GAP1IP4BP contains a novel Group I pleckstrin homology domain that directs constitutive plasma membrane association.

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Abstract

The Group I family of pleckstrin homology (PH) domains are characterised by their inherent ability to specifically bind phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃) and its corresponding inositol head-group inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄). In vivo this interaction results in the regulated plasma membrane recruitment of cytosolic Group I PH domain-containing proteins following agonist stimulated PtdIns(3,4,5)P₃ production. Amongst Group I PH domain-containing proteins, the Ras GTPase-activating protein GAP1IP4BP is unique in being constitutively associated with the plasma membrane. Here we show that although the GAP1IP4BP PH domain interacts with PtdIns(3,4,5)P₃ it also binds, with a comparable affinity, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) (Kₐ’s of 0.5 ± 0.2 and 0.8 ± 0.5 µM respectively). Intriguingly, whereas this binding site overlaps with that for Ins(1,3,4,5)P₄, consistent with the constitutive plasma membrane association of GAP1IP4BP resulting from its PH domain-binding PtdIns(4,5)P₂, we show that in vivo depletion of PtdIns(4,5)P₂, but not PtdIns(3,4,5)P₃, results in dissociation of GAP1IP4BP from this membrane. Thus the Ins(1,3,4,5)P₄-binding PH domain from GAP1IP4BP defines a novel class of Group I PH domains that constitutively targets the protein to the plasma membrane and may allow GAP1IP4BP to be regulated in vivo by Ins(1,3,4,5)P₄ rather than PtdIns(3,4,5)P₃.
Background

Pleckstrin homology (PH) domains are protein modules of approximately 120 amino acids that were initially identified as regions of weak sequence homology repeated in pleckstrin (1,2). Subsequently PH domains have been identified in more than 100 different proteins that despite possessing low sequence similarities (10-20%) have been shown, or predicted to have, very similar overall topologies (3). Many of these PH domain-containing proteins are involved in intracellular signalling, where the majority require membrane association for their function. Recent work has highlighted a pivotal role for PH domains in this membrane targeting (4,5).

Although some PH domains have been reported to bind other proteins (6-8), a consensus is emerging that many PH domains are designed to bind phosphoinositides, particularly phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃), and their corresponding water-soluble inositol head-groups, inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄) respectively (9). For example, the PH domain of phospholipase C-δ₁ (PLC-δ₁) binds with high affinity and stereospecificity to both PtdIns(4,5)P₂ and Ins(1,4,5)P₃. The interaction of PLC-δ₁ with PtdIns(4,5)P₂ results in its plasma membrane association which, since PLC-δ₁ hydrolyses PtdIns(4,5)P₂ to generate Ins(1,4,5)P₃, is a reversible process governed by whether the PH domain is bound to PtdIns(4,5)P₂ or cytosolic Ins(1,4,5)P₃. Indeed, this competition has been proposed to have an important role in regulating PLC-δ₁ activity in vivo (reviewed in 10).
More recent studies have demonstrated that certain PH domains specifically bind 3-phosphoinositides generated following agonist stimulation of class I phosphatidylinositol 3-kinases (PI 3-kinases) (11,12). For instance, the so-called Group I PH domains from Bruton’s tyrosine kinase (Btk) (13-15) and the ADP-ribosylation factor-exchange factors, cytohesin-1, general receptor for phosphoinositides-1 (GRP-1) and ARF nucleotide-binding site opener (ARNO; 16-18), are all characterised by their ability to specifically bind PtdIns(3,4,5)P3 and Ins(1,3,4,5)P4, in preference to either phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2) or PtdIns(4,5)P2 (and the corresponding inositol 1,3,4-trisphosphate (Ins(1,3,4)P3) and Ins(1,4,5)P3 head-groups (19-22)). This ability of Group I PH domains to specifically bind PtdIns(3,4,5)P3 is manifested in vivo by the PH domain-dependent plasma membrane recruitment of these cytosolic proteins following agonist stimulation of PI 3-kinase (20-23).

Other proteins that bind Ins(1,3,4,5)P4 in vitro, with an affinity and isomeric specificity typical of Group I PH domains, are those of the GAP1 family of Ras GTPase-activating proteins (GAPs) (24,25). Of these proteins GAP1IP4BP and GAP1m both contain, besides N-terminally coupled C2 domains (26) and a highly conserved central Ras GAP domain (24), a C-terminal Ins(1,3,4,5)P4-binding PH domain that is linked to a 26 amino acid Btk motif (PH/Btk) (25,27). In keeping with the other Group I PH domain-containing proteins, GAP1m is a cytosolic protein that undergoes rapid plasma membrane recruitment following agonist activation of PI 3-kinase (28). Given the high sequence identity (63%) and similar Ins(1,3,4,5)P4-
binding characteristics of the GAP1m and GAP1IP4BP PH/Btk domains (29), one would expect that GAP1IP4BP may behave in a similar manner. Surprisingly however a number of studies have suggested that rather than being localised within the cytosol, both endogenous (30,31) and overexpressed GAP1IP4BP (32), along with its Drosophila homologue GAP1 (33), are constitutively associated with the plasma membrane. The precise manner of this membrane association is unclear although there is no evidence that GAP1IP4BP contains either transmembrane spanning domains or post-translational membrane targeting sequences (29).

