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Regulation of ASAP1 by phospholipids is dependent on the interface between the PH and Arf GAP domains

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Abstract

ASAP1 is an Arf GAP with a PH domain immediately N-terminal to the catalytic Arf GAP domain. PH domains are thought to regulate enzymes by binding to specific phosphoinositide lipids in membranes, thereby recruiting the enzyme to a site of action. Here, we have examined the functional relationship between the PH and Arf GAP domains. We found that GAP activity requires the cognate PH domain of ASAP1, leading us to hypothesize that the Arf GAP and PH domains directly interact to form the substrate binding site. This hypothesis was supported by the combined results of protection and hydrodynamic studies. We then examined the role of the PH domain in the regulation of Arf GAP activity. The results of saturation kinetics, limited proteolysis, FRET and fluorescence spectrometry support a model in which regulation of the GAP activity of ASAP1 involves a conformational change coincident with recruitment to a membrane surface, and a second conformational change following the specific binding of phosphatidylinositol 4,5-bisphosphate.

Keywords: ADP-ribosylation factor; PH domains; GTPase-activating proteins

1. Introduction

Pleckstrin homology (PH) domains are common to many signalling and membrane trafficking proteins (reviewed in [1–6]). This protein motif is comprised of approximately 100 amino acids and, although the primary sequences of different PH domains often have low levels of identity, the protein fold is conserved. PH domains form a seven-stranded β sandwich capped by an α-helix. Basic residues on several loops cluster into a patch of positive charge, which mediates binding through electrostatic interactions to both lipids and proteins. The ability to bind specific components of membranes is central to the proposed functions of PH domains.

One function that has been extensively examined is site-specific targeting through binding of low abundance

Abbreviations: DH, dbl homology; FRET, fluorescence resonance energy transfer; GAP, GTPase-activating protein; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); NHS, *N*-hydroxysuccinimide; PA, phosphatidic acid; PdZA, a chimeric protein consisting of the PH domain of PLC δ1 and the Arf GAP and ANK repeat domains of ASAP1; PH, pleckstrin homology; PI, phosphatidylinositol; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PI(3,4,5)P3, phosphatidylinositol 3,4,5-triphosphate; PLC, phospholipase C; PS, phosphatidylserine; PZA, protein comprised of the PH, Arf GAP and ANK repeat domains of ASAP1; PZA2, protein comprised of PH, Arf GAP and ANK repeat domains of ASAP2/PAP; ZA, protein comprised of Arf GAP (which contains zinc binding motif) and ANK repeats of ASAP1.

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membrane components derived from the phosphorylation of phosphatidylinositol (PI) [1,3,5]. Kinases that catalyze the production of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2), phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2) and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3) are regulated. Production of a particular phosphoinositide within a membrane compartment results in the recruitment of proteins containing PH domains specific for that phosphoinositide [4,7–14]. Localization dependent on PH domain interaction with specific proteins has also been studied [15–17]. For instance, the PH domain of PLC β2 and PLC β3 binds Rac•GTP [18] and the PH domain of four phosphate adaptor protein (FAPP) binds Arf1•GTP [17]. Coincident binding of lipids and proteins may increase the spatial and temporal specificity of targeting.

Studies of guanine nucleotide exchange factors for Rho-family proteins support the idea that PH domains have roles in addition to a targeting function [19–21]. Rho-family GTP-binding proteins [22–24] are members of the Ras superfamily that regulate actin cytoskeleton remodeling and cell growth. The function of Rho proteins depends on the controlled binding of GTP [25–27], which, in turn, is catalyzed by guanine nucleotide exchange factors. Those exchange factors specific for Rho-family proteins contain a catalytic dbl-homology (DH) domain and, invariably, a PH domain occurs C-terminal to it [19,20]. Crystallographically determined structures reveal that the PH domain folds together with the DH domain and, further, that the PH domain contributes residues that interact with the substrate Rho family protein [20], so it can be considered to form part of the substrate binding site.

AZAP family Arf GAPs are another example of proteins containing PH domains that may function in a capacity other than site-specific targeting. The substrates of Arf GAPs are Arfs, which, like Rho proteins, are members of the Ras superfamily of GTP binding proteins [28–31]. Arfs regulate membrane traffic and also contribute to actin remodeling [32–34]. As for other Ras family proteins, the function of Arfs is dependent on the controlled binding and hydrolysis of GTP. Because Arfs have no detectable intrinsic GTPase activity, GTPase-activating proteins (GAPs) are critical to their function. The first Arf GAP identified, Arf GAP1, was found to contain a catalytic domain comprised of a zinc finger motif [35,36] and subsequently, 24 genes have been found to encode the Arf GAP domain [37,38]. Of these, the products of 6 gene subtypes have been found to have GAP activity. Two subtypes, Arf GAP1/3 and Git1/2, have the catalytic domain at the N-terminus of the proteins; four subtypes (ASAPs, ACAPs, AGAPs and ARAPs), collectively called AZAPs, have a PH domain immediately N-terminal to the Arf GAP domain. The PH domain of AZAP proteins has a critical role in regulating Arf GAP activity. The most extensively studied, in this regard, is ASAP1, which was first identified as a PI(4,5)P2-dependent Arf GAP. Efficient stimulation of GAP activity was found to require PI(4,5)P2 in the context of an acidic lipid environment that could include phosphatidylserine or phosphatidic acid [39,40]. A combination of PA and PI(4,5)P2 activates ASAP1 10,000-fold [40].

