Phosphoinositide-dependent Activation of the ADP-ribosylation Factor GTPase-activating Protein ASAP1

EVIDENCE FOR THE PLECKSTRIN HOMOLOGY DOMAIN FUNCTIONING AS AN ALLOSTERIC SITE*

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The ADP-ribosylation factor (Arf) family of GTP-binding proteins are regulators of membrane traffic and the actin cytoskeleton. Both negative and positive regulators of Arf, the centaurin β family of Arf GTPase-activating proteins (GAPs) and Arf guanine nucleotide exchange factors, contain pleckstrin homology (PH) domains and are activated by phosphoinositides. To understand how the activities are coordinated, we have examined the role of phosphoinositide binding for Arf GAP function using ASAP1/centaurin β4 as a model. In contrast to Arf exchange factors, phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P₂) specifically activated Arf GAP. D3 phosphorylated phosphoinositides were less effective. Activation involved PtdIns-4,5-P₂ binding to the PH domain; however, in contrast to the Arf exchange factors and contrary to predictions based on the current paradigm for PH domains as independently functioning recruitment signals, we found the following: (i) the PH domain was dispensable for targeting to PDGF-induced ruffles; (ii) activation and recruitment could be uncoupled; (iii) the PH domain was necessary for activity even in the absence of phospholipids; and (iv) the Arf GAP domain influenced localization and lipid binding of the PH domain. Furthermore, PtdIns-4,5-P₂ binding to the PH domain caused a conformational change in the Arf GAP domain detected by limited proteolysis. Thus, these data demonstrate that PH domains can function as allosteric sites. In addition, differences from the published properties of the Arf exchange factors suggest a model in which feedforward and feedback loops involving lipid metabolites coordinate GTP binding and hydrolysis by Arf.

Arf proteins are ubiquitous and essential GTP-binding proteins in eukaryotes. They were first identified as cofactors for cholera toxin-catalyzed ADP-ribosylation of Gₛ (1). The relationship between this activity and the normal cellular function of Arf is not clear. The best described activity of Arf is the regulation of membrane traffic (2, 3). More recently, potential roles in reorganization of the actin cytoskeleton have been identified for Arf1 and Arf6 (4–7). Neither the molecular mechanism by which Arf proteins regulate these pathways nor the means by which Arf itself is regulated have been delineated; however, phospholipids probably function in both capacities.

The relationship between Arf and phospholipids is complex. Arf activates phospholipase D and phosphatidylinositol 4-phosphate 5-kinase, resulting in the production of phosphatic acid (PA) and PtdIns-4,5-P₂ (8–11). Phosphoinositides and phosphatic acid also regulate Arf function. Arf itself binds PtdIns-4,5-P₂ (12). Phosphoinositides have a role in converting Arf-GTP to Arf-GDP by the centaurin β family of Arf GAPs. Four members of this family have been identified (13, 14), and one member of the family, ASAP1 (also called centaurin β4), was purified as an Arf GAP that is coordinately activated by PA and phosphoinositides (13). The ArfPtdIns-4,5-P₂ complex was found to be the substrate for ASAP1 (12). The centaurin βs themselves contain a PH domain, a likely site of phosphoinositide binding (13, 14). Phosphoinositides also have a role in the activation of Arf. One family of Arf exchange factors has been found to contain PH domains, and members of this family are activated by phosphoinositides (15–17). These Arf- and phospholipid-dependent reactions could establish a system of feedforward and feedback loops that would provide robust regulation of Arf (18, 19). However, such a system would require some means to coordinately regulate the exchange factor and GAP.

Phosphoinositide-dependent stimulation of several Arf exchange factors containing PH domains has been examined (15, 17, 20). The sequence of the PH domain is most similar to type I PH domains, and, as anticipated based on other type I PH domains, the Arf GEFs have been found to bind PtdInsP₃ in preference to PtdIns-4,5-P₂ (15, 20). The PH domain mediates recruitment of the exchange factor to membranes and functions independently of the catalytic domain (17). If, for instance, an Arf mutant that is not recruited to membranes is used as a substrate, phosphoinositides have no effect on activity, and the...
exchange factor lacking the PH domain is as active as the protein with the PH domain. As has been found for other PH domains, binding to vesicles requires an anionic surface that can be provided by a second acid phospholipid such as phosphatidylserine or PA (16).