In this study we have examined the molecular interactions required for the constitutive plasma membrane association of GAP1IP4BP. In particular we describe experiments that highlight a unique and pivotal role for the GAP1IP4BP PH/Btk domain in this membrane association.
Experimental Procedures

General materials

\[ D-\text{Ins}(1,3,4,5)P_4 \text{ and dipalmitoyl PtdIns}(3,4,5)P_3 \text{ were kind gifts from Professor Robin Irvine F.R.S. (Department of Pharmacology, University of Cambridge) and Dr’s Len Stephens and Phill Hawkins (The Babraham Institute, Cambridge) respectively. CHO-M3 cells were a kind gift from Dr John Challis and Dr Andrew Tobin (Department of Pharmacology, University of Leicester). Unless otherwise stated all other reagents were obtained from Sigma.} \]

Generation of \( \text{GAP1}^{1\text{P4BP}} \) and \( \text{GAP1}^m \) chimeras.

Site-directed mutagenesis was used to introduce the following unique restriction sites in both \( \text{GAP1}^m \) and \( \text{GAP1}^{1\text{P4BP}} \): at the start of the PH domain an \( \text{AgeI} \) site and at the end of the Btk motif an \( \text{AscI} \) site. The \( \text{AgeI} \) site was introduced into \( \text{GAP1}^{1\text{P4BP}} \) using the Transformer KitTM (Clontech) and pCI-neo-GAP1^{1P4BP} as template (Primer BP-AgeI \[ 5'\text{-CAAGAGTGTTGAGCAACCGGTTCGCTGCTTAAAGAAG-3'}\]). The \( \text{AscI} \) site was subsequently introduced using the Quikchange KitTM (Stratagene) (Primers BP-AscI \[ 5'\text{-CCCTGCACTGGCGCGCGCCAGCCAACATCCAG-3'}\] and BP-AscIrc \[ 5'\text{-CTGGATGTTGGCTGGCGCCGCGCCAGTGCAGGG-3'}\]). Similarly the corresponding mutations in \( \text{GAP1}^m \) were sequentially introduced using the Quikchange KitTM with pCI-neo-GAP1^m being the initial template (Primers M-
AgeI [5'-AGTGGTACGAGTGAACCGGTGCACCTGAAGAGG-3'], M-AgeIrc [5'-CCTTCTTTCAGGTGCAACCGGTTCCTCGATCCACT-3'], M-AscI [5'-CCATGTACTGCAGCGCGCCTGCAGACATCCAA-3'] and M-AscIrc [5'-TTGGATGTCTGCAGCGCGCCTGACGATCATGG-3']). The underlined region in each primer indicates the restriction site and 'rc' designates reverse compliment. Subcellular localisation of these mutants, visualised using transient transfection and indirect immunofluorescence, were identical to wild-type. Using a double digest of AgeI and AscI the PH/Btk motifs were swapped to give GAP1IP4BP/GAP1m-PH/Btk and GAP1m/GAP1IP4BP-PH/Btk.

Site-directed mutagenesis of the GAP1IP4BP PH/Btk domain.

This was performed as previously described (32) using the Transformer Kit\textsuperscript{TM} (Clontech) and pCIneo-GAP1IP4BP as template. Primers used were as follows:

- K585→R, 5'-'GAAGGGTTTCATGATCAGGGCCCAAGGACG-3'; A587→F, 5'-TTCCATGATCAAGAGTTTCAAGGACGGACGC-3'; N597→D, 5'-TTTGGGATGAAGATTAAAGAGATGG-3'; F598→Q, 5'-TTTGGGATGAAGATTAAAGAGATGG-3'; R601→K, 5'-GATGAAGAATTAAAGAGATGGTTC-3'; R601→C, 5'-GATGAAGAATTAAAGAGATGGTTC-3'; k614→E, 5'-GAATTTACCTACCAAGACAGCGGAGAGGGACCAG-3'. In each case the underlined codon describes the particular mutation. For prokaryotic expression of the
various GAP1\textsuperscript{IP4BP} site-directed mutants, the corresponding cDNAs were removed from the pCI\textsuperscript{neo} vector by digestion with \textit{SalI} and \textit{NotI}. The liberated cDNAs were gel purified and ligated into the corresponding sites within pGEX4T-2 (\textit{Pharmacia}). Expression and purification of GAP1\textsuperscript{IP4BP} and GAP1\textsuperscript{IP4BP} site-directed mutants were as previously described (35).