The PH domain of ASAP1 was also found to be necessary for the protein to have any GAP activity, independent of the presence of phosphoinositides. Truncation of the PH domain did not appear to affect the folding of the Arf GAP domain. ASAP1 truncation mutants were soluble and did not have aberrant chromatographic behavior. Furthermore, the Arf GAP and ANK repeats of an ASAP1 homolog, ASAP2/PAP, were found to be structurally similar to Arf GAP1 in crystallographic analysis [41]. However, ASAP1 and PAP mutants lacking the PH domain had less than 10^{-5} the activity of protein with the PH domain [40]. The activities of other AZAPs, including ACAP1, ARAP1 and AGAP1, are also highly dependent on the presence of the PH domain immediately N-terminal to the Arf GAP domain. These results have led us to consider that the PH domain of ASAP1 may interact with the Arf GAP domain to form the catalytic interface with the substrate Arf1•GTP, in a manner similar to that described for Rho GEFs [20]. With the PH and Arf GAP domains closely apposed, we also propose that conformational changes in the PH domain that occur on binding the ligand [42] could be directly transmitted to the Arf GAP domain.

Here we test the hypotheses that (i) an interface between the PH and Arf GAP domains contributes to formation of the substrate binding site, and that (ii) PIP2 binding to the PH domain of ASAP1 induces a conformational change resulting in stimulation of GAP activity. We tested for an interface using protection assays, modeling and hydrodynamic analysis. Conformational changes were detected by changes in protease sensitivity, FRET and fluorescence spectrometry. The results are consistent with a model in which ASAP1 is recruited to a surface with a coincident conformational change. On specifically binding PIP2, ASAP1 then undergoes an additional conformational change that results in maximum stimulation of GAP activity.

2. Experimental procedures

2.1. Plasmids

Bacterial expression vectors for Arf1 [43], [L8K]Arf1 [44], [338–431]ASAP1 (PH), [325–724]ASAP1 (PZA) and [452–724]ASAP1 (ZA) [40] have been previously described. An expression vector for a chimeric protein consisting of residues 1 to 134 of PLC δ1 [45] and residues 441 to 724 of ASAP1 was constructed by amplifying the reading frame encoding residues 441 to 724 of ASAP1 with *Nde*I and *Not*I restriction sites on the 5′ end of the ORF and

*Xho*I site at 3′ end. This DNA was ligated into pET19 using the *Nde*I and *Xho*I sites. The reading frame encoding residues 1 to 134 of PLC δ1 was amplified incorporating *Nde*I and *Not*I sites. After restriction digestion, this amplified DNA was ligated into the first construct using the *Nde*I and *Not*I sites. The protein expression product is called PdZA. Bacterial expression vectors for his10 tagged [367–724]ASAP1, his10 [381–724]ASAP1 and his10 [394–724]ASAP1 were constructed using pET19 and standard methods. Cameleon [278–694]ASAP2/PAPα (called cameleon PZA2 in the text) was constructed by inserting the appropriate reading frame into the SphI and SacI sites of yellow cameleon in a bacterial expression plasmid pRSET [46]. Point mutations in [325–724]ASAP1, including cysteine 388 to serine, valine 390 to glutamate, lysine 391 to glutamate and glutamate 395 to lysine, were introduced into DNA using Stratagene's Quikchange kit (Stratagene, LaJolla, CA). [K348N, 325–724]ASAP1, [K355N, 325–724]ASAP1, [R360Q, 325–724]ASAP1 and [K365N,325–724]ASAP1 have been previously described [40].

2.2. Proteins

Arf1, [L8K]Arf1, [338–431]ASAP1 (PH), [325–724]ASAP1 (PZA), point mutants of [325–724]ASAP1, [278–694]ASAP2/PAPα and the chimeric protein comprised of the PH domain of PLC δ1 ([1–134]PLC δ1) and [441–724]ASAP1 (chimera called PdZA) were expressed and purified from bacteria as described [43,47,48]. Cameleon [278–694]ASAP2/PAPα (called cameleon PZA2 in the text) was purified using the Talon® kit from Clontech (BD Biosciences, Palo Alto, CA).

2.3. Lipids and lipid derivatives

Egg phosphatidylcholine, phosphatidic acid derived from egg PC, and bovine brain PI(4,5)P2 were from Avanti Polar Lipids (Alabaster, AL). Distearoyl phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2), distearoyl phosphatidylinositol 3,4,5-trisphosphate (PIP3), dibutanoyl PI(3,5)P2, dibutanoyl PI(4,5)P2 and inositol 1,4,5-trisphosphate were from Echelon Biosciences Incorporated (Salt Lake City). Triton X-100 was from Sigma-Aldrich (St. Louis, MO).