ASAP1 Arf GAP activity is stimulated by phosphoinositides and PA (13). The PH domain is, like the exchange factors, most similar in sequence to type I PH domains (21, 22). Therefore, ASAP1 was anticipated to be most efficiently activated by PtdIns-4,5-P2, and consequently, the opposing exchange and GAP activities would be activated by the same signal. To determine if this were the case, we have examined the role of phosphoinositides in ASAP1 function, using natural phospholipids and a comprehensive structural set of synthetic phosphoinositides. We found that ASAP1 differs from the previously studied PH domain-containing Arf GEFs in both specific lipid requirements and mechanism of regulation. PtdIns-4,5-P2 more efficiently activated the Arf-GAP than did D3 phosphorylated phosphoinositides. Although the PH domain contributed to membrane targeting, this PH domain influenced Arf GAP activity through additional mechanisms. The PH domain interacted with the Arf GAP domain. PtdIns-4,5-P2 binding to the PH domain resulted in a conformational change in the Arf GAP domain. The differences in lipid specificity may explain how the GAPs and GEFs are coordinated. Furthermore, these results indicate that the regulatory mechanism of ASAP1's PH domain differs from the currently accepted paradigm of PH domain function and supports the hypothesis that PH domains can function as allosteric sites.

**EXPERIMENTAL PROCEDURES**

**Protein Expression in and Purification from Bacteria**—To express the PH domain, ZA and PZA, reading frames for proteins consisting of residues 338–431 (PH domain), 452–724 (referred to here as ZA), and 325–724 (PZA) of ASAP1 were ligated into the NdeI/XhoI sites of pet19c (Novagen) to express histidine-tagged proteins and pet21 (Novagen) to express proteins without additional residues. Proteins were expressed, extracted, and purified over a HiTrap Q column (Amerham Pharmacia Biotech) as described (13). His-tagged proteins were further fractionated on HiTrap chelating columns (Amerham Pharmacia Biotech) as described (13). Experiments were performed with both the histidine-tagged and the untagged proteins with identical results. Unless otherwise indicated, the results shown were obtained using untagged proteins. To prepare myristoylated PZA, PZA containing the myristoylation site of PKA (GNAAAAKKGSEQES) was constructed by polymerase chain reaction using primers that included the sequence encoding the myristoylation site. The fusion protein was coexpressed with n-myristoyl transferase as described for Arf (23). Myristoylated PZA was extracted from inclusion bodies in 7M urea and was refolded by diluting the protein 50-fold, to a concentration of 0.014 mg/ml, into 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM MgCl2 containing 0.1% Triton X-100 1 h prior to use in the GAP assay. Nonmyristoylated Arf (24) and myristoylated Arf (25) were prepared as described.

**Protein Expression in Mammalian Cells and Immunofluorescence**—Expression vectors for ASAP1 tagged with the Myc and FLAG epitopes are described (13). NIH 3T3 cells were transfected using Fugene (Roche Molecular Biochemicals) as described by the manufacturer. Twenty-four hours following transfection, cells were removed from plates using trypsin/EDTA and replated in Opti-MEM (Life Technologies, Inc.) with no serum on fibronectin-coated coverslips. After an additional 5–7-h incubation, the cells were treated with PDEGF for 5 min and fixed in 2% formaldehyde in phosphate-buffered saline for 10 min. Cells were prepared for immunofluorescence as described (4), visualizing ASAP1 and the protein of interest. Incubation with Perkin-Elmer reagents. Actin was labeled with rhodamine-conjugated phalloidin and the FLAG epitope was accomplished by incorporating the appropriate sequence in an oligonucleotide used for polymerase chain reaction. Actin was labeled with rhodamine-conjugated phalloidin (Molecular Probes, Inc.).

**Mutagenesis**—All mutants were derived from ASAP1b (GenBank™ accession no. AF075462). To assist in selecting residues for site-directed mutagenesis, the PH domain was compared with other PH domains (Fig. 3C), and a homology model of the PH domain of ASAP1, based on the bARK PH domain, was constructed (Fig. 3D). Based on comparison with pleckstrin, PLC-δ, and Btk, lysine 348 was anticipated to contribute to binding PtdIns-4,5-P2 and PtdIns-3,4,5-P3, lysine 355 to binding PtdIns-4,5-P2, and arginine 360, which aligns with arginine 28 of Btk, to binding PtdIns-3,4,5-P3. Lysine 365 is not conserved among the PH domains that have been characterized, although mutation of a threonine residue at this position in Btk is inactivating (27). Point mutations were introduced as described (13) using reagents from a kit (Quikchange; Stratagene). The mutations were in residues predicted to be on the surface of the protein or on the interior surface of a possible inositol binding pocket (Fig. 3D) and, based on solubility and chromatographic behavior, had no gross structural effects. Furthermore, the far-UV CD spectra of the mutants were indistinguishable from wild type PZA, which, based on the deconvoluted spectra, was 17% a-helix, 27% β-sheet, 22% turn, and 35% undefined secondary structure. Truncations were accomplished by polymerase chain reaction using Vent polymerase. Fusion of the FLAG epitope was accomplished by incorporating the appropriate sequence in an oligonucleotide used for polymerase chain reaction. Mutations were labeled by sequencing using an ABI 373 instrument with Perkin-Elmer reagents.