\textit{Sucrose-loaded liposome assays.}

Lipid mixtures phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PtdIns) (all in CHCl\textsubscript{3}) supplemented with either phosphatidylinositol 4-monophosphate (PtdIns(4)P), PtdIns(4,5)P\textsubscript{2} (both in 90\% CHCl\textsubscript{3}, 9\% MeOH, 1\% H\textsubscript{2}O) or PtdIns(3,4,5)P\textsubscript{3} (in H\textsubscript{2}O) were dried down to form a thin film in a 0.5 ml minifuge tube (Beckmann) and then bath sonicated in 0.2M sucrose, 20 mM KCl, 20 mM Hepes pH 7.4, 0.01\% azide to yield a 10x dense lipid stock. This was diluted 10-fold in reaction buffer (0.12 M NaCl, 1 mM EGTA, 0.2 mM CaCl\textsubscript{2} (free Ca\textsuperscript{2+} concentration of approximately 50 nM), 1.5 mM MgCl\textsubscript{2}, 1 mM DTT, 5 mM KCl, 20 mM Hepes pH 7.4, 1 mg/ml BSA) containing 250-500 ng recombinant GAP1\textsuperscript{IP4BP}. GAP1\textsuperscript{IP4BP}-lipid complexes were allowed to form by incubation at 30\textdegree{C} for 4 minutes prior to centrifugation (100,000g for 30 minutes). After spinning, supernatants were carefully removed and the pellets retrieved by addition of an equal volume of 60\textdegree{C} SDS sample buffer and subsequent bath sonication. GAP1\textsuperscript{IP4BP} present in both the supernatant and lipid vesicles were separated by SDS-PAGE and visualised by Western blotting. Detection was
performed using the ECL Western blotting system (*Amersham*) according to the manufacturer’s recommendations. Developed films were analysed by volume integration using ImageQuant software (version 3.3, *Molecular Dynamics Inc.*) in a similar manner to that previously described (31).

**Transient transfection and indirect immunofluorescence.**

COS-7 and HeLa cells, culture as described (32), were plated on glass coverslips and transfected with vector DNA at 50-60% confluency by lipofection using Lipofectamine (Gibco BRL) at a ratio of 0.15 μg DNA/μl cationic lipid. At 48 hours post-transfection, cells were serum starved for 2 hours prior to being fixed and stained for GAP1IP4BP or GAP1m expression as described previously (32). Immunofluorescence staining was visualised by a Leica TCS-NT confocal microscope equipped with a Kr/Ar laser. The confocal was attached to a Leica DM RBE upright epifluorescence microscope.

**Live cell laser scanning confocal microscopy.**

48 hours post-transfection serum starved cells, expressing GFP-tagged proteins, were analysed at 37°C in Krebs-Ringer phosphate buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO4, 1.25 mM CaCl2, 5 mM sodium phosphate) containing 2 mM NaHCO3 and 25 mM Hepes, pH 7.4. Fluorescence imaging was performed with either a Leica DM IRBE inverted confocal microscope controlled with TCS-NT4 software (*Leica*) or an UltraViewTM MultiUser confocal optical scanner (*EG&G Wallac*).
Results

The GAP1IP4BP PH/Btk domain is the sole requirement for plasma membrane association.

To address whether regions outside the GAP1IP4BP PH/Btk domain are necessary for plasma membrane association we generated various GAP1IP4BP/GAP1m chimeras in which the PH/Btk domain from GAP1IP4BP was replaced with the corresponding domain from GAP1m (GAP1IP4BP/GAP1m-PH/Btk) and the PH/Btk domain from GAP1m was replaced with that from GAP1IP4BP (GAP1m/GAP1IP4BP-PH/Btk) (Fig. 1A). Expression of these constructs in serum starved COS-7 cells, followed by analysis of their resultant subcellular distribution revealed that whereas GAP1IP4BP/GAP1m-PH/Btk was cytosolic, GAP1m/GAP1IP4BP-PH/Btk was constitutively associated with the plasma membrane (Fig. 1B). Co-expression of GAP1IP4BP/GAP1m-PH/Btk with a membrane targeted, constitutively active Class I PI 3-kinase catalytic subunit (p110CAAX) resulted in the recruitment of this chimera specifically to the plasma membrane (Fig. 1B); an observation consistent with the GAP1m PH/Btk domain-binding 3-polypolysphoinositides (28). Thus, not only is GAP1IP4BP devoid of its PH/Btk domain unable to associate with the plasma membrane, but the GAP1IP4BP PH/Btk domain is sufficient, when substituting the corresponding domain from GAP1m, to direct plasma membrane targeting.

Site-directed mutagenesis of the GAP1IP4BP PH/Btk domain.
Given that the PH/Btk domain of GAP1IP4BP also comprises the \textit{in vitro} Ins(1,3,4,5)P$_4$-binding site (27), the results described above raise the possibility that binding of the PH/Btk domain to phosphoinositides may be required for plasma membrane association of GAP1IP4BP. To examine this we generated a number of site-directed mutants within the GAP1IP4BP PH/Btk domain designed to inhibit \textit{in vitro} Ins(1,3,4,5)P$_4$-binding. Two groups of mutants were produced. Firstly, those located within the positively charged potential Ins(1,3,4,5)P$_4$-binding pocket (GAP1IP4BP-K$_{585}$\textrightarrow{}R; -R$_{601}$\rightarrow{}C; -R$_{601}$\rightarrow{}K and -K$_{614}$\rightarrow{}E) and secondly, mutants analogous to those found in the PH domain of Btk that are known to inhibit Ins(1,3,4,5)P$_4$-binding (GAP1IP4BP-A$_{587}$\rightarrow{}F; -N$_{597}$\rightarrow{}D and -F$_{598}$\rightarrow{}Q (15)). Ins(1,3,4,5)P$_4$-binding to GAP1IP4BP-K$_{585}$\rightarrow{}R, -A$_{587}$\rightarrow{}F, -F$_{598}$\rightarrow{}Q and -R$_{601}$\rightarrow{}C was inhibited by greater than 90\% compared to wild-type GAP1IP4BP, whereas binding to the -N$_{597}$\rightarrow{}D, -R$_{601}$\rightarrow{}K and significantly -K$_{614}$\rightarrow{}E mutants was inhibited by 81.4 $\pm$ 3.1, 75.4 $\pm$ 4.1 and only 15.2 $\pm$ 3.1\% respectively ($n$ = 3; data not shown).