2.4. Limited proteolysis

Limited proteolysis was performed by a modification of the previously described method [40]. Eight micrograms of [325–724]ASAP1 were incubated with 200 ng trypsin in 20 mM HEPES, pH 8.0, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM GTP and 0.1% Triton X-100 with either phosphatidic acid and/or phosphatidylinositol 4,5-bisphosphate (PIP2) or phosphatidylinositol 3,5-trisphosphate (PI(3,5)P2), as indicated, in a total volume of 75 μl at 30 °C. At the indicated times, 10 μl aliquots of the reaction were quenched with trypsin inhibitor from egg. The reaction products were further diluted for use in the GAP assay. For analysis of the proteolysis products by SDS-PAGE and N-terminal sequencing, all proteins were used at 4-fold higher concentrations.

2.5. N-terminal sequencing

Edman degradation was performed on protein fragments fractionated by SDS-PAGE and transferred to PVDF membranes using an Applied Biosystem Model Procise protein sequencer (Foster City, CA) following the manufacturers protocol.

2.6. Protection studies and peptide mapping

Trypsin digestion of [325–724]ASAP1 (PZA), [338–431]ASAP1 (PH) and [452–724]ASAP1 (ZA) treated with 20 to 250 μM biotin *N*-hydroxysuccinimide ester (NHS-biotin) was performed as previously described [49]. A Micromass QTF Ultima Global (Micromass, Manchester, UK) in electrospray mode, interfaced with an Agilent HP1100 CapLC (Agilent Technologies, Palo Alto, CA) was used for LC-MS/MS analysis. Six microliters of each digest was loaded onto a Vydec C18 MS column (100×0.15 mm: Grace Vydec, Hesperia, CA) and chromatographic separation was performed at 1 μl/min using the following gradient: 0–10% B over 5 min; 10–40% B over 60 min; 40–95% B over 5 min; 95% B held for 5 min (solvent A: 0.2% HCOOH in H_2O; solvent B: 0.2% HCOOH in acetonitrile). The three most abundant ions (≥threshold of 10 counts/s) were selected for CID fragmentation (*m*/*z* 50–1990). MS/MS data were processed by MassLynx (version 4.0) and subsequent search was performed using an in-house licensed Mascot Daemon (http:/biospec.nih.gov) to identify biotinylated lysyl-containing peptides from the NCBI nr database. Manual inspection of MS/MS spectra was also necessary. Once the location of biotinylation had been determined, a similar LC-MS experiment was carried out for quantitative analysis by including a synthesized tryptic peptide (500 fmol) as an internal standard (ALDATGNGLISSADLR, +2 charge at *m*/*z* 787.4). Quantitation of biotinylated peptide peaks was performed using extracted ion chromatograms for ions of interest (with a mass window of ±0.05 Da) under which the areas were integrated. The areas were normalized to the internal standard. This ratio, expressed as relative abundance, was plotted against increasing concentrations of NHS-biotin.

2.7. Modeling

A homology model of the PH domain of ASAP1 was constructed using the PH domain of the G protein-coupled receptor kinase 2 (beta-adrenergic receptor kinase 1, pdb

code 1BAK [50] as reported previously [40]. A homology model of the Arf-GAP and ankyrin repeats of ASAP1 was constructed with the Modeler module of InsightII (Accelrys, San Diego CA) using the X-ray structure of PYK2-associated protein beta (PAPβ, also called ASAP2, pdb code 1DCQ [41]). The PH domain was docked interactively at the PH domain/ArfGAP ankyrin repeat interface such that the protected surface lysines were at or near the interface. The linker between the PH and ArfGAP domains was constructed using the Homology module of InsightII. Energy minimization, 200 ps of dynamics (GB/SA implicit solvent, 20Å cut-off, 300K), and reminimization were performed using MacroModel (Schoedinger, Portland OR), to relax and insure stability of the docked complex. ArfGAP and ankyrin repeat residues >12Å from the interface were held fixed. For comparison with the hydrodynamic experiments, the his-tag sequence added in the cloning process was appended to the complex with random backbone angles; the his-tag was also added to the PH and the ArfGAP ankyrin repeat homology model structures. An 'extended' PH/ArfGAP/ankyrin repeat complex was also constructed for comparison with the hydrodynamic experiments by moving the PH domain away from the ArfGAP/ankyrin repeats and constructing an extended conformation linker between them.

2.8. Hydrodynamics

Sedimentation velocity analytical ultracentrifugation experiments were conducted with an Optima XLI/A (Beckman Coulter, Fullerton, CA). 400 μl of protein samples at 0.3 mg/ml, dialyzed against PBS were filled in standard double-sector Epon centerpieces equipped with sapphire windows, and inserted in an An60 Ti four-hole rotor. Interference optical fringe profiles were recorded at a rotor speed of 59,000 rpm at 23 °C. Data were analyzed with the software SEDFIT (www.analyticalultracentrifugation.com) using the c(s) method of distributions of Lamm equation solutions [51]. A single major peak was observed for the monomeric proteins, which was integrated and transformed to an $s_{20,w}$ value. The protein partial-specific volume was predicted from amino acid composition, and buffer corrections were applied with the software SEDNTERP (kindly provided by Dr. John Philo). Theoretical $s_{20,w}$ values for each protein and for the different theoretical conformations were calculated by bead modeling from atomic coordinates [52] using the program HYDROPRO (kindly provided by Dr. José García de la Torre).