**Lipids**—All lipids purified from mammalian sources, including bovine brain PtdIns-4P and PtdIns-4,5-P2, were obtained from Sigma. Bovine brain PtdIns-4,5-P2 was also obtained from Roche Molecular Biochemicals. D3 phosphorylated phosphoinositides, including PtdIns-3,4,5-P3, PtdIns-3,4-P2, and phosphatidylinositol 5,6-bisphosphate, were prepared by unambiguous
Fig. 2. Characterization of the GAP activity of PZA. A, PtdIns-P2 concentration dependence. GAP activity in 0.5 nM PZA was determined as described in Fig. 1A B, comparison of PZA and ZA. GAP activity of PZA (circles and inverted triangles) and ZA (triangles) at the indicated concentrations was determined in the presence of 1.4 Triton X-100 (open symbols) or Triton X-100 containing 360 μM PA and 90 μM bovine brain PtdIns-P2 (filled symbols) using either wild type Arf1 (circles and triangles) or an Arf mutant lacking 13 N-terminal amino acids ([Δ13]Arf1, inverted triangles) as substrates. C, PtdIns-P2-Arf-GTP is the substrate for PZA. The PtdIns-P2 dependence of GAP activity of 0.5 nM PZA using either wild type Arf or the indicated Arf mutants in the presence of Triton X-100 micelles containing 360 μM PA and the indicated concentration of PtdIns-P2 was determined. D, the phosphoinositide specificity of PZA is similar to that of native ASAP. The activity of 0.5 nM PZA was determined using mixed micelles as described for Fig. 1B. *, greater than no addition, p < 0.05; ‡, greater than no addition, PtdIns-3,4-P2, PtdIns-4P, and PtdIns-P3, PtdIns-P, and PtdIns-4P, p < 0.05; *, greater than no addition, PtdIns-3,4-P2, PtdIns-P2, PtdIns-4P, and diC16PtdIns-P2, p < 0.01. E, activation of PZA by phosphoinositides in vesicles. GAP activity was measured in the presence of 50 μM phosphoinositides in phospholipid vesicles containing 700 μM PC and 300 μM PA. +, greater than no addition, PtdIns-3,4-P2, PtdIns-P3, and PtdIns-4P, p < 0.05; *, greater than no addition, PtdIns-3,4-P2, PtdIns-P2, PtdIns-4P, and diC16PtdIns-P2, p < 0.01.

Spectra were recorded on a JASCO model J-715 circular dichroism spectropolarimeter (JASCO, Easton, MD) at room temperature using 1-mm path length quartz cuvettes. Four consecutive scans were accumulated, and the average spectra were stored. The spectra were deconvoluted using the program Selenoc.

Homology Modeling—The sequences of all PH domains whose structures have been solved (Protein Data Bank accession codes 1AWE, 1BAK, 1DYN, 1MAI, 1MPH, 1PLS, 1PMS, and 2DYN) were aligned with the sequence of the PH domain of ASAP1. The four PH domain structures whose sequence alignments had the highest identity and similarity scores (1BAK, 1MAI, 1MPH, and 1PLS) were used to generate homology model structures for the ASAP1 PH domain using GenoMine software (Molecular Applications Group, Palo Alto, CA) (33). The protein threading algorithm Profiles3D (Molecular Simulations Inc., San Diego, CA) (34) was used to assess the plausibility of the three-dimensional structure of each homology model generated. The PH domain of the G protein-coupled receptor kinase 2, or shork (1BAK) (35), scored highest and was used to aid the selection of potential mutation sites on the surface of the ASAP1 PH domain.

Sequence Analysis—Amino acid sequences corresponding to the PH domains of a number of known D3 phosphoinositide-binding proteins were aligned with equivalent sequences from ASAP1/centaurin β4 and PAF1/centaurin β3 as well as other known phosphoinositide binding PH domains including PLCβ and spectra as well as with the N-terminal PH domain from pleckstrin itself. The alignment was performed using the Clustalw function of the Multiple Sequence Alignment programs, available on the World Wide Web.

Miscellaneous—GAP activity was measured as described (19). Lipid binding to Arf (12) and PZA (32) was determined as described in the same buffer used to measure GAP activity. The amount of PZA bound to vesicles was determined by fractionating the protein by SDS-polyacrylamide gel electrophoresis and staining with Coomassie Blue. Protein
Recruitment-independent Function of the ASAP1 PH Domain

**RESULTS**

Activation of ASAP1 by Phospholipids Is Specific for PtdIns-4,5-P_2—Although ASAP1 and Arf exchange factors are both regulated by phosphoinositides, differences in the particular phosphoinositide could provide independent regulation. To examine this possibility, we determined the phospholipid specificity of ASAP1. As previously reported, GAP activity of ASAP1 purified from bovine brain was activated by PtdIns-P_2 in the presence of PA (Fig. 1A). The activation was independent of lipid presentation. Similar results were observed whether PtdIns-P_2 and PA were in mixed micelles or vesicles (not shown).

