a 5 Å (1 Å = 0.1 nm) layer of water and applying constraints to preserve the basic backbone structure as copied from Btk [the backbone constraints were 100 kcal/Å² for 200 iterations, 50 kcal/Å² for 200 iterations, 20 kcal/Å² for 200 iterations, 5 kcal/Å² for 500 iterations, 0.5 kcal/Å² for 500 iterations and 0 kcal/Å² for 4000 iterations (1 cal ≡ 4.184 J)]. The automatic parameter function of InsightII was used to assign force-field parameters to the Ins(1,3,4,5)P₄ molecule, and these were checked to ensure that appropriate potentials were used. The final structure was checked for abnormalities and inconsistencies using the Protein Health option in Quanta.

Materials

Inositol phosphates were generously given by Professor Robin Irvine (Department of Pharmacology, University of Cambridge, U.K.), Professor Barry Potter (Department of Pharmacology, University of Bath, U.K.) and Dr Young-Tae Chang (Department of Chemistry, Pohang University of Science and Technology, Pohang 790-784, South Korea).

RESULTS

Analysis of the affinity and inositol phosphate specificity of [³²P]Ins(1,3,4,5)P₄-binding to the PH domains from Btk and GAP1^{IP4BP}

Table 1 describes the affinity and inositol phosphate specificity of Ins(1,3,4,5)P₄-binding to the PH domain of recombinant GAP1^{IP4BP}. From these experiments the following were readily apparent. Firstly, D/L-Ins(1,3,4,5)P₄ was half as potent as D-Ins(1,3,4,5)P₄, consistent with L-Ins(1,3,4,5)P₄ being weaker than the D-isomer. Secondly, any inositol tetrakisphosphate with a phosphate on the 2-axial position was essentially inactive. Thirdly, there was some tolerance for a 6-phosphate [Ins(1,3,4,5,6)P₅ compared with Ins(1,3,4,5)P₄], and a 1-phosphate considerably strengthened binding [Ins(3,4,5,6)P₄ compared with Ins(1,3,4,5,6)P₅ [44] and also Ins(3,4,5)P₃ compared with Ins(1,3,4,5)P₄ [45]]. Finally, both a 3-, 4- and 5-phosphate were essential [Ins(1,4,5)P₃ compared with Ins(1,3,4,5)P₄, Ins(1,3,5)P₃ compared with Ins(1,3,4,5)P₄, and Ins(1,3,4)P₃ compared with Ins(1,3,4,5)P₄], although addition of a 6-phosphate slightly increased the binding affinity [Ins(1,4,5)P₃ compared with Ins(1,4,5,6)P₄]. Thus the Ins(1,3,4,5)P₄-binding site within GAP1^{IP4BP} primarily recognized the 1-, 3-, 4- and 5-phosphates,

Table 1 Comparison of the affinity and inositol phosphate specificity of the Ins(1,3,4,5)P₄ binding sites present within the PH domains of GAP1^{IP4BP} and Btk

[³²P]Ins(1,3,4,5)P₄-binding was performed as described in the Experimental section. In each case IC₅₀ values (means ± S.E.M.) were obtained from three individual experiments performed on distinct protein preparations. n.d., not determined.

Inositol phosphate	IC ₅₀ (nM)	
	GST–Btk PH domain	GST–GAP1 ^{IP4BP}
D-Ins(1,3,4,5)P ₄	57.2 ± 3.4	11.3 ± 3.2
D/L-Ins(1,2,4,5)P ₄	4572 ± 321	2231 ± 112
D/L-Ins(1,2,4,6)P ₄	2874 ± 129	1086 ± 56.1
Ins(1,2,3,5)P ₄	> 10 000	> 5000
Ins(1,3,4,6)P ₄	> 10 000	2793 ± 231
Ins(2,4,5,6)P ₄	> 10 000	2658 ± 321
D/L-Ins(1,2,5,6)P ₄	> 10 000	5000
D/L-Ins(1,2,3,4)P ₄	> 10 000	5000
D/L-Ins(1,4,5,6)P ₄	3212 ± 235	632.9 ± 189
D/L-Ins(1,3,4,5)P ₄	92.4 ± 9.4	26.4 ± 9.1
D-Ins(1,4,5)P ₃	> 10 000	> 5000
D-Ins(1,3,4)P ₃	> 10 000	> 5000
D-Ins(1,3,5)P ₃	n.d.	> 1000
D-Ins(1,3,5,6)P ₄ [= L-Ins(1,3,4,5)P ₄]	n.d.	96.3 ± 9.1
D-Ins(1,3,4,5,6)P ₅	442.3 ± 14.3	85.0 ± 4.5
InsP ₆	833.2 ± 15.7	646.7 ± 28.7

with no tolerance for a 2-axial phosphate and limited tolerance for a 6-phosphate.

Importantly, by directly comparing the pharmacological profile of Ins(1,3,4,5)P₄-binding to GAP1^{IP4BP} with that of the Btk PH domain, it was readily apparent that, of the inositol phosphates tested, a similar specificity was observed (see Table 1). This suggests that the manner through which the PH domains of GAP1^{IP4BP} and Btk bind Ins(1,3,4,5)P₄ are very similar.