2.9. FRET

Fluorescence resonance energy transfer (FRET) was measured using a Jobin Yvon Horiba FluorMax-3. Cameleon [278–694]ASAP2/PAPα (cameleon PZA2), at 60 nM, in phosphate buffered saline with 0.1% Triton X-100 and the indicated phospholipids, was excited with 432 nm light and an emission spectrum from 440 to 580 nm was determined. The excitation slit was 2 nm. The emission slit was 5 nm.

2.10. Miscellaneous

Lipid binding was performed as described [40] using vesicles comprised of phosphatidylcholine (PC) and the indicated concentrations of phosphatidic acid (PA) and PI(4,5)P2 or PI(3,5)P2. Total lipid concentration in the assay was 1 mM. GAP assays were performed as previously described [48]. For saturation kinetics, time courses for GTP hydrolysis at the indicated Arf1•GTP concentrations were determined from which initial hydrolysis rates were estimated. An Arf mutant, [L8K]Arf1, was used as a substrate because it loads efficiently with GTP and the k_{cat}/K_m is otherwise indistinguishable from wild type Arf1 when using PZA as an enzyme[1] [44]. Tryptophan fluorescence spectra were obtained using a Jobin Yvon Horiba Fluoromax 3 fluorescence spectrophotometer. The excitation wavelength was 280 nm and emission spectra from 310 nm–450 nm were collected. Protein concentrations were estimated using the BioRad assay (BioRad, Hercules, CA). Curve fitting and statistics were performed with GraphPad Prism (GraphPad Software, San Diego).

3. Results

We have proposed that the PH domain of ASAP1 has functions other than or in addition to being a targeting domain [40]. We began the current studies by determining if the cognate PH domain was, in fact, necessary for ASAP1 GAP activity. Previously, [325–724]ASAP1 (we call this protein PZA, see Fig. 1A for schematic of proteins) was found to contain GAP activity, whereas a protein comprised of the Arf GAP and ANK repeat domains of ASAP1 ([452–724] ASAP1, called ZA) was found to lack Arf GAP activity (less than 1/100,000 of the activity of PZA) (Fig. 1C and [40]). In the work described here, the PH domain of ASAP1 was exchanged for the PH domain of PLC δ1 [53–55,45] resulting in a chimeric protein we call PdZA (Fig. 1A). This protein was soluble and, as anticipated, efficiently bound to lipid vesicles containing phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) or phosphatidic acid (PA) and PI(4,5)P2 (Fig. 1B), conditions in which maximum enzymatic activity of PZA is observed [40]. If the function of the PH domain were simply to recruit ASAP1's catalytic domain to the

[1] We have only tested [L8K]Arf1 as a substrate for [325–724]ASAP1, [278–694]ASAP2, AGAP1 and Arf GAP1. The properties described may not extrapolate to other proteins with Arf GAP activity.