The phosphorylation of ASAP1’s PH domain was dependent on both the phosphoinositide head group and the acyl groups. Specificity for PtdIns-4,5-P_2 (Fig. 1B) was observed when comparing dipalmitoyl dioctanoyl PtdIns-4,5-P_2 binding. The PtdIns-P_2 dependence of GAP activity was determined using mixed micelles of Triton X-100, 360 μM PA, and the indicated concentrations of PtdIns-P_2. Results shown are for histidine-tagged proteins and are indistinguishable from the results for the untagged proteins. GAP activity depended on the phosphoinositide head group and the acyl groups, with diC_16PtdIns-4,5-P_2 having a greater effect than PA (Fig. 1A, B).

GAP Activity Depends on Phosphoinositide Binding to Both Arf and ASAP1—The PtdIns-P_2 dependence of GAP activity could reflect a requirement for PtdIns-P_2 binding to Arf, ASAP1, or both. Previously, PtdIns-P_2 binding to Arf was found necessary for GAP activity; mutant Arf proteins with reduced PtdIns-P_2 affinity require more PtdIns-P_2 for interaction with GAP (12). Here we tested if PtdIns-P_2 binding to the PH domain of ASAP1 is also necessary for activity. We used a bacterially expressed protein containing the PH domain, Arf GAP domain, and ANK repeats (henceforth referred to as PZA) to examine the role of PtdIns-4,5-P_2 binding to ASAP1. This protein was soluble and, unlike the native protein, stable under the conditions of the binding assay. In addition, using a bacterially expressed protein eliminated the possible contribution of associated mammalian proteins to phospholipid binding. The GAP activity of PZA was similar to that of the native protein PtdIns-P_2 and PA, which together result in the formation of the ternary complex (Ref. 13 and Fig. 2A) 10,000-fold (Fig. 2B). PZA and the native protein had similar Arf specificities (13). The PZA GAP activity required PtdIns-P_2 binding to Arf (Fig. 2C); the EC_{50} for PtdIns-P_2 was increased if mutant Arf proteins with reduced PtdIns-P_2 affinity were used as substrates. PZA and the

was quantified by densitometry using NIH Image. Bovine plasma fibronectin was from Sigma. Data were analyzed by nonlinear regression and analysis of variance with the Tukey-Kramer post-test, as appropriate, using GraphPad Prism. All experiments were performed at least twice with similar results. Limited proteolysis experiments were performed seven times with PZA and four times with [K348N]PZA with similar results. Representative experiments are shown.
Bacterially expressed PZA with either no mutation or the indicated point mutations in the PH domain were examined. GAP activity was determined in mixed micelles containing 1.4 mM Triton X-100 and 360 μM phosphatidic acid. The PtdInsP₂ dependence of binding to vesicles containing 700 μM phosphatidyicholine and 300 μM phosphatidic acid was determined. The EC₅₀ and Kₛ were estimated by nonlinear least square fitting of PtdInsP₂-dependences and are reported with the S.E.

### Table I

| Protein      | Activity | Binding |
|--------------|----------|---------|
|              | EC₅₀     | Rate    | Kₛ     | Fraction |
| Wild type    | 14 ± 3   | 0.18 ± 0.03² | 49 ± 10 | 0.53 ± 0.05² |
| K345N        | NA       | 0.005 ± 0.004 | NB      | 0.02 ± 0.02 |
| K355N        | >100⁴    | 0.077 ± 0.015⁴ | >100⁴ | 0.15 ± 0.07⁴ |
| R360Q        | NA       | 0.009 ± 0.002⁵ | NB      | 0.02 ± 0.02 |
| K365N        | 13 ± 4   | 0.185 ± 0.025⁶ | 43 ± 13 | 0.45 ± 0.03⁶ |

- The mean rate (min⁻¹) in the presence of 50 μM PtdInsP₂, with the S.D., for three determinations are reported.
- The mean fraction of protein bound to vesicles containing 100 μM PtdInsP₂, with the S.D., for four determinations are reported.
- Greater than [K348N]PZA, [K355N]PZA, and [R360Q]PZA, p < 0.05.
- NA, no activity.
- NB, no binding observed.
- Activity and binding were linearly related to PtdInsP₂ concentration up to 100 μM.
- Greater than [K348N]PZA and [R360Q]PZA, p < 0.05.