Molecular modelling of the GAP1^{IP4BP} PH domain

In an attempt to explain, at the molecular level, the pharmacological details of Ins(1,3,4,5)P₄-binding to GAP1^{IP4BP} we have generated a molecular model of the GAP1^{IP4BP} PH domain in complex with Ins(1,3,4,5)P₄. As various PH domain structures have been solved, there was a choice of which structure to use as the basis for the modelling. Structures of three PH domains have been determined as complexes with inositol phosphates: PLC-δ₁ [7] and β-spectrin [9] in complex with Ins(1,4,5)P₃ and Btk [38] in complex with Ins(1,3,4,5)P₄. PLC-δ₁ and β-spectrin bound the Ins(1,4,5)P₃ in different pockets, located on opposite sides of the β1/β2 loop. In the PLC-δ₁–Ins(1,4,5)P₃ complex there are more interactions with the Ins(1,4,5)P₃ than in the β-spectrin/Ins(1,4,5)P₃ structure, an observation possibly explaining why Ins(1,4,5)P₃ binds with a much higher affinity to PLC-δ₁ than it does to β-spectrin [6–9]. In the Btk PH domain structure the Ins(1,3,4,5)P₄ was bound in an equivalent pocket with many similar interactions to that observed for Ins(1,4,5)P₃ in PLC-δ₁ [38]. However, the orientation of Ins(1,3,4,5)P₄ in the Btk structure is rotated 180° about the P1–P4 axis when compared with Ins(1,4,5)P₃ in PLC-δ₁ [38]. Table 2 shows that PLC-δ₁ and Btk have some residues, which interact with the substrate, that are conserved and some that are similar. Both PH domains also have additional residues that interact with the substrates, which may help to give the different specificities of the proteins. Since the data in Table 1 demonstrated that Btk interacted with Ins(1,3,4,5)P₄ with a similar affinity and pharmacology as

Table 2 Comparison of the residues involved in binding of the inositol phosphate substrates in PLC- δ_1 , Btk and the model GAP1^{IP4BP}

Bold type indicates residues that interact with the substrate and normal type indicates residues that do not interact with the substrate. In parentheses are shown the phosphates with which that residue interacts and whether it is via the side chain (sc) or backbone (bb) of that residue.

PLC- δ_1	Btk	GAP1 ^{IP4BP}
Lys³⁰ (4 + 5, sc)	Lys¹² (3 + 4, sc)	Lys⁵⁸⁵ (3 + 4, sc)
Lys³² (4, sc)	Ser ¹⁴	Ala ⁵⁸⁷
Ser ³³	Gln¹⁵ (4, bb)	Gln⁵⁸⁸ (4, bb)
Ser ³⁴	Gln¹⁶ (5, bb)	Gly⁵⁸⁹ (5, bb)
—	Lys¹⁷ (5, bb)	Arg⁵⁹⁰ (5, bb)
—	Lys¹⁸ (5, sc + bb)	Lys⁵⁹¹ (5, sc + bb)
—	Lys ¹⁹	Arg ⁵⁹²
—	Ser²¹ (5, sc)	Gly ⁵⁹⁴
Trp³⁶ (1, sc)	Asn²⁴ (1, sc)	Asn⁵⁹⁷ (1, sc)
Arg³⁸ (1, sc)	Lys²⁶ (1, sc)	Lys⁵⁹⁹ (1, sc)
Arg⁴⁰ (5, sc)	Arg²⁸ (3, sc)	Arg⁶⁰¹ (3, sc)
Gln ⁵³	Tyr³⁹ (4, sc)	Tyr⁶¹² (4, sc)
Ser⁵⁵ (5, sc)	Glu ⁴¹	Lys ⁶¹⁴
Arg⁵⁶ (5, sc)	Tyr ⁴²	Ser ⁶¹⁵
Lys⁵⁷ (4 + 5, sc)	Asp ⁴³	Lys ⁶¹⁶

GAP1^{IP4BP}, and a comparison of the residues involved in binding the substrates showed that GAP1^{IP4BP} had greater similarity to Btk than PLC- δ_1 (Table 2), it was decided to use the recently solved structure of the Btk PH domain–Ins(1,3,4,5)P₄ complex (solved to 2.4 Å resolution [38]) to create the model GAP1^{IP4BP}–Ins(1,3,4,5)P₄ complex structure.

The sequences used for modelling were 1–170 of Btk and 574–714 of GAP1^{IP4BP}. Although the alignment of the sequence for the PH domain of GAP1^{IP4BP} (Figure 1) did not show a high degree of identity with Btk (32%), it was possible to produce a model structure with the typical PH domain fold (Figure 2) and similar Ins(1,3,4,5)P₄-binding sites.

As stated above, although PLC- δ_1 and Btk bound the substrates in equivalent pockets, the orientation of the inositol

phosphates differed by being rotated 180° about the P1–P4 axis. Because of the symmetry of Ins(1,3,4,5)P₄ about the P1–P4 axis, either orientation would have little effect on the position of the phosphates in the Btk binding site. This is highlighted by many interactions between the protein and substrate being conserved or similar in PLC- δ_1 and Btk; that is many interactions are conserved even though the substrates are in different orientations. It is possible to place Ins(1,3,4,5)P₄ into the GAP1^{IP4BP} model structure in either orientation and retain all of the interactions with the phosphates. However, another effect of the rotation is to swap the position of the 2- and 6-hydroxyls; as the 2-hydroxyl is axial and the 6-hydroxyl is equatorial, any interactions with the hydroxyls in one orientation of the Ins(1,3,4,5)P₄ are unlikely to be retained in the other orientation. PLC- δ_1 has no direct interactions with the hydroxyls of Ins(1,4,5)P₃, but in Btk Ser²¹ interacts with the 6-hydroxyl, and Lys¹² and Asn²⁴ interact with the 2-hydroxyl. In GAP1^{IP4BP}, Lys¹² and Asn²⁴ of Btk are conserved (Lys⁵⁸⁵ and Asn⁵⁹⁷ in GAP1^{IP4BP}), but Ser²¹ of Btk is replaced by Gly⁵⁹⁴ in GAP1^{IP4BP}. As two of the residues that interact with the hydroxyls in Btk are conserved in GAP1^{IP4BP}, and because the binding affinities of Btk and GAP1^{IP4BP} for Ins(1,3,4,5)P₄ are similar, it was decided to use the orientation of the Ins(1,3,4,5)P₄ in Btk for the model GAP1^{IP4BP}–Ins(1,3,4,5)P₄ complex structure.