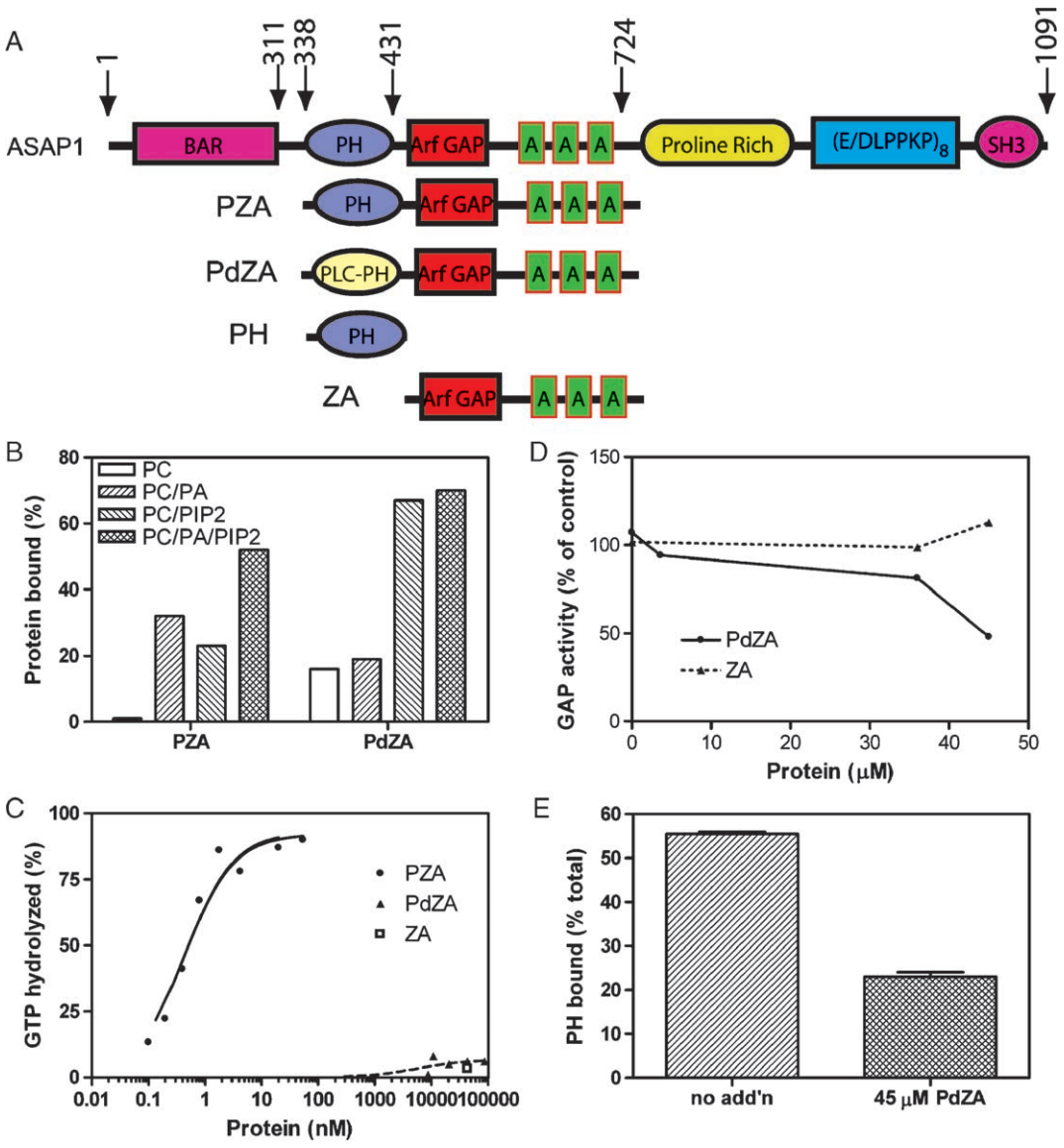


Fig. 1. Activity of chimeric PLCδ1/ASAP1. A. Schematic of proteins used. B. Protein binding to phospholipids vesicles. PZA and PdZA, at 1.5 mg/ml, were incubated with sucrose-loaded vesicles of four compositions: (i) 1 mM PC; (ii) 700 μM PC/300 μM PA; (iii) 1 mM PC/50 μM PI(4,5)P2; or (iv) 700 μM PC/300 μM PA/50 μM PI(4,5)P2. Protein bound to lipid was rapidly separated from bulk solution by centrifugation. Proteins were separated by SDS-PAGE, visualized by Coomassie blue staining and quantified by densitometric scanning. C. Relative activity of wild type and chimeric protein. Arf1 was loaded with [α^{32}P] GTP and used in GAP assay to measure GTP hydrolysis as described under "Experimental Procedures." PZA, PdZA and ZA were titrated into the assay. D. Effect of inactive GAP proteins on GAP activity of ASAP1. ZA and PdZA, at the indicated concentrations, were added to a GAP assay containing 0.5 nM PZA. E. Effect of PdZA chimeric protein on PH domain binding to lipids. PdZA, 45 μM, was included in assays determining PH binding to PC/PA/PIP2 vesicles.

membrane surface, PdZA would be expected to have activity in the presence of PA and PI(4,5)P2. However, based on titrations, PdZA had less than 1/100,000 the activity of wild type ASAP1 (Fig. 1C). Thus, recruitment of the Arf GAP domain is not sufficient to stimulate Arf GAP activity.

We considered that the PH domain might contribute directly to binding the substrate Arf1•GTP, similar to the role of the PH domain in some DH-PH proteins [19,20]. To test this possibility, we determined if the inactive ASAP1 proteins inhibit an active GAP. If inhibition were observed, it would presumably be due to sequestering Arf1•GTP. In fact, ZA did not inhibit activity at concentrations up to 45 μM (Fig. 1D). PdZA inhibited activity by 50% at a concentration of 45 μM (Fig. 1D); however, the inhibition had a PdZA concentration dependence that was different than expected for sequestration. We considered that the inhibition could be a consequence of PdZA sterically blocking access of PZA to the vesicle surface. To examine this possibility, we determined the effect of PdZA on binding of the isolated ASAP1 PH domain ([338–431] ASAP1, called PH) to

vesicles. At 45 μM, PdZA inhibited binding of PH by 50% (Fig. 1E), fully accounting for the inhibition of GAP activity that we had observed. Taken together, these results support the conclusion that the Arf GAP domain requires association with its cognate PH domain to bind Arf1•GTP.

Given the requirement for the cognate PH domain for GAP activity, a reasonable hypothesis is that the two domains are closely apposed or integrally folded. To test for the presence of a structural interface between the PH and Arf GAP domains that would occur in this case, we treated PH, ZA and PZA with NHS-biotin, a reagent of some bulk that is known to covalently modify any exposed lysine amino group. Proteolysis provided a series of peptides and tandem mass spectrometry (MS/MS) provided their location. Eight lysines were found to be less biotinoylated in PZA than in PH or ZA (see Table 1). Seven of these were part of ZA with the greatest protection found for lysines 513, 555, 570 and 711. Using the previously determined homology model structure for the PH domain of ASAP1 [40], along with a homology model for ZA (the Arf-GAP/ANK repeat domains of ASAP1, see Methods, Fig. 2A), we found these confined to one face of the predicted ZA structure, defining a possible interface between the two domains. To examine this further and to ensure that the positioning of the PH domain at this interface was geometrically feasible given the length of the inter-domain linker, a model with the PH domain docked at this interface was constructed (Fig. 2B). In the model structure, lysines 513, 555, 570, and 711 are protected to the greatest extent, consistent with the data.