\textit{Mutations within the PH/Btk domain that inhibit Ins(1,3,4,5)P$_4$-binding result in GAP1IP4BP dissociating from the plasma membrane.}

In order to determine the correlation between Ins(1,3,4,5)P$_4$-binding to GAP1IP4BP and the ability to associate with the plasma membrane, the subcellular localisation of the GAP1IP4BP mutants was determined. As seen in Figure 2, GAP1IP4BP mutants with dramatically reduced abilities to bind Ins(1,3,4,5)P$_4$, -K$_{585}$\rightarrow{}R, -
A587→F, N597→D, R598→Q, R601→K and R601→C, no longer associate with the plasma membrane but were instead primarily cytosolic. This contrasted with GAP1P4BP-K614→E which, consistent with its ability to bind Ins(1,3,4,5)P4, retained a predominant plasma membrane localisation (Fig. 2). However, detailed image analysis revealed a detectable increase in cytosolic fluorescence compared to wild type (Fig. 3). Thus there does appear to be a strong correlation between the ability of the PH/Btk domain to bind phosphorylated forms of inositol and an ability to associate with the plasma membrane. Such a relationship suggests a potential molecular explanation for the plasma membrane localisation of GAP1P4BP; namely that the PH/Btk domain may directly bind phosphoinositides present within the inner plasma membrane leaflet.

**GAP1P4BP binds PtdIns(4,5)P2 and PtdIns(3,4,5)P3 with similar affinities via a binding site that overlaps with that for Ins(1,3,4,5)P4.**

To test whether GAP1P4BP is capable of interacting with phosphoinositides, we generated sucrose-loaded liposomes composed of a mixture of phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PtdIns) (respective ratio of 28.5:28.5:28.5:14.5% (w/w); total lipid content of 70 μM), to which was added 7% (w/w) of either PtdIns, PtdIns(4)P, PtdIns(4,5)P2 or PtdIns(3,4,5)P3. As shown in Figure 4, although GAP1P4BP binding to mixed liposomes containing PtdIns or PtdIns(4)P was barely detectable a clear association was seen with liposomes containing PtdIns(4,5)P2 or
PtdIns(3,4,5)P₃ (Fig. 4A). Under these conditions, the association of GAP₁IP⁴BP occurred via a
binding site that appeared to overlap with the *in vitro* Ins(1,3,4,5)P₄-binding site
since addition of Ins(1,3,4,5)P₄ resulted in the dissociation of GAP₁IP⁴BP.

Furthermore, a GAP₁IP⁴BP mutant with a significantly reduced ability to bind
Ins(1,3,4,5)P₄ and the plasma membrane (GAP₁IP⁴BP-R601→C, see Fig. 2) failed to
associate with the liposomes (Fig. 4B).

By sequentially lowering PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ levels, but
maintaining the same total lipid content by increasing the mass of PtdIns, the
specificity of this association was determined. Assuming an even distribution of the
lipids within the aqueous environment, GAP₁IP⁴BP bound PtdIns(4,5)P₂ and
PtdIns(3,4,5)P₃ with apparent association constants of 0.8 ± 0.5 and 0.5 ± 0.2 µM
respectively (Fig. 4C). Given that PtdIns(4,5)P₂ is far more abundant than
PtdIns(3,4,5)P₃ even after agonist stimulation (34), these affinities suggest that the
plasma membrane association of GAP₁IP⁴BP may result from its PH/Btk domain
predominantly binding PtdIns(4,5)P₂ rather than PtdIns(3,4,5)P₃.

Unlike GAP₁IP⁴BP, GAP₁ᵐ specifically binds PtdIns(3,4,5)P₃.

A prediction arises from the conclusions drawn from Figures 1 and 4. If the
plasma membrane association of GAP₁IP⁴BP is a consequence of an ability to
directly bind PtdIns(4,5)P₂ then given that GAP₁₅ is cytosolic in unstimulated cells, its affinity for PtdIns(4,5)P₂ should be lower than that of GAP₁IP₄BP. In parallel experiments the association constants of GAP₁₅ for PtdIns(4)P, PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ were determined to be >24.5 (3.5 ± 1.5% association at this concentration), >24.5 (21.1 ± 8.3% association) and 0.4 ± 0.2 μM respectively (Fig. 5). Thus although GAP₁₅ and GAP₁IP₄BP have almost identical affinities for PtdIns(3,4,5)P₃, the affinity of GAP₁₅ for PtdIns(4,5)P₂ is at least 30-fold lower than that of GAP₁IP₄BP for PtdIns(4,5)P₂.

In unstimulated cells GAP₁IP₄BP is not localised to the plasma membrane via an ability to bind basal PtdIns(3,4,5)P₃.