A comparison of the residues from Btk, PLC- δ_1 and the model GAP1^{IP4BP} that are important in binding the inositol phosphates (Table 2) showed similarities between these high-affinity binding sites. Some of these residues are conserved in all three proteins (Lys¹² and Arg²⁸ of Btk; Lys⁵⁸⁵ and Arg⁶⁰¹ of GAP1^{IP4BP}; Lys³⁰ and Arg⁴⁰ of PLC- δ_1), while others, although not identical, have similar functions (Asn²⁴ and Lys²⁶ of Btk; Asn⁵⁹⁷ and Lys⁵⁹⁹ of GAP1^{IP4BP}; Trp³⁶ and Arg³⁸ of PLC- δ_1). PLC- δ_1 has extra residues that interact with the 4- and 5-phosphates of Ins(1,4,5)P₃ that are not in Btk or GAP1^{IP4BP}, which is consistent with PLC- δ_1 having a higher affinity for Ins(1,4,5)P₃ compared with the affinity of either Btk or GAP1^{IP4BP} for Ins(1,4,5)P₃. The residues in the binding site of the model GAP1^{IP4BP} have a higher similarity to Btk than PLC- δ_1 , which is consistent with the substrate specificities and, as stated above, was one of the

Figure 1 Sequence alignment of the PH domains from PLC- δ_1 , GAP1^{IP4BP} and Btk

The alignment between PLC- δ_1 and Btk is structure based, with the GAP1^{IP4BP} PH domain being aligned based on sequence. β -sheets are highlighted by solid boxes and α -helical regions by dashed boxes. The secondary-structural features conserved in PH domains are labelled β 1– β 7 and α 1. Residues mutated in GAP1^{IP4BP} are indicated by ●.

Figure 2 Schematic representation of the backbone of (a) R28C Btk crystal structure, (b) crystal structure of wild type Btk in complex with Ins(1,3,4,5)P₄, (c) crystal structure of PLC- δ_1 in complex with Ins(1,4,5)P₃ and (d) the model structure of GAP1^{IP4BP} in complex with Ins(1,3,4,5)P₄

The structural features conserved in PH domains are labelled $\beta 1$ – $\beta 7$ and $\alpha 1$. The $\beta 1/\beta 2$ - and $\beta 3/\beta 4$ -loops shown to be involved in Ins(1,4,5)P₃ binding by PLC- δ_1 and Ins(1,3,4,5)P₄ binding by Btk are highlighted. The Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ groups are shown as ball-and-stick structures. The Btk residues 83–88 were not clearly visible in the electron density map. The * in (b) indicates the gap this region would occupy. This Figure was generated using Molscript [49].

reasons for modelling the structure of GAP1^{IP4BP} on to Btk rather than PLC- δ_1 .

The major differences between the model of the GAP1^{IP4BP} PH domain and the Btk crystal structure occurred in the loop regions, particularly the $\beta 3/\beta 4$ - and $\beta 5/\beta 6$ -loops (Figure 2). The $\beta 5/\beta 6$ -loop in Btk has an α -helical insertion that is not found in GAP1^{IP4BP}, but instead there is just a short loop between the $\beta 5$ - and $\beta 6$ -strands. In Btk the $\beta 3/\beta 4$ -loop is 12 residues long and forms a random coil with no interactions with the Ins(1,3,4,5)P₄. In contrast, the $\beta 3/\beta 4$ -loop of GAP1^{IP4BP} is smaller, being only seven residues in length, and contains basic residues that could interact with the substrate. However, modelling this region on the Btk structure orientated the loop away from the Ins(1,3,4,5)P₄ binding site (Figure 2) and therefore there were no interactions between this loop and Ins(1,3,4,5)P₄ in the model structure. This is in contrast with the $\beta 3/\beta 4$ -loop of PLC- δ_1 , which forms part of the binding site and provides interactions to the bound Ins(1,4,5)P₃. The $\beta 3/\beta 4$ -loop of GAP1^{IP4BP} is more similar in size

to PLC- δ_1 , which may suggest that it could interact with the substrate. However, as the binding characteristics for GAP1^{IP4BP} were very similar to Btk, it was decided to leave the $\beta 3/\beta 4$ -loop based on the Btk structure.

To account for the much higher affinity of GAP1^{IP4BP} for Ins(1,3,4,5)P₄ than Ins(1,4,5)P₃ (K_d of 11.3 ± 3.2 compared with > 5000 nM), there must be some interactions which only occur when the 3-phosphate is present. Based on the model structure, these interactions are probably from the $\beta 1/\beta 2$ -loop, which in the GAP1^{IP4BP} PH–Ins(1,3,4,5)P₄ model structure is adjacent to the 5-phosphate. As the structure of the PLC- δ_1 –Ins(1,4,5)P₃ complex was solved prior to the Btk–Ins(1,3,4,5)P₄ structure, it was proposed that the 3-phosphate would interact with the $\beta 1/\beta 2$ -loop. Based on this, two characteristics of the $\beta 1/\beta 2$ -loop were proposed to play important roles in binding 3-phosphorylated inositol products [43]. Firstly a non-bulky residue at the start of the loop is required to allow space for the additional phosphate (e.g. Ser¹⁴ in Btk) and secondly the loop

Figure 3 Mutational analysis of Ins(1,3,4,5)P₄-binding to the PH domain of GAP1^{IP4BP}

The ability of the GAP1^{IP4BP} mutants to bind [³²P]Ins(1,3,4,5)P₄ is shown. Binding was performed as described in the Experimental section on protein purified from prokaryotic expression. Data are means $\pm$ S.E.M. ($n = 3$).