To examine the function of the putative interface, we introduced mutations in the expression vector for PZA that would result in changes of amino acids thought to contribute to stabilizing the interface. Proteins completely lacking the interface, including the isolated PH domain, ZA and the chimeric PdZA, were soluble. PZA with cysteine 388 changed to serine was soluble and indistinguishable from wild type protein in GAP activity (not shown). However, disrupting other residues, in particular reversing their hydrophobicity, within the putative interface, such as changing valine 390 to alanine or glutamate, lysine 391 to alanine or glutamate, glutamate 395 to alanine or lysine, and lysine 397 to alanine or glutamate, resulted in insoluble protein. ASAP1 mutants with partial deletion of the PH domain ([367–724]ASAP1, [381–724]ASAP1 and [394–724]ASAP1) were also insoluble. These results are taken as evidence for the interface; misfolding occurred if the interface was partially disrupted.

Table 1
Relative biotinoylation of lysines in ASAP1 as determined by mass spectrometry

Residue	PZA	PH	ZA
K348	++	++	NA
K349	–	++	NA
K355	++	++	NA
K382	++	++	NA
K398	++	++	NA
K513	–	NA	++
K555	–	NA	++
K570	–	NA	++
K578	+	NA	++
K587	++	NA	++
K693	+	NA	++
K696	+	NA	++
K711	–	NA	++

The indicated proteins were biotinylated and the products analyzed as described in the text. –, +, ++ indicate relative levels of biotinylation, going from least to greatest. NA, not applicable because residues are not present in the indicated structure.

Hydrodynamic properties of PZA were determined as a test of the homology model. Using hydrodynamic bead modeling, theoretical sedimentation coefficients ($s_{20,w}$) for PZA in an extended conformation (no interaction between PH and Arf GAP domains) or a closed conformation (proposed structure based on protection studies and homology modeling) could be calculated and compared to the experimentally determined value. We validated this approach using PH and ZA. For both, the calculated s-values were within 4% of those determined experimentally by sedimentation velocity analytical ultracentrifugation (Table 2); this is within the typical error of hydrodynamic modeling [52]. In comparison, the theoretical s-values for the closed or extended model differ by 20%, far exceeding the error of the hydrodynamic modeling. For PZA, the experimentally determined sedimentation coefficient was consistent only with the most compact conformation as calculated for the closed model.

These results support the idea that the PH domain folds together with the GAP domain to form the substrate binding site of ASAP1. However, they do not address the mechanism by which PIP2 binding to the PH domain influences GAP activity. We considered two possibilities: (i) PIP2 could induce a conformational change in the catalytic structure affecting K_m and/or k_{cat} for the reaction; (ii) as described in the prevailing paradigm, PIP2 could recruit the GAP to the same surface as the substrate, which would be manifest as a change in K_m. As one step in discriminating among these possibilities we determined kinetic parameters for recombinant PZA and PZA with mutations within the PH domain, some of which affected PIP2 binding (Table 3). These proteins have been previously described [40]. The results of saturation kinetics were consistent with a change in PIP2 binding leading to a change in affinity for the substrate. For the wild type protein under optimal lipid conditions, we estimated a K_m of 5.6 μM and a k_{cat} of 31/s (Fig. 3A, Table 3). The K_m and k_{cat} of [K365N]PZA, which binds PIP2 with similar affinity as the wild type protein, were indistinguishable from those of wild type ASAP1. The K_ms for [K348N]PZA, [K355N]PZA and