To address whether the plasma membrane association of GAP₁IP₄BP may result from binding to a low resting level of PtdIns(3,4,5)P₃, we transiently transfected COS-7 cells with a plasmid encoding a green fluorescent protein (GFP)-tagged GAP₁IP₄BP construct (GFP-GAP₁IP₄BP). Using live cell confocal imaging we observed the plasma membrane localisation of GFP-GAP₁IP₄BP prior to and during incubation with wortmannin at a concentration that specifically inhibits PI 3-kinase (100 nM). Under these conditions no detectable dissociation of GFP-GAP₁IP₄BP from the plasma membrane was observed during the course of a 10 minute incubation with wortmannin (data not shown). To circumvent the unlikely possibility
that the dissociation rate of PtdIns(3,4,5)P$_3$ from GAP1IP$_4$BP is slow (i.e. in the order of minutes) serum starved COS-7 cells were microinjected with the plasmid encoding for GFP-GAP1IP$_4$BP and cultured for 4 hours in the continual presence of either LY294002 (50 μM changed every 30 minutes) or wortmannin (100 nM similarly changed every 30 minutes) (Fig. 6). Again GFP-GAP1IP$_4$BP remained predominantly localised to the plasma membrane. Together these data emphasise that the plasma membrane association of GAP1IP$_4$BP is unlikely to result from its PH/Btk domain-binding resting levels of PtdIns(3,4,5)P$_3$.

In vivo GAP1IP$_4$BP dissociates from the plasma membrane following depletion of PtdIns(4,5)P$_2$.

If the plasma membrane association of GAP1IP$_4$BP is a consequence of its PH/Btk domain-binding PtdIns(4,5)P$_2$, then manipulation of the concentration of this lipid should result in the dissociation of GAP1IP$_4$BP from the plasma membrane. Two methods were used to manipulate PtdIns(4,5)P$_2$ levels. Firstly, HeLa cells expressing GFP-GAP1IP$_4$BP, were incubated with ionomycin (1 μM) in the presence of external Ca$^{2+}$ (1.2 mM). Under these conditions ionomycin induces a large increase in cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) that activates endogenous PLC and results in a subsequent decrease in plasma membrane PtdIns(4,5)P$_2$ levels (35). As shown in Figure 7, addition of ionomycin caused the dissociation of GFP-
GAP1\textsuperscript{IP4BP} from the plasma membrane and a simultaneous appearance of GFP fluorescence in the cytosol (Fig. 7A). This dissociation was rapid and paralleled control experiments using the well characterised \textit{in vivo} PtdIns(4,5)\textsubscript{P$_2$}-binding PH domain from PLC-δ\textsubscript{1} (Fig. 7B (35,36)). To test the role of PLC in the ionomycin-induced GAP1\textsuperscript{IP4BP} dissociation we incubated GFP-GAP1\textsuperscript{IP4BP} expressing HeLa cells with the relatively specific PLC inhibitor U73122 (37). Before ionomycin addition, the localisation of GFP-GAP1\textsuperscript{IP4BP} was unaltered and upon ionomycin addition U73122-treated cells failed to show the plasma membrane dissociation observed in control cells (Fig. 7C).

As a second, independent, approach we made use of the observation that high concentrations of wortmannin (1 \textmu M) induce a gradual fall in the levels of plasma membrane PtdIns(4,5)\textsubscript{P$_2$} due to an inhibition of type III PtdIns 4-kinase (38,39). Incubation of GFP-GAP1\textsuperscript{IP4BP} transfected HeLa cells with 1 \textmu M wortmannin resulted in a slow dissociation of GFP-GAP1\textsuperscript{IP4BP} from the plasma membrane (Fig. 8A). Importantly, this rate of dissociation was significantly enhanced by stimulation of PtdIns(4,5)\textsubscript{P$_2$} hydrolysis via histamine-induced activation of PLC (100 \textmu M; Fig. 8B).

\textit{Agonist stimulated production of cytosolic Ins(1,3,4,5)\textsubscript{P$_4$} does not result in the plasma membrane dissociation of GAP1\textsuperscript{IP4BP}.}

In the above experiments, agonist and ionomycin stimulation of PLC induces
not only hydrolysis of PtdIns(4,5)P₂ but also Ins(1,4,5)P₃ production. This cytosolic second messenger, which triggers the well-characterised release of Ca²⁺ from intracellular stores, is the substrate for an Ins(1,4,5)P₃ 3-kinase that generates the potential second messenger Ins(1,3,4,5)P₄ (40). Given that the high affinity PtdIns(4,5)P₂-binding site overlaps with that for Ins(1,3,4,5)P₄ (see Fig. 4), one interpretation of the data in Figure 7 and 8 is that GAP1IP4BP may undergo a regulated dissociation from the plasma membrane as a consequence of Ins(1,3,4,5)P₄ competing with PtdIns(4,5)P₂ for binding to the PH/Btk domain. To test this we transiently transfected CHO cells stably overexpressing the M3 muscarinic receptor (CHO-M3) with GFP-GAP1IP4BP and observed its subcellular localisation prior to, and during, carbachol stimulation. In control cells overexpressing GFP-PLC-δ₁ a clear transient dissociation of GFP-PLC-δ₁ was observed, with maximal dissociation occurring at about 60 seconds after carbachol addition (Fig. 9A). However, no such dissociation was detectable in cells expressing GFP-GAP1IP4BP (Fig. 9B). Similar data were also obtained following histamine stimulation of HeLa cells stably overexpressing the B-isoform of Ins(1,4,5)P₃ 3-kinase and transiently transfected with the construct encoding GFP-GAP1IP4BP (Millard and Banting, unpublished data).
Discussion