has to be at least six residues in length to be flexible enough to bend around the 3-phosphate [43]. Although the Btk–Ins(1,3,4,5)P₄ structure showed that it was the 5-phosphate which was orientated towards the $\beta 1/\beta 2$ -loop, the same characteristics of the loop would need to be fulfilled. In GAP1^{IP4BP} the $\beta 1/\beta 2$ -loop comprises 11 amino acids, the first being an alanine residue (Ala⁵⁸⁷), which allows space for the 5-phosphate. In the Btk–Ins(1,3,4,5)P₄ complex structure, the 5-phosphate has interactions with the backbone nitrogens of Gln¹⁶, Lys¹⁷ and Lys¹⁸, and also with the side chain of Lys¹⁸. The $\beta 1/\beta 2$ -loop of GAP1^{IP4BP} modelled well on to the Btk structure; the backbone interactions with the 5-phosphate were retained (Gly⁵⁸⁹, Arg⁵⁹⁰ and Lys⁵⁹¹ in GAP1^{IP4BP}) and Lys¹⁸ of Btk is conserved (Lys⁵⁹¹ in GAP1^{IP4BP}).

The first structure of Btk solved was of the R28C mutant. In this structure the $\beta 1/\beta 2$ -loop is pointing away from the binding pocket and is flush with the surface of the protein. However, in the Btk–Ins(1,3,4,5)P₄ complex, the loop is perpendicular to the protein surface and interacts with the 5-phosphate. The majority of the interactions with the 5-phosphate are from the backbone nitrogens, with only Lys¹⁸ providing a side-chain interaction. This suggests that as Ins(1,3,4,5)P₄ enters the binding site the 5-phosphate initially interacts with the $\beta 1/\beta 2$ -loop main chain, causing a conformational change in this loop. This conformational change is probably similar to the difference observed in the $\beta 1/\beta 2$ -loop between the structures of the R28C mutant and the Btk–Ins(1,3,4,5)P₄ complex. Based on the similarities between the Btk–Ins(1,3,4,5)P₄ crystal structure and the model GAP1^{IP4BP}–Ins(1,3,4,5)P₄ structure, a similar conformational change in the $\beta 1/\beta 2$ -loop of GAP1^{IP4BP} may occur upon Ins(1,3,4,5)P₄ binding. If Ins(1,4,5)P₃ entered the binding site of Btk or GAP1^{IP4BP}, then, with no 3-phosphate present, a similar orientation of substrate as observed in the PLC- δ_1 –Ins(1,4,5)P₃ complex would be likely (that is, maximize the interactions with residues already in the binding site). Therefore there would be no phosphate to cause the conformational change in the $\beta 1/\beta 2$ -loop, and so overall there would be fewer interactions between protein and substrate. In other words the higher affinity of GAP1^{IP4BP} for Ins(1,3,4,5)P₄ over Ins(1,4,5)P₃ arises because of the interactions between the $\beta 1/\beta 2$ -loop and the 5-phosphate of Ins(1,3,4,5)P₄, but the interactions will only occur

if the 3-phosphate is present to allow the substrate to adopt the rotated conformation.

[³²P]Ins(1,3,4,5)P₄-binding to site-directed mutants within the PH domain of GAP1^{IP4BP}

Prior to generating the model of the GAP1^{IP4BP} PH–Ins(1,3,4,5)P₄ complex we have previously isolated a number of site-directed mutants within the GAP1^{IP4BP} PH domain that corresponded to mutations known to effect Ins(1,3,4,5)P₄-binding to Btk [37,41,46]. These mutants, expressed as full-length GST–GAP1^{IP4BP} fusion proteins, were analysed with respect to their ability to bind Ins(1,3,4,5)P₄. As shown in Figure 3 [³²P]Ins(1,3,4,5)P₄-binding to the GAP1^{IP4BP} K585R, A587F, F598Q, R601C and W668F mutants was dramatically inhibited whereas binding to the N597D and R601K mutants was inhibited by approximately 81.4 ± 3.1 and 75.4 ± 4.1 %, respectively ($n = 3$). The lack of Ins(1,3,4,5)P₄-binding to these mutants made the subsequent detailed analysis of the affinity and specificity of Ins(1,3,4,5)P₄ practically impossible. However, in the case of GAP1^{IP4BP} K614E, in which Ins(1,3,4,5)P₄-binding was only inhibited by 15.2 ± 3.1 %, a detailed pharmacological analysis of this binding activity was performed.

[³²P]Ins(1,3,4,5)P₄ bound to recombinant GAP1^{IP4BP} K614E with a K_d value for Ins(1,3,4,5)P₄ of 109.1 ± 5.2 nM ($n = 3$), with the other inositol phosphates tested, namely Ins(1,3,4,5,6)P₅, InsP₆, Ins(1,4,5)P₃ and Ins(1,3,4)P₃, having K_d values of 401.2 ± 8.1 , > 10000 nM, > 10000 nM and > 10000 nM, respectively ($n = 3$, for each inositol phosphate). Detailed Scatchard analysis revealed a single class of binding site (results not shown). With respect to these parameters expressed GAP1^{IP4BP} K614E had approximately a 10-fold lower affinity compared with Ins(1,3,4,5)P₄-binding to wild-type GAP1^{IP4BP} (see Table 1).