Native protein also had similar phosphoinositide specificities (compare Fig. 2, D and E, with Fig. 1B). PtdInsP₂ purified from bovine brain stimulated activity to a greater extent than did bovine brain PtdIns₄P or diC16PtdIns₄,5-P₂; PtdIns₄,5-P₂ stimulated activity to a greater extent than did D₃ phosphorylated phosphoinositides. Phosphoinositide specificity was the same whether the lipids were presented as mixed micelles (Fig. 2D) or vesicles (Fig. 2E) and, therefore, was unlikely to be the result of differences in how the lipids were incorporated into the lipid interface.

We began testing the hypothesis that PtdIns₄,5-P₂ binding to ASAP1 was necessary for activity by correlating binding with activity. Like activity, PtdIns₄,P₂ binding to PZA was dependent on a second anionic phospholipid (Fig. 3A). In addition, a protein containing the Arf GAP domain and ANK repeats (referred to as ZA) that did not bind PtdIns₄-P₂ (Fig. 3B) was not active as an Arf GAP in the presence of PtdIns₄-P₂ (Fig. 2B). ZA’s lack of activity could be the result of the catalytic domain misfolding. Therefore, to further test the hypothesis that PtdIns₄-P₂ binding to the PH domain stimulates GAP activity, the effects of point mutations in the PH domain that reduced PtdIns₄,5-P₂ binding were examined. Four residues were selected: lysine 348, lysine 355, arginine 360, and lysine 365. The mutations did not detectably affect protein folding (see “Experimental Procedures” for the rationale for selecting the residues to be mutated and the structural analysis of the proteins). Examination of these mutant PZAs revealed that alterations in PtdIns₄,5-P₂ binding correlated with changes in PtdIns₄,5-P₂ dependence of activity (Fig. 3, E and F, and Table I). PtdIns₄,5-P₂ binding to PZA was neither bound to nor activated [K348N]PZA or [R360Q]PZA. PtdIns₄,5-P₂ binding and activation were reduced in [K355N]PZA relative to the wild type protein. Mutating residue 365 had little or no effect on either PtdIns₄,5-P₂ binding or PtdIns₄,5-P₂-dependent activity. These results support the hypothesis that PtdIns₄,5-P₂ binding to ASAP1 is required for GAP activity.

### Recruitment Can Be Uncoupled from GAP Activation

—The currently accepted paradigm is that phosphoinositides regulate activity of enzymes by recruitment. Based on this model, the lower activity of D₃ phosphorylated phosphoinositides compared with PtdIns₄,5-P₂ and the lower activity of dipalmitoyl PtdIns₄,5-P₂ compared with bovine brain PtdIns₄-P₂ should reflect differences in binding to either Arf or PZA. We therefore compared binding of the phosphoinositide isomers to Arf and PZA. In contrast to their effects on GAP activity and contrary to the prediction, all phosphoinositides tested bound Arf and PZA to a similar extent (Fig. 4, A and B). The Kₛ for PZA binding to diC16PtdIns₄,5-P₂ (65 ± 25 μM) was indistinguishable from the Kₛ for bovine brain PtdIns₄,5-P₂. Therefore, recruitment was not sufficient to activate the GAP ASAP1, raising the possibility that, in addition to recruitment, PtdIns₄,5-P₂ binding could be inducing a conformational change in one or both proteins that was necessary for Arf-GTP to productively interact with ASAP1.

We tested if ASAP1 recruitment to membranes was necessary and sufficient for GAP activation in three ways. First, the activity of PZA and ZA was compared in the absence of phospholipids (Fig. 2B). If the role of phosphoinositides were simply recruitment, then, in the absence of phospholipids, the activity of PZA and ZA should be similar. Instead, PZA had activity and ZA was devoid of activity. Second, the effect of PtdIns₄,5-P₂ on GAP activity of PZA was determined using a mutant Arf, [Δ13]Arf1, that is soluble in the GTP bound form (36, 37) and does not bind PtdIns₄-P₂ (Fig. 4A). If PtdIns₄,5-P₂-dependent activity were the result of recruitment of GAP and Arf to the same surface, then PtdIns₄-P₂ should have no effect on GAP activity. Instead, the GAP activity of PZA was stimulated by PtdIns₄-P₂ (Figs. 3B and 5A). The extent of activation was less than that observed for wild type Arf1, consistent with PtdIns₄-P₂-Arf-GTP being the preferred substrate. As a third test, activity of myristoylated PZA using myristoylated Arf as a substrate was examined. Both proteins are recruited to hydrophobic surfaces, and therefore, PtdIns₄-P₂ should have no effect if recruitment were the sole mechanism of activation. PA had a greatly reduced effect for the myristoylated proteins (Fig. 5B) compared with nonmyristoylated PZA (Fig. 2A), suggesting that PA has a role in recruitment. However, in contrast to PA and contrary to the prediction of the recruitment model, PtdIns₄-P₂ stimulated activity (Fig. 5B). These results show that recruitment is not sufficient for activation of GAP activity and support the hypothesis that PtdIns₄-P₂ induces a conformational change in either ASAP1 or Arf or both proteins.