In this study we have examined the molecular interactions required for the constitutive plasma membrane association of the Ras GTPase-activating protein GAP1 IP4BP. Initially through the generation of GAP1 IP4BP/GAP1 chimera GAP1/IP4BP m chimeras we have shown that the GAP1 IP4BP PH/Btk domain is the sole determinant required to direct plasma membrane association. Furthermore by site-directed mutagenesis we have highlighted a correlation between the ability of the GAP1 IP4BP PH/Btk domain to bind phosphorylated forms of inositol, as characterised by in vitro Ins(1,3,4,5)P4-binding, and the resultant plasma membrane association of GAP1 IP4BP. Such a relationship clearly suggests that the plasma membrane association of GAP1 IP4BP results from the ability of its PH/Btk domain to directly bind inositol-containing lipids present in the inner leaflet.

Previously the PH/Btk domain of GAP1 IP4BP has been classified, along with those from Btk, GAP1 m and the ARF-exchange factors GRP1, ARNO and cytohesin-1, as a member of the Group I family of PtdIns(3,4,5)P3 and Ins(1,3,4,5)P4-binding PH domains (14,41). In this study however, through the use of artificial liposomes, we have shown that although the PH/Btk domain of GAP1 IP4BP does indeed bind PtdIns(3,4,5)P3 with an affinity similar to that of the PH/Btk domain of GAP1 m (0.5 ± 0.2 and 0.4 ± 0.2 µM respectively), unlike this protein GAP1 IP4BP does not have a 30-fold lower affinity for PtdIns(4,5)P2. On the contrary,
GAP1IP4BP binds PtdIns(4,5)P₂ with an almost identical affinity to PtdIns(3,4,5)P₃ (0.8 ± 0.5 µM). The PH/Btk domain of GAP1IP4BP is therefore a unique Ins(1,3,4,5)P₄-binding PH domain which, we propose, defines a novel class of Group I PH domains (14,41). Based on these results we suggest that the Group I PH domains should be subdivided into Group IA, comprising those that specifically bind PtdIns(3,4,5)P₃ (i.e. Btk, ARNO, GRP1, cytohesin-1 and GAP1m), and Group IB which, although retaining an ability to bind Ins(1,3,4,5)P₄ (a characteristics distinct from Group II PH domains), nevertheless bind PtdIns(3,4,5)P₃ and PtdIns(4,5)P₂ with comparable affinities (i.e. GAP1IP4BP).

The similar affinity of GAP1IP4BP for PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ suggests, given that plasma membrane PtdIns(4,5)P₂ is more abundant than PtdIns(3,4,5)P₃ even after agonist stimulation (34), that the constitutive plasma membrane association of GAP1IP4BP may occur as a consequence of its PH/Btk domain-binding plasma membrane PtdIns(4,5)P₂. If such a mechanism were correct then one would expect that since GAP1m is not constitutively associated with the plasma membrane it should have a lower affinity for PtdIns(4,5)P₂. As stated above, this is indeed the case. Furthermore, any mechanism that induces a significant depletion of plasma membrane PtdIns(4,5)P₂ should result in the dissociation of GAP1IP4BP. Experimentally we have presented direct evidence that reducing
PtdIns(4,5)P_2 content within the inner leaflet of the plasma membrane, does indeed result in the plasma membrane dissociation of GAP1IP4BP. Together therefore these data strongly suggest that, in an unstimulated cell, the plasma membrane association of GAP1IP4BP results from its PH/Btk domain binding PtdIns(4,5)P_2.

A major issue regarding GAP1IP4BP is whether it functions as a receptor for PtdIns(3,4,5)P_3 or Ins(1,3,4,5)P_4. From this study the similar affinity of GAP1IP4BP for PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 suggests that, given the cellular levels of PtdIns(4,5)P_2 exceed those of PtdIns(3,4,5)P_3 even after agonist stimulation (34), in vivo GAP1IP4BP may not significantly bind, and hence be regulated by, PtdIns(3,4,5)P_3. The only other potential in vivo ligand is therefore Ins(1,3,4,5)P_4.

Support for a role of GAP1IP4BP as an Ins(1,3,4,5)P_4 receptor comes from two sources. Firstly, in the presence of artificial liposomes, membrane associated GAP1IP4BP is inactive as a RasGAP (24). Under these conditions Ins(1,3,4,5)P_4, but not PtdIns(3,4,5)P_3, can specifically stimulate the RasGAP activity (24). Secondly, GAP1IP4BP can enhance the well-documented ability of Ins(1,3,4,5)P_4 to regulate Ca^{2+} homeostasis in permeabilised L1210 cells (42-44).