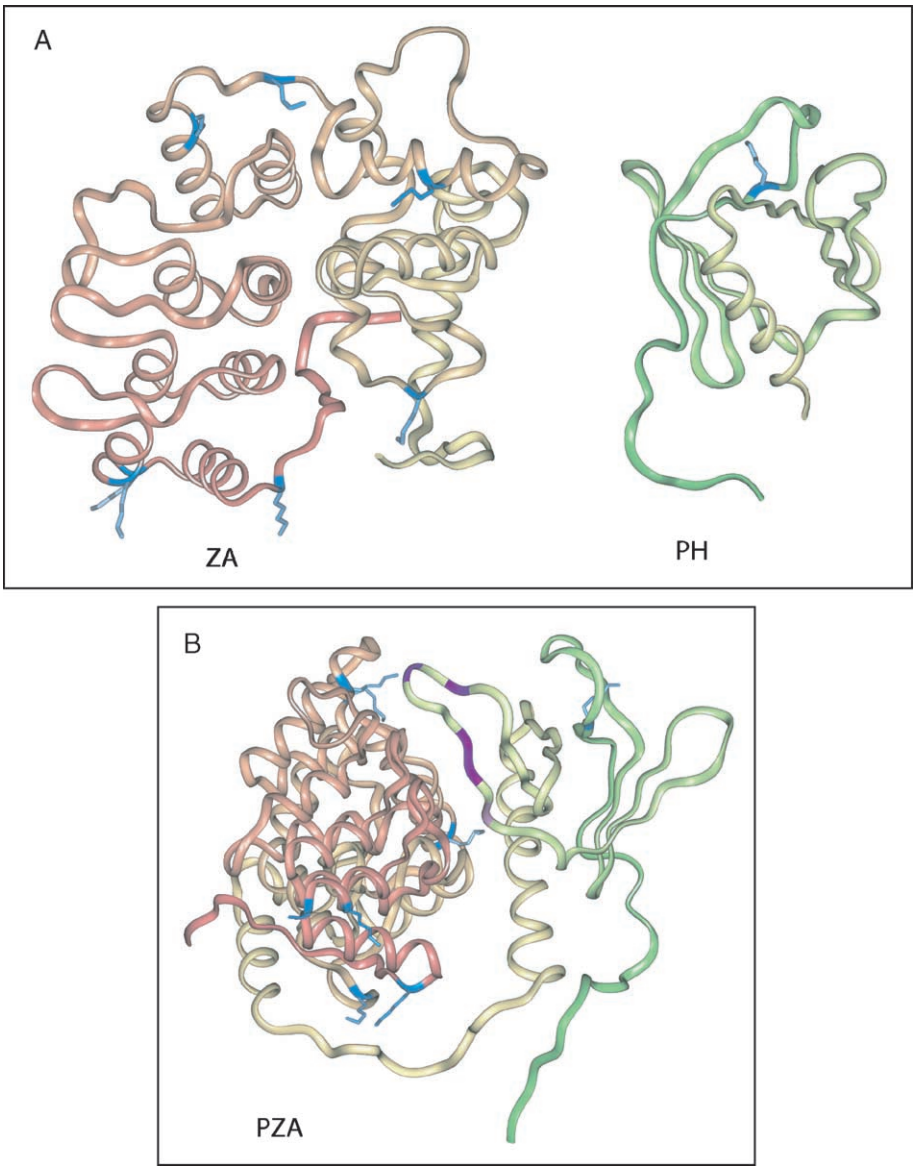


Fig. 2. Computer modeling of domain structure and proposed interface. A. Location of protected lysines on predicted structures. The Arf GAP/ANK repeats are shown on the left, the PH domain on the right. The eight protected lysines (349, 513, 555, 570, 578, 693, 696 and 711), with side chains, are shown in blue. The putative interface regions of the domains are facing the viewer. The backbone is shaded from green at the N-terminus of the PH domain to red and the C-terminus of the ankyrin repeats. B. Proposed docking of PH and Arf GAP domains. In this artist's rendition of the proposed interface, protected lysines are blue. Valine 390 and glutamate 395 are purple. Cysteine 388 is pink.

[R360Q]PZA, all of which have a lower affinity for PIP2 than wild type PZA, were greater than 10 μM; we could not determine values for either K_m or k_{cat} because saturation was not achieved. We also determined $K_{d,Arf1•GTP\gamma S}$, the affinity of the enzyme for the substrate, by using Arf1•GTPγS as a competitive inhibitor of Arf GAP activity. We observed the same trends (Table 2, Fig. 3B). Wild type PZA and [K365N]PZA, with similar affinities for PIP2, also had similar affinities for Arf1•GTPγS. [K355N]PZA had a lower affinity for PIP2 and for Arf1•GTPγS than did wild type PZA and [R360Q]PZA, which had the lowest affinity for PIP2 among the proteins tested, had the lowest affinity for Arf1•GTPγS. We also found that $K_{d,Arf1•GTP\gamma S}$ for wild type PZA increased with decreasing PIP2 concentration (Fig. 3C, Table 3). These data clearly indicate PIP2 binding affected affinity of GAP for Arf1•GTP. However, this result is consistent with either induction of a conformational change or recruitment of the GAP. For this reason, we sought other approaches to distinguish between these possible mechanisms (Table 4).

We used several strategies to probe for PIP2-induced conformational changes in ASAP1. In one approach, we examined rates of proteolytic inactivation of PZA (Fig. 4A). Catalytic activity of PZA that had been treated with low concentrations of trypsin was determined. We found

Table 2
Hydrodynamic analysis of PH, ZA and PZA

Structure	Predicted $s_{20,w}$ (f/f_0)	Experimentally determined $s_{20,w}$ (f/f_0)
PH	1.52 *s* (1.44)	1.58 *s* (1.44)
ZA	2.88 *s* (1.31)	2.98 *s* (1.27)
PZA, open structure	2.96 *s* (1.58)	
PZA, closed structure	3.48 *s* (1.31)	3.35 *s* (1.40)

Theoretical $s_{20,w}$ and f/f_0 of PH, ZA and the two models for PZA were calculated as described in "Experimental Procedures." The hydrodynamic parameters were then determined experimentally.

that there was a change in trypsin sensitivity dependent on recruitment to a vesicle surface. PA, which is a poor activator of GAP activity but does bind to PZA (see Fig. 1B [40]), increased the rate of inactivation (Fig. 4A). Including either PI(4,5)P2 or PI(3,5)P2 in the incubation with trypsin further increased the rate of inactivation. Both lipids bind PZA but only PI(4,5)P2 efficiently stimulates GAP activity [40]. The increased rate of inactivation was consistent with a conformational change exposing a proteolytic site.

To determine the proteolysis sites exposed on binding lipid, we examined the peptide fragments generated by limited proteolysis (Fig. 4B). Analysis by SDS-PAGE identified two fragments with M_r~39 kDa that were generated more rapidly in the presence of phospholipids (Fig. 4B). Automated Edman degradation revealed that the they resulted from cleavage between arginine 407 and threonine 408 within the PH domain and between arginine 439 and glycine 440 found in the amino acids sequence linking the PH and Arf GAP domains.