**PtdIns₄-P₂ Induces a Conformational Change in ASAP1**

—Given the result with [Δ13]Arf1, PtdIns₄-P₂ was expected to affect at least PZA. We tested for a conformation change in PZA by examining protease sensitivity (Fig. 6). PZA was incubated with trypsin in the presence of micelles containing PA with either PtdIns₄-P₂ from bovine brain, which recruits and stimulates, or diC16PtdIns₄-P₂, which recruits but does not efficiently stimulate, GAP activity (Fig. 6A). Two peptide fragments of 39 and 14 kDa and a minor fragment of 37 kDa were generated in the presence of PA. In the presence of the activating lipid, PtdIns₄-P₂, there was also a time-dependent increase in two closely spaced peptide fragments of 19 and 20 kDa. The production of these two fragments was dependent specifically on PtdIns₄-P₂; PtdIns₄-P₂ had little effect on production of the 19- and 20-kDa fragments (Fig. 6A). The generation of the 19- and 20-kDa fragments also depended on PtdIns₄-P₂ binding to PZA. PtdIns₄-P₂ did not bind to [K348N]PZA or stimulate the GAP activity of [K348N]PZA (Fig. 3, E and F); likewise, PtdIns₄-P₂ did not increase proteolysis of [K348N]PZA, leading to the 19- and 20-kDa peptide fragments (Fig. 6D). [K348N]PZA did yield 39 and 14-kDa fragments when trypsinized in the presence of PA, indicating, together with the CD spectrum, that the mutation did not affect protein folding (Fig. 6D). A faint 8-kDa band was observed when either PtdIns₄-P₂ or PtdIns₄-P₂ were present using either PZA or [K348N]PZA as a substrate for proteolysis; however, the generation of this frag-
ment was not clearly time-dependent, and amounts generated were variable between experiments (Fig. 6). Thus, the generation of the 8-kDa fragment probably occurs during sample preparation and is not associated with GAP activation. In contrast, under conditions in which GAP activity was stimulated specifically by PtdIns-4,5-P2, proteolysis sites yielding peptide fragments of 19 and 20-kDa were unmasked specifically by PtdIns-4,5-P2.

The protease-sensitive sites were mapped by comparing the cleavage of an amino-terminally tagged PZA with untagged PZA (Fig. 6, B and C). Cleavage in the presence of PA of the tagged PZA yielded two peptide fragments, one at 39 kDa as for the untagged protein, and one at 18 kDa, larger than the 14-kDa fragment seen with the untagged protein by approximately the size of the N-terminal tag (2840.9). Therefore, we could map the major proteolytic site to be between the PH and Arf GAP domains at approximately residue 450 (site B in Fig. 6E). The size of the PtdIns-P2-dependent proteolysis products increased to 23 and 24 kDa, indicating that PtdIns-P2 caused exposure of a trypsin site around residue 510 of ASAP1, which is within the Arf GAP domain (site C in Fig. 6E). By the same analysis, the cleavage site giving the faint 8-kDa band could be mapped to within the PH domain at approximately residue 390 (site A). These data taken together are consistent with a PtdIns-P2-dependent conformational change in the Arf GAP domain of ASAP1.

Differences in Determinants of PtdIns-4,5-P2 and PtdIns-P3 Binding—Given that PtdIns-P3 binding does not correlate as strongly with activation as does PtdIns-P2 binding, differences in binding sites for these lipids were anticipated. Differential effects of mutations in specific residues on binding of PtdIns-P2 and PtdIns-P3 are consistent with this prediction. Mutation of lysine 355 partially reduced PtdIns-4,5-P2 binding (Fig. 3E) but entirely abolished PtdIns-P3 binding (Fig. 7). Similarly, mutating lysine 365 had little effect on PtdIns-4,5-P2 binding (Fig. 3E) but decreased PtdIns-P3 binding (Fig. 7). These findings support the suggestion that there are differences between the binding of PtdIns-4,5-P2 and D3 phosphorylated phosphoinositides to ASAP1.

The PH Domain Is Dispensable for Targeting to the Plasma Membrane—PH domains have been shown to act as targeting domains. We therefore examined whether the PH domain of ASAP1 has a similar function (Fig. 8). When overexpressed, wild

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4 The phosphoinositide dependence of the generation of the 8-kDa product could result from a phosphoinositide-protein interaction dependent on SDS and occurring in the SDS-containing sample buffer used for electrophoresis.
Late with exposure of a specific protease cleavage site. Eight micelles of Triton X-100 and 360 ng of PZA containing either anionic lipid, and synergy between the two lipids was not observed. The data are consistent with binding of the Arf GAP domain to the PH domain influencing the interaction with lipids and conferring specificity for PtdIns-4,5-P$_2$.