The conclusion that GAP1IP4BP constitutes a receptor for Ins(1,3,4,5)P_4 rather than PtdIns(3,4,5)P_3 has a significant baring on the molecular events that may follow Ins(1,3,4,5)P_4-binding to GAP1IP4BP. In this study we have presented
evidence that the PtdIns(4,5)P₂-binding site located within the GAP₁IP₄BP PH/Btk domain overlaps with the Ins(1,3,4,5)P₄-binding site. For instance, PtdIns(4,5)P₂-binding to this site can be blocked by competition with Ins(1,3,4,5)P₄ and is also significantly reduced by the introduction of the R⁶⁰¹→C mutation. Based simply on these results it may be predicted that following agonist stimulated Ins(1,3,4,5)P₄ production, GAP₁IP₄BP could dissociated from the plasma membrane as a result of cytosolic Ins(1,3,4,5)P₄ competing for binding with PtdIns(4,5)P₂. Such a conclusion is not supported however by the following experimental evidence. Firstly, endogenous GAP₁IP₄BP, when associated with the plasma membrane of platelets, is capable of binding Ins(1,3,4,5)P₄ with nanomolar affinity (Kₐ of 6.3 nM) via the characterised Ins(1,3,4,5)P₄-binding site (29,45). Under these conditions Ins(1,3,4,5)P₄-binding does not lead to the dissociation of GAP₁IP₄BP from the membrane (Reynolds and Cullen, unpublished data). Secondly, in the current study we have been unable to detect any plasma membrane dissociation of GAP₁IP₄BP following agonist stimulated Ins(1,3,4,5)P₄ production. Together these data strongly argue that Ins(1,3,4,5)P₄, on competing with PtdIns(4,5)P₂, does not induce the plasma membrane dissociation of GAP₁IP₄BP. Currently a molecular explanation for this observation is lacking however it is tempting to speculate that the formation of the GAP₁IP₄BP/Ins(1,3,4,5)P₄ complex may result in it interacting with either a plasma membrane associated protein and/or lipid.
In summary therefore we have presented experimental evidence of the molecular interactions that occur in order for GAP1IP4BP to constitutively associate with the plasma membrane. We have highlighted the vital and unique role of the PH/Btk domain and furthermore suggests some of the events that may occur following the agonist stimulated production of Ins(1,3,4,5)P4 and/or PtdIns(3,4,5)P3. Future experiments will need to address the downstream consequences of these interactions on GAP1IP4BP regulated Ras signalling.
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Figure Legends

Figure 1. The PH/Btk domain of GAP1IP4BP is the sole determinant required for constitutive plasma membrane association.
(a). Depiction of the GAP1 chimeras generated as described in Experimental procedures.
(b). COS-7 cells were transiently transfected with either pCIneo-GAP1IP4BP/GAP1m-PH/Btk (i and ii) or pCIneo-GAP1m/GAP1IP4BP-PH/Btk (iii and iv) along with in

ii and iv p110CAAX. After 48 hours cells were fixed and stained using either GAP1IP4BP (i and ii) or GAP1m (iii and iv) specific antisera and anti-rabbit IgG TRITC conjugate. Fixation and imaging were as described in Experimental procedures.

Figure 2. The subcellular localisation of GAP1IP4BP PH/Btk domain mutants expressed in HeLa cells.
COS-7 cells were transiently transfected with the desired pCIneo-GAP1IP4BP mutant. 48 hours post-transfection cells were fixed and GAP1IP4BP detected by indirect immunofluorescence using GAP1IP4BP specific antiserum and anti-rabbit IgG TRITC conjugate. Fixation and imaging were performed as described in Experimental procedures. Data is representative of at least 150 cells imaged after three separate transfections.

Figure 3. Image analysis of the subcellular localisation of the GAP1IP4BP mutants expressed in HeLa cells.
Indirect immunofluorescence of cells expressing GAP1IP4BP, GAP1IP4BP-R601→C or GAP1IP4BP-K614→E was performed as described in Fig. 2. Staining intensities measured according to pixel brightness were quantified along cell transepts for each construct on the depicted cell. The solid black line corresponds to the location of the nucleus. Similar data was obtained in a further 10 cells for each mutant.

Figure 4. GAP1IP4BP associates with nanomolar affinity to liposomes supplemented with either PtdIns(4,5)P2 or PtdIns(3,4,5)P3 via a binding site that overlaps with the Ins(1,3,4,5)P4-binding site.

(a). Dried down phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol (PtdIns) (respective ratio of 28.5:28.5:28.5:14.5% (w/w) total lipid content of 70 μM) were supplemented with either 7% (w/w) PtdIns (basal), PtdIns(4)P (PI(4)P), PtdIns(4,5)P2 (PI(4,5)P2) or PtdIns(3,4,5)P3 (PIP3). Sucrose loaded liposomes, formed as described in Experimental procedures, were incubated with 250-500 ng of recombinant GAP1IP4BP for 4 minutes at 30°C. The resultant GAP1IP4BP/lipid complexes were pelleted by centrifugation and the resultant supernatants (S) and pellets (P) separated prior to being resolved by SDS/PAGE and Western blotting using GAP1IP4BP specific antiserum.

(b). Liposomes supplemented with 7% (w/w) PtdIns(4,5)P2 were incubated with either GAP1IP4BP, GAP1IP4BP-R601→C (GAP1IP4BP(R601C)) or GAP1IP4BP in the presence of 50 μM Ins(1,3,4,5)P4. Formation of liposomes and the resolution of
the resultant protein/lipid complex were achieved as described above. Similar data was seen in PtdIns(3,4,5)P₃ supplemented liposomes (data not shown).

(c). Specificity of GAP1IP4BP binding to the various phosphoinositides (PI) was determined by lowering the specific content of the required PI whilst maintaining the total lipid content by increasing the amount of PtdIns. Formation of liposomes and the resolution of the resultant protein/lipid complex were achieved as described above.