Molecular explanation of the mutagenic studies

From Figure 3 it can clearly be seen that mutation of Arg⁶⁰¹, either R601C or R601K, had the effect of inhibiting Ins(1,3,4,5)P₄-binding to GAP1^{IP4BP} by greater than 75 %. This was not surprising, given that from the GAP1^{IP4BP} model structure, mutations to Arg⁶⁰¹ would remove some of the interactions with the 3-phosphate of Ins(1,3,4,5)P₄ (Figure 4). For example, Cys⁶⁰¹ would be unable to interact with the 3-phosphate, whereas Lys⁶⁰¹ could retain some hydrogen bonds, explaining why R601C inhibited Ins(1,3,4,5)P₄-binding to a greater extent than R601K (90 % compared with 75 %). The importance of Arg⁶⁰¹ in Ins(1,3,4,5)P₄-binding was further highlighted by the F598Q mutation. Phe⁵⁹⁸ of GAP1^{IP4BP}, which is conserved in Btk (Phe²⁵) and so was modelled in the same position, points away from the Ins(1,3,4,5)P₄-binding site and appeared to have no interaction with Ins(1,3,4,5)P₄ in the model complex. Therefore it was not initially obvious why mutating this residue to glutamine should dramatically reduce Ins(1,3,4,5)P₄-binding activity. However, the difference in size between phenylalanine and glutamine, or if glutamine hydrogen bonds to a neighbouring residue, could cause a localized conformational change. Arg⁶⁰¹ is only three residues away and so any change in its conformation could cause the effects observed.

With respect to K585R, although this could retain the interaction with the 4-phosphate of Ins(1,3,4,5)P₄, the bulkier arginine residue may, by steric hindrance, cause the Ins(1,3,4,5)P₄ molecule to alter its position in the binding site, effectively having to move further out. This would weaken, or remove, the other interactions between Ins(1,3,4,5)P₄ and GAP1^{IP4BP}, thereby lowering the binding activity. Likewise, the mutation N597D, which

Figure 4 Two views of the Ins(1,3,4,5)P₄-binding site in the model GAP1^{IP4BP} structure, showing the relative positions of the residues chosen to study by site-directed mutagenesis

The $\beta 1/\beta 2$ - and $\beta 3/\beta 4$ -loops are shown as ribbons. Ins(1,3,4,5)P₄ and amino acid side chains are shown as stick structures. Other residues in and around the binding site have been omitted for clarity.

inhibited Ins(1,3,4,5)P₄ binding by $81.4 \pm 3.1\%$, would not only remove hydrogen bonding with the substrate, but also, by introducing a negative charge close to the 1-phosphate, may repel the Ins(1,3,4,5)P₄. Similarly, although the K614E mutation only inhibited Ins(1,3,4,5)P₄ binding by $15.2 \pm 3.1\%$, the 10-fold lower affinity may indicate that either the lysine residue had a weak interaction that was removed by the mutation or, if there was no such interaction, then the introduction of a negative charge may again repel the Ins(1,3,4,5)P₄ molecule from the binding site. The model structure did not have any direct interactions between Lys⁶¹⁴ and Ins(1,3,4,5)P₄ (Figure 4), indicating electrostatic repulsion between K614E and Ins(1,3,4,5)P₄.

In the model structure Ala⁵⁸⁷ of GAP1^{IP4BP} corresponded to Lys³² of PLC- δ_1 . This was interesting, since Lys³² of PLC- δ_1 is at the start of the $\beta 1/\beta 2$ -loop and has been previously suggested to prevent substrates containing a 3-phosphate from binding [43,47] [based on the Btk–Ins(1,3,4,5)P₄ complex it is actually the 5-phosphate that will be close to Ala⁵⁸⁷ in GAP1^{IP4BP}]. Therefore although a change from Lys³² in PLC- δ_1 to Ala⁵⁸⁷ in GAP1^{IP4BP} reduced the number of interactions with Ins(1,3,4,5)P₄, such a change may be essential for Ins(1,3,4,5)P₄ binding. In the model GAP1^{IP4BP} structure Ala⁵⁸⁷ was close to the 5-phosphate (Figure 4), so it was not surprising that the A587F mutation caused steric hindrance, which inhibited Ins(1,3,4,5)P₄ binding by 90%.

Figure 5 Schematic representation of the model GAP1^{IP4BP} PH–Ins(1,3,4,5)P₄ complex with Trp⁶⁶⁸, and the residues Lys⁵⁷⁹ and Met⁵⁸³ with which it interacts, shown as ball-and-stick structures

Ins(1,3,4,5)P₄ is shown as a ball-and-stick structure and the structural features conserved in PH domains are labelled $\beta 1$ – $\beta 7$ and $\alpha 1$. This Figure was generated using Molscript [49].

The only invariant residue in PH domains is a tryptophan in the α -helix (Trp⁶⁶⁸ in GAP1^{IP4BP}). This residue has been suggested to form a structural role in the orientation of the α -helix and β -sheet [48]. In the model GAP1^{IP4BP} structure, and also the Btk and PLC- δ_1 structures, this tryptophan is sandwiched between two residues (Lys⁵⁷⁹ and Met⁵⁸³) from the $\beta 1$ -strand (Figure 5). The W668F mutation in GAP1^{IP4BP} was therefore likely to cause a structural change, probably in either the $\beta 1$ -strand or $\beta 1/\beta 2$ -loop, which resulted in a reduction in the ability of the protein to bind Ins(1,3,4,5)P₄.

DISCUSSION

In the present study we have compared, in some detail, the Ins(1,3,4,5)P₄-binding sites present within the PH domains of recombinant GAP1^{IP4BP} and Btk, an approach which has highlighted that these two proteins bind Ins(1,3,4,5)P₄ with very similar affinities and inositol phosphate specificities. With this in mind, we have used the crystal structure of the Btk PH domain in complex with Ins(1,3,4,5)P₄ [38] to generate a molecular model of the potential Ins(1,3,4,5)P₄-binding site within GAP1^{IP4BP}.