Second, we examined the effect of the Arf GAP domain on in vivo targeting by the PH domain. Although the PH domain was not required for targeting the protein to ruffles, the isolated PH domain and [1–431]ASAP1, which contained the amino terminus and PH domain, were found concentrated in membrane extensions (Fig. 9, arrows). [1–338]ASAP1, which lacked the PH domain, did not localize to membrane extensions, consistent with localization to the membrane extensions being mediated by the PH domain. However, the addition of the Arf GAP domain to the PH domain, as observed for both [1–600]ASAP1 (Fig. 9) and [325–725]ASAP1 (not shown), excluded the protein from membrane extensions. The effect of the Arf GAP domain on localization could be either the result of the Arf GAP domain interacting with another protein or the result of an intramolecular interaction between the Arf GAP and PH domains. Thus, the result is consistent with the in vitro data supporting the intramolecular association of the PH and Arf GAP domains.

**DISCUSSION**

Both positive and negative regulators of the GTP-binding protein Arf contain PH domains and are presumably regulated, at least in part, by phosphoinositide signaling. Here, we have examined the lipid specificity and molecular mechanism of activation of an Arf GAP, ASAP1, to determine how the GAP and exchange factors may be coordinated. We found that ASAP1 was specifically activated by PtdIns-4,5-P$_2$. Differential binding could not account for specificity. Furthermore, activation of the GAP was not simply the result of recruitment to the plasma membrane. Instead, PtdIns-4,5-P$_2$ induced a specific interaction between the Arf GAP and PH domains, which do not bind phosphoinositides, were also recruited to the PH domain, as observed for both 

**Fig. 6. Phosphoinositide binding and activation of GAP correlate with exposure of a specific protease cleavage site.** Eight micrograms of PZA, histidine-tagged PZA, and [K348N]PZA were incubated under conditions used for the GAP and binding assays with mixed micelles of Triton X-100 and 360 $\mu$M PA containing 50 $\mu$M of the indicated phosphoinositide (bbPtdIns$_2$ was used for these experiments) and 50 ng of trypsin for the indicated time at 30 °C. Reactions were stopped by the addition of trypsin inhibitor from egg, and protease products were fractionated by SDS-polyacrylamide gel electrophoresis and visualized with a colloidal blue stain. A, time course of proteolysis of PZA. B, proteolysis of histidine-tagged PZA. C, time course of proteolysis of histidine-tagged PZA. D, proteolysis of [K348N]PZA. E, map of proteolysis sites.

**Fig. 7. PtdIns-P$_2$ binding to PZA with mutations in the PH domain.** Vesicles contained 700 $\mu$M PC, 300 $\mu$M PA, and, where indicated, 50 $\mu$M PtdIns-P$_2$. Means and S.E. for five experiments (except [R360Q]PZA, which was examined in three experiments) are reported. *, significantly greater than no PtdIns-P$_2$, $p < 0.05$. Type ASAP1 was recruited to dorsal membrane ruffles induced by PDGF (arrowheads). The isolated PH domain could also be found in the PDGF-induced ruffles but did not concentrate in the ruffles to the same extent as did the full-length protein. Furthermore, [K348N]ASAP1 (not shown), [K355N]ASAP1, and [R360Q]ASAP1, which do not bind phosphoinositides, were also recruited to the ruffles, as was a deletion mutant, [1–338]ASAP1, that completely lacked the PH domain. Thus, although the PH domain may contribute to targeting, it is not required for targeting to the plasma membrane ruffles.

**Interaction between the PH and Arf GAP Domain—** Our studies support the hypothesis that the PH domain of ASAP1 functionally interacts with the Arf GAP domain. To further test for an interaction between these domains, we determined if the Arf GAP domain could influence the behavior of the PH domain.

We first compared phospholipid binding of a protein consisting of the isolated PH domain of ASAP1, [338–431]ASAP1, with PZA (Fig. 3B). PZA binding to vesicles was codependent on PA and PtdIns-P$_2$. In contrast, the isolated PH domain could bind to vesicles containing either anionic lipid, and synergy between the two lipids was not observed. The data are consistent with binding of the Arf GAP domain to the PH domain influencing the interaction with lipids and conferring specificity for PtdIns-4,5-P$_2$.
uncoupled from recruitment, suggesting that the function of this PH domain differs from currently accepted models.