**Figure 5.** GAP1ᵐ associates with nanomolar affinity to liposomes supplemented with PtdIns(3,4,5)P₃ but not PtdIns(4,5)P₂.

(a). Dried down phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol (PtdIns) (respective ratio of 28.5:28.5:28.5:14.5% (w/w) total lipid content of 70 ¼M) were supplemented with either 7% (w/w) PtdIns (basal), PI(4)P, PI(4,5)P₂ or PIP₃. Sucrose loaded liposomes, formed as described in Experimental procedures, were incubated with 250-500 ng of recombinant GAP1ᵐ for 4 minutes at 30°C. The resultant GAP1ᵐ/lipid complexes were pelleted by centrifugation and the resultant supernatants (S) and pellets (P) separated prior to being resolved by SDS/PAGE and Western blotting using GAP1ᵐ specific antiserum. (b). Specificity of GAP1ᵐ binding to the various phosphoinositides (PI) was determined as described in Fig. 5.

**Figure 6.** Expression of a microinjected GFP-GAP1IP4BP construct in COS-7 cells cultured in the continual presence of the PI 3-kinase inhibitors LY294002 and
wortmannin.

Serum-starved COS-7 cells were microinjected with pEGFP-GAP1\textsuperscript{IP4BP} and immediately cultured in media with no addition (A) or in the continuous presence of either 50 \(\mu\)M LY294002 (B) or 100 nM wortmannin (C). In B and C the media was changed every 30 minutes with fresh media containing PI 3-kinase inhibitors. After 4 hours the subcellular localisation of GFP-GAP1\textsuperscript{IP4BP} was visualised in live cells by confocal laser scanning microscopy as described in Experimental procedures. Similar data was obtained in at least five other cells for each condition.

**Figure 7.** GFP-GAP1\textsuperscript{IP4BP} dissociates from the plasma membrane of HeLa cells following stimulation of PLC by addition of ionomycin.

HeLa cells were transiently transfected with either pEGFP-GAP1\textsuperscript{IP4BP} (A, C) or pEGFP-\textsuperscript{PLC-δ1PH} (B). After culturing for 48 hours the subcellular localisation of GFP-GAP1\textsuperscript{IP4BP} or GFP-\textsuperscript{PLC-δ1PH} was visualised in live cells by confocal laser scanning microscopy at 37\(^\circ\)C in Krebs-Ringer phosphate buffer (containing 1.25 mM Ca\(^{2+}\)). In each case ionomycin (1 \(\mu\)M) was added immediately after capture of the zero time image. Subsequent images were captures every 15 seconds. In C, GFP-GAP1\textsuperscript{IP4BP} expressing cells were preincubated for 15 minutes with the PLC inhibitor U73122 (10 \(\mu\)M). Similar data was obtained in at least five other cells for each condition.

**Figure 8.** Incubation of HeLa cells expressing GFP-GAP1\textsuperscript{IP4BP} with 1 \(\mu\)M
wortmannin causes the gradual plasma membrane dissociation GFP-GAP1IP4BP; an event enhanced by histamine stimulation of PLC.

HeLa cells were transiently transfected with pEGFP-GAP1IP4BP and 48 hours later GFP-GAP1IP4BP was visualised in live cells by confocal laser scanning microscopy. Wortmannin (1 μM; A), or wortmannin (1 μM) plus histamine (100 μM; B), were added immediately after the capture of the zero second image. Subsequent images were captured as the stated times (in seconds). Similar data was obtained in at least ten other cells for each condition.

Figure 9. Stimulation of Ins(1,4,5)P₃/Ins(1,3,4,5)P₄ production does not induce the plasma membrane dissociation of GFP-GAP1IP4BP.

CHO-M3 cells stably overexpressing the M3 receptor were transiently transfected with either pEGFP- PLC-δ₁PH (A) or pEGFP-GAP1IP4BP (B). Cells were cultured for 48 hours after which GFP- PLC-δ₁PH (A) or GFP-GAP1IP4BP (B) were visualised in live cells by confocal laser scanning microscopy at 37°C in Krebs-Ringer phosphate buffer. In each case carbachol (100 μM) was added immediately after capture of the 10 second image. Subsequent images were captured at the stated times (in seconds). Similar data was obtained in at least five other cells for each condition.
Fig. 1

(a).

GAP1<sub>m</sub>

GAP1<sub>IP-BP</sub>

GAP1<sub>m</sub>/GAP1<sub>IP-BP</sub> PH/Btk

GAP1<sub>IP-BP</sub>/GAP1<sub>m</sub> PH/Btk

(b).

GAP1<sub>IP-BP</sub>/GAP1<sub>m</sub> PH/Btk

+ p110<sup>CAAX</sup>

(i)

(ii)

(iii)

(iv)
Fig. 3
(a).

basal

PI(4)P

PI(4,5)P₂

PIP₃

+ 7% (w/w)

(b).

GAP₁⁻BP

GAP₁⁻BP

(R501C)

GAP₁⁻BP

+ 50 μM IP₄

+ 7% (w/w) PI(4,5)P₂

(c).

% of GAP₁⁻BP associated with liposomes

PI added (% (w/w) of total lipid)

Fig. 4
**GAP1IP4BP contains a novel Group I pleckstrin homology domain that directs constitutive plasma membrane association**

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