The model structure of the GAP1^{IP4BP} PH–Ins(1,3,4,5)P₄ complex predicts an inositol phosphate-binding site in which the inositol ring is partially buried within a pocket comprised of the $\beta 1/\beta 2$ - and $\beta 3/\beta 4$ -loops. Importantly, we propose that the high-affinity binding site is formed following a conformational change in the $\beta 1/\beta 2$ -loop that is induced upon Ins(1,3,4,5)P₄-binding. Our model suggests that the pharmacology of inositol phosphate-binding to GAP1^{IP4BP}, as detailed in Table 1, arises for the following reasons. Firstly, for high-affinity binding a

minimum of four phosphate groups are necessary in order to induce the conformational changes required to bring crucial residues, some of which we have highlighted by mutagenesis, into proximity with the phosphates [compare the affinities of D-Ins(1,4,5)P₃, D-Ins(1,3,4)P₃ and D-Ins(1,3,5)P₃ with D-Ins(1,3,4,5)P₄]. These four phosphates need to be in equatorial positions, since any inositol tetrakisphosphate containing a phosphate on the axial 2-hydroxyl has a low affinity (see Table 1). Furthermore, three of the phosphates need to be in a row, with the fourth phosphate immediately opposite, such that the remaining hydroxyls separate it from the three phosphates [see the affinities of D-Ins(1,3,4,5)P₄ and D-Ins(1,3,5,6)P₄ [= L-Ins(1,3,4,5)P₄] in Table 1]. Such a requirement is predicted from the model, since the three phosphates [3-, 4- and 5- in the case of D-Ins(1,3,4,5)P₄] are shown to interact with residues that lie in the bottom of the binding pocket and are therefore buried; the remaining phosphate [the 1-phosphate of D-Ins(1,3,4,5)P₄] is orientated towards the top of the binding site. In this context the high affinity of GAP1^{IP4BP} for D-Ins(1,3,4,5,6)P₅ ($K_d = 85 \pm 4.5$ nM) is interesting, since we would predict from the model that this binds in the same orientation as D-Ins(1,3,4,5)P₄ and that sufficient space may be available to accommodate the 6-phosphate.

Both PLC- δ_1 and Btk have had their crystal structures solved in complex with inositol phosphates, and they have shown that they bind the inositol phosphates in equivalent binding sites. Of the residues involved in binding the substrates some are conserved, others although not identical do perform similar functions and there are unique residues that are probably responsible for the distinct affinities of the two PH domains. As the binding sites

are similar, it is surprising that they bound the inositol phosphates in different orientations, which are rotated 180° about the P1–P4 axis. This gave a choice of which orientation to use for the model GAP1^{IP4BP}–Ins(1,3,4,5)P₄ structure. Based on the sequence and substrate-specificity similarities between GAP1^{IP4BP} and Btk it was decided to use the Btk orientation. However, until the crystal structure of GAP1^{IP4BP} in complex with Ins(1,3,4,5)P₄ has been solved the orientation of the Ins(1,3,4,5)P₄ will be open to debate.

Therefore, in summary, we have generated a credible model structure describing the Ins(1,3,4,5)P₄-binding site within the PH domain of GAP1^{IP4BP}. This model explains the molecular nature of the high-affinity isomer-specific binding of Ins(1,3,4,5)P₄ to GAP1^{IP4BP} and highlights important residues required for high-affinity binding, some of which we have confirmed by site-directed mutagenesis.

We thank Professor Robin Irvine and Professor Tony Clarke for helpful discussions. This work is funded by Grants from the BBSRC Integration of Cellular Responses Initiative and the MRC.J.S.R. is in receipt of a BBSRC Studentship. P.J.C. is a Lister Institute Research Fellow.