The ASAP1 PH domain appears to be an integral part of the protein and acts as an allosteric binding site. The results of three experiments provided evidence for interaction of the PH and Arf GAP/zinc finger domains. First, the isolated PH domain and PZA had different binding specificity, indicating that the folding of the PH domain was influenced by adjacent domains. Second, PZA had activity in the absence of phospholipids, whereas ZA was devoid of activity, suggesting that the PH domain contributed to folding of the catalytic site. Third, the Arf GAP domain affected PH domain-dependent localization to membrane extensions. Other experiments excluded recruitment as the sole mechanism for activation. PtdIns-P$_3$ recruited but did not efficiently activate the protein. Myristoylated proteins were not active in the absence of PtdIns-P$_2$. PtdIns-P$_2$ stimulated activity when a soluble Arf was used as a substrate. These experiments together with the effect of PtdIns-P$_2$ on protease sensitivity provided evidence for PtdIns-4,5-P$_2$-dependent changes in the conformation of the protein. Homology models of the PH (Fig. 3) and zinc finger domains$^5$ reveal possible electrostatic interactions between the PH and zinc finger domains. Previous reports have suggested that PH domains may function as allosteric sites. For instance, PH domains have been found to fold integrally with adjacent protein domains (27, 41). Particularly relevant for ASAP1 is BTK (27), in which the PH domain folds with the carboxyl-terminal BTK domain, a zinc finger, as a single domain. Furthermore, point mutations in the PH domains of Btk and PLC$\delta$ lead to in-

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$^5$ J. Gruschus and J. Ferretti, unpublished observations.
creased enzymatic activity, consistent with an allosteric interaction between the PH and catalytic domains (42, 43).

These experiments have not addressed whether Arf may also undergo a conformational change when PtdIns-P2 binds and how that might contribute to phosphoinositide specificity of GAP activity. Arf mutants with impaired PtdIns-P2 binding were poor substrates for the GAP even when recruited by PA, consistent with Arf also undergoing a conformational change on PtdIns-4,5-P2 binding. The need for Arf to bind PtdIns-P2 could explain the relatively low PtdIns-4,5-P2-dependent activation of GAP (20-fold) detected when a soluble Arf, [Δ13]Arf1, was used as a substrate. This is reminiscent of previously reported phosphoinositide binding to both enzyme and substrate (44). However, in the present case, PtdIns-4,5-P2-induced conformational changes in both substrate and enzyme are involved and could provide further regulation, resulting in the observed 10,000-fold activation and, consequently, function essentially as a binary switch.

Although most similar to class I PH domains that specifically bind PtdIns-P3 (21, 22), the ASAP1 PH domain did not preferentially bind PtdIns-P3. Considering relative cellular concentrations of PtdIns-4,5-P2 and PtdIns-P3, activation is probably mediated by PtdIns-4,5-P2. However, determinants of PtdIns-4,5-P2 and PtdIns-P3 binding need to be examined in detail. Our preliminary mutational analysis showed that residues aligning with those specifically affecting PtdIns-P3 binding in other PH domains affected both PtdIns-4,5-P2 and PtdIns-P3 binding in ASAP1. The observed effects of mutating arginine 360 and lysine 365 on PtdIns-4,5-P2 binding were unanticipated. Mutation of the residue corresponding to arginine 360 in GRP-1 leads to diminished PtdIns-P3 binding, while equivalent mutations in Btk, cytohesin 1, ARNO, and centaurin α PH domains prevent phosphatidylinositol 3-kinase-dependent membrane recruitment (45–49). These findings have led to speculation that the presence of an arginine at this position may mark a PH domain as specific for D3 phosphorylated phosphoinositides. Our findings indicate that in ASAP1 this residue is involved in binding both PtdIns-4,5-P2 and Pt-

FIG. 9. The Arf GAP domain affects localization mediated by the PH domain. NIH 3T3 cells were transfected with plasmids directing expression of the indicated proteins with FLAG epitope tags. The cells were plated on fibronectin-coated coverslips and maintained in serum-free medium for 6 h prior to fixation and preparation for immunofluorescence. Membrane extensions are indicated with arrows. A schematic diagram of the deletion mutants used is shown.
PtdIns-4,5-P$_2$ is probably the relevant activating lipid. Arf exchange factors have been found to be recruited to ruffles and purified from bovine brain. There is a rapid increase to a plateau that may be maintained for many minutes. However, recruitment-independent Function of the ASAP1 PH Domain

Fig. 10. Hypothetical scheme of feedforward and feedback loops allowing coordinate regulation of Arf exchange factors and GAPs. In this speculative scheme, solid lines indicate chemical conversions. Dotted lines indicate regulatory interactions.

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Recruitment-independent Function of the ASAP1 PH Domain

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Phosphoinositide-dependent Activation of the ADP-ribosylation Factor GTPase-activating Protein ASAP1: EVIDENCE FOR THE PLECKSTRIN HOMOLOGY DOMAIN FUNCTIONING AS AN ALLOSTERIC SITE

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