REFERENCES

- Haslam, R. J., Koide, H. B. and Hemmings, B. (1993) Pleckstrin domain homology. *Nature* **363**, 629–630.
- Mayer, B. J., Ren, R., Clark, K. L. and Baltimore, D. (1993) A putative molecular domain present in diverse signalling proteins. *Cell* **73**, 629–630.
- Lemmon, M. A., Ferguson, K. M. and Schlessinger, J. (1996) PH domains: diverse sequences with a common fold recruit signalling molecules to the cell surface. *Cell* **85**, 621–624.
- Lemmon, M. A., Falasca, M., Ferguson, K. M. and Schlessinger, J. (1997) Regulatory recruitment of signalling molecules to the cell membrane by PH domains. *Trends Cell Biol.* **7**, 237–242.
- Bottomley, M. J., Salim, K. and Panayotou, G. (1998) Phospholipid-binding protein domains. *Biochim. Biophys. Acta* **1436**, 165–183.
- Hirata, M., Kanematsu, T., Sakuma, K., Koga, T., Watanabe, Y., Ozaki, S. and Yagisawa, H. (1994) D-myo-inositol 1,4,5-trisphosphate binding domain of phospholipase C-delta 1. *Biochem. Biophys. Res. Commun.* **205**, 1563–1571.
- Ferguson, K. M., Lemmon, M. A., Schlessinger, J. and Sigler, P. B. (1995) Structure of the high affinity complex of inositol trisphosphate with phospholipase C PH domain. *Cell* **83**, 1037–1046.
- Lemmon, M. A., Ferguson, K. M., Sigler, P. B. and Schlessinger, J. (1995) Specific and high affinity binding of inositol phosphates to an isolated PH domain. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10472–10476.
- Hyvönen, M., Macias, M. J., Nilges, M., Oshkinat, H., Saraste, M. and Willmans, M. (1995) Structure of the binding-site for inositol phosphates in a PH domain. *EMBO J.* **14**, 4676–4685.
- Paterson, H. F., Savopoulos, J. W., Perisic, O., Cheung, R., Ellis, M. V., Williams, R. L. and Katan, M. (1995) Phospholipase C_{β1} requires a PH domain for interaction with the plasma membrane. *Biochem. J.* **312**, 661–666.
- Stauffer, T. P., Ahn, S. and Meyer, T. (1998) Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P₂ concentration monitored in living cells. *Curr. Biol.* **8**, 343–346.
- Varnai, P. and Balla, T. (1998) Visualisation of phosphoinositides that bind PH domains: calcium- and agonist-induced dynamic changes and relationship to myo-[³H]-inositol-labeled phosphoinositide pools. *J. Cell Biol.* **143**, 501–510.
- Fukuda, M., Kojima, T., Kabayama, H. and Mikoshiba, K. (1996) Mutation of the PH domain of Bruton's tyrosine kinase in immunodeficiency impaired inositol 1,3,4,5-tetrakisphosphate binding capacity. *J. Biol. Chem.* **271**, 30303–30306.
- Rameh, L. E., Arvidsson, A., Carraway, K. L. R., Couvillon, A. D., Rathbun, G., Crompton, A., VanRenterghem, B., Czech, M. P., Ravichandran, K. S., Burakoff, S. J. et al. (1997) A comparative analysis of the phosphoinositide-binding specificity of PH domains. *J. Biol. Chem.* **272**, 22059–22066.
- Varnai, P., Rother, K. I. and Balla, T. (1999) PI 3-kinase-dependent membrane association of the Bruton's tyrosine kinase PH domain visualised in single living cells. *J. Biol. Chem.* **274**, 10983–10989.
- Venkateswarlu, K., Oatey, P. B., Tavare, J. M. and Cullen, P. J. (1998) Insulin-dependent translocation of ARNO to the plasma membrane of 3T3 L1 adipocytes requires PI 3-kinase. *Curr. Biol.* **8**, 463–466.
- Lockyer, P. J., Wennstrom, S., Kupzig, S., Venkateswarlu, K., Downward, J. and Cullen, P. J. (1999) Identification of the Ras GTPase-activating protein GAP1TM as a phosphatidylinositol 3,4,5-trisphosphate-binding protein *in vivo*. *Curr. Biol.* **9**, 265–268.
- Cullen, P. J. (1998) Bridging the GAP in inositol 1,3,4,5-tetrakisphosphate signalling. *Biochim. Biophys. Acta* **1436**, 35–47.
- Allen, M., Chu, S., Brill, S., Stotter, C. and Buckler, A. (1998) Restricted tissue expression pattern of a novel human rasGAP related gene and its murine ortholog. *Gene* **218**, 17–25.
- Fukuda, M. and Mikoshiba, K. (1996) Structure-function relationships of the mouse GAP1TM. *J. Biol. Chem.* **271**, 18838–18842.
- Lockyer, P. J., Bottomley, J. R., Reynolds, J. S., Venkateswarlu, K., Potter, B. V. L., Dempsey, C. E. and Cullen, P. J. (1997) Distinct subcellular distribution of the putative inositol 1,3,4,5-tetrakisphosphate receptors GAP1^{IP4BP} and GAP1TM result from the GAP1^{IP4BP} PH domain directing plasma membrane targeting. *Curr. Biol.* **7**, 1007–1010.
- Bottomley, J. R., Reynolds, J. S., Lockyer, P. J. and Cullen, P. J. (1998) Structure and functional analysis of the putative inositol 1,3,4,5-tetrakisphosphate receptors GAP1^{IP4BP} and GAP1TM. *Biochem. Biophys. Res. Commun.* **250**, 143–149.
- O'Rourke, F., Matthews, E. and Feinstein, M. B. (1996) Isolation of inositol 1,3,4,5-tetrakisphosphate and inositol hexakisphosphate binding proteins from human platelets: inositol 1,3,4,5-tetrakisphosphate promotes Ca²⁺ efflux from inside-out plasma membrane vesicles containing 104 kDa GAP1^{IP4BP} protein. *Biochem. J.* **315**, 1027–1034.
- Lockyer, P. J., Vanlingen, S., Reynolds, J. S., McNulty, T. J., Irvine, R. F., Parys, J. B. and Cullen, P. J. (1999) Tissue specific expression and endogenous subcellular distribution of the inositol 1,3,4,5-tetrakisphosphate-binding proteins GAP1^{IP4BP} and GAP1TM. *Biochem. Biophys. Res. Commun.* **255**, 421–426.
- Cullen, P. J., Hsuan, J. J., Troung, O., Letcher, A. J., Jackson, T. R., Dawson, A. P. and Irvine, R. F. (1995) Identification of a specific inositol 1,3,4,5-tetrakisphosphate-binding protein as a member of the GAP1 family. *Nature (London)* **376**, 527–530.
- Loomis-Husselbee, J. W., Cullen, P. J., Dreikhausen, U. E., Irvine, R. F. and Dawson, A. P. (1996) Synergistic effects of inositol 1,3,4,5-tetrakisphosphate on inositol 2,4,5-trisphosphate-stimulated Ca²⁺ release do not involve direct interaction of inositol 1,3,4,5-tetrakisphosphate with inositol trisphosphate-binding sites. *Biochem. J.* **314**, 811–816.
- Loomis-Husselbee, J. W., Walker, C. D., Bottomley, J. R., Cullen, P. J., Irvine, R. F. and Dawson, A. P. (1998) Modulation of inositol 2,4,5-trisphosphate-stimulated Ca²⁺ mobilisation by inositol 1,3,4,5-tetrakisphosphate: enhancement by activated G-proteins, and evidence for the involvement of a GAP1 protein, a putative inositol 1,3,4,5-tetrakisphosphate receptor. *Biochem. J.* **331**, 947–952.
- Yoon, H. S., Hajduk, P. J., Petros, A. M., Olejniczak, E. T., Meadows, R. P. and Fesik, S. W. (1994) Solution structure of a PH domain. *Nature (London)* **369**, 672–675.
- Macias, M. J., Musacchio, A., Ponstingl, H., Nilges, M., Saraste, M. and Oshkinat, H. (1994) Structure of the PH domain from β-spectrin. *Nature (London)* **369**, 675–677.
- Zhang, P., Talluri, S., Deng, H. Y., Branton, G. and Wagner, G. (1995) Solution structure of the PH domain of *Drosophila* β-spectrin. *Structure* **3**, 1185–1195.
- Downing, A. K., Driscoll, P. C., Gout, I., Salim, K., Zvebil, M. J. and Waterfield, M. D. (1994) Three-dimensional solution structure of the PH domain from dynamin. *Curr. Biol.* **4**, 884–891.
- Ferguson, K. M., Lemmon, M. A., Schlessinger, J. and Sigler, P. B. (1994) Crystal structure at 2.2 Å resolution of the PH domain from human dynamin. *Cell* **79**, 199–209.
- Fushman, D., Cahill, S., Lemmon, M. A., Schlessinger, J. and Cowburn, D. (1995) Solution structure of the PH domain of dynamin by heteronuclear NMR spectroscopy. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 816–820.
- Timm, D., Salim, K., Gout, I., Guruprasad, L., Waterfield, M. and Blundell, T. (1994) Crystal structure of the PH domain from dynamin. *Nat. Struct. Biol.* **1**, 782–788.
- Zheng, J., Chen, R. H., Corblan-Garcia, S., Cahill, S. M., Bar-Sagi, D. and Cowburn, D. (1997) The solution structure of the PH domain of human SOS1: A possible structural role for the sequential association of diffusion and PH domains. *J. Biol. Chem.* **272**, 30240–30344.
- Koshiba, S., Kigawa, T., Kim, J. H., Shirouzu, M., Bowtell, D. and Yokoyama, S. (1997) The solution structure of the PH domain of mouse Son-of-sevenless (mSOS1). *J. Mol. Biol.* **269**, 579–591.
- Hyvönen, M. and Saraste, M. (1997) Structure of the PH domain and Btk motif from Bruton's tyrosine kinase: molecular explanation for X-linked agammaglobulinemia. *EMBO J.* **16**, 3396–3404.
- Baraldi, E., Carugo, K. D., Hyvonen, M., LoSurdo, P., Riley, A. M., Potter, B. V. L., O'Brien, R., Ladbury, J. E. and Saraste, M. (1999) Structure of the PH domain from Bruton's tyrosine kinase in complex with inositol 1,3,4,5-tetrakisphosphate. *Structure* **7**, 449–460.

- 39 Fushman, D., Najmabadi-Haske, T., Cahill, S., Zheng, J., LeVine, H. and Cowburn, D. (1998) The solution structure and dynamics of the PH domain of G protein-coupled receptor kinase 2 (β -adrenergic receptor kinase 1) — a binding partner of $G\beta\gamma$ subunits. *J. Biol. Chem.* **273**, 2835–2843
- 40 Zheng, J., Cahill, S. M., Lemmon, M. A., Frushman, J., Schlessinger, J. and Cowburn, D. (1996) Identification of the binding site for acidic phospholipids on the PH domain of dynamin: implications for stimulation of GTPase activity. *J. Mol. Biol.* **255**, 14–21
- 41 Salim, K., Bottomley, M. J., Querfurth, E., Zvelebil, M. J., Gout, I., Scaife, R., Margolis, R. L., Gigg, R., Smith, C. I., Driscoll, P. C. et al. (1996) Distinct specificity in the recognition of phosphoinositides by the PH domains of dynamin and Bruton's tyrosine kinase. *EMBO J.* **15**, 6241–6250
- 42 Harlan, J. E., Yoon, H. S., Hajduk, P. J. and Fesik, S. W. (1995) Structural characterisation of the interaction between a PH domain and phosphatidylinositol 4,5-bisphosphate. *Biochemistry* **34**, 9859–9864
- 43 Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Brice, M. D., Rogers, J. R., Kenard, O., Shimanouchi, T. and Tasumi, M. (1997) The Protein Data Bank: a computer-based archival file for macromolecular structures. *J. Mol. Biol.* **112**, 535–542
- 44 Cullen, P. J., Chung, S.-K., Chang, Y.-T., Dawson, A. P. and Irvine, R. F. (1995) Specificity of the purified inositol 1,3,4,5-tetrakisphosphate-binding protein from porcine platelets. *FEBS Lett.* **358**, 240–243
- 45 Bradford, P. G. and Irvine, R. F. (1987) Specific binding-sites for [3 H]inositol 1,3,4,5-tetrakisphosphate on membranes of HL60 cells. *Biochem. Biophys. Res. Commun.* **149**, 680–685
- 46 Kojima, T., Fukuda, M., Watanabe, Y., Haamamoto, F. and Mikoshiba, K. (1997) Characterisation of the PH domain of Btk as an inositol polyphosphate and phosphoinositide binding domain. *Biochem. Biophys. Res. Commun.* **236**, 333–339
- 47 Isakoff, S., Cardozo, T., Andreev, J., Li, Z., Ferguson, K. M., Abagyan, R., Lemmon, M. A., Aronheim, A. and Skolnik, E. Y. (1998) Identification and analysis of PH domain-containing targets of phosphatidylinositol 3-kinase using a novel *in vivo* assay in yeast. *EMBO J.* **17**, 5374–5387
- 48 Shaw, G. (1996) The PH domain: an intriguing multifunctional protein module. *BioEssays* **18**, 35–46
- 49 Kraulis, P. J. (1991) MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* **24**, 946–950

Received 24 January 2000/21 March 2000; accepted 7 April 2000