Fluorescence resonance energy transfer (FRET) was used as a second approach to detect conformational changes coincident with binding to lipids. In these experiments, a recombinant protein consisting of the PH, Arf GAP and ANK repeat domains of ASAP2/PAP (we refer

Table 3
Kinetic parameters of ASAP1 mutants with altered PIP2 binding

Protein	C50 (nM)	K_m (μM)	k_{cat} (/s)	K_d (μM)	$K_{d,PIP2}$ (μM)*
PZA	0.3±0.06	4±2	29±5	5.6±0.2	49±10
[K348N]PZA	31±2.8	ND[a]	ND	ND	NB[b]
[K355N]PZA	2±0.3	ND	ND	14±1	>100[c]
[R360Q]PZA	4±0.3	ND	ND	>35[d]	NB
[K365N]PZA	0.5±0.05	3.5±2	37±4	3.4±0.3	43±13

C50, the concentration of GAP inducing 50% hydrolysis of GTP bound to Arf in 3 min, was determined by titration of the GAP. K_m and k_{cat} were determined by saturation kinetics using the enzymes at or near the C50. K_d was determined for [L8K]Arf1•GTPγS by using it as a competitive inhibitor. All reactions contained 360 μM PA and 90 μM PIP2.

[a] ND, not determined because saturation could not be achieved.

[b] NB, no binding was observed at 100 μM PIP2.

[c] Binding was linear to 100 μM PIP2.

[d] ≈30% inhibition observed at 35 μM [L8K]Arf1•GTPγS.

* Data taken from [40].

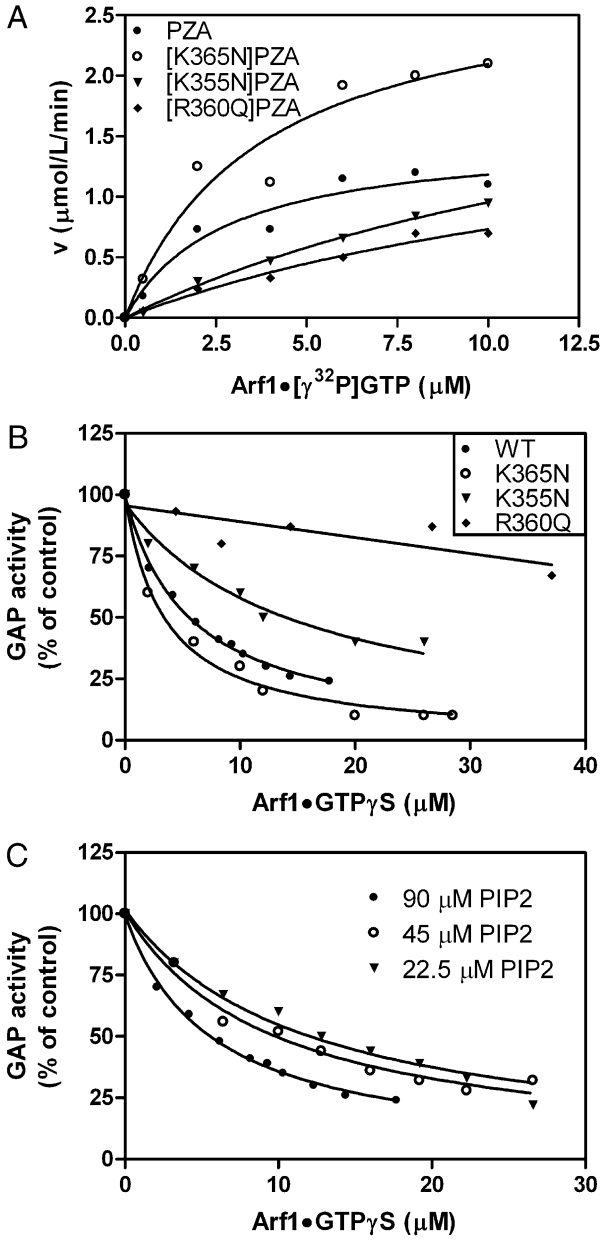


Fig. 3. Kinetic consequences of PIP2 binding to ASAP1 A. Saturation kinetics for 4 mutants. Initial reaction rates were determined using [L8K]Arf1•[γ^{32}P]GTP at the indicated concentrations. The initial rates vs. Arf concentration were fit to a hyperbolic equation. B. $K_{d,Arf1•GTP\gamma S}$ of mutants. [L8K]Arf1•GTPγS was titrated into reactions containing ≈10 nM Arf1•[α^{32}P]GTP and 0.5 nM PZA. Incubations were continued for a fixed time. C. PIP2 dependence of $K_{d,Arf1•GTP\gamma S}$. $K_{d,Arf1•GTP\gamma S}$ was determined for wild type PZA in GAP assays containing 360 μM PA and the indicated concentrations of PI(4,5)P2.

to this protein as "PZA2") fused to cyan fluorescent protein at the N-terminus and citrin at the C-terminus (cameleon PZA2, Fig. 5B) [46] was prepared. ASAP2 is similar to ASAP1 in structure (see alignment of PH and Arf GAP domains in Fig. 5A), activity and lipid binding [56]. The fused fluorescent proteins did not affect these properties related to GAP activity. Cameleon PZA2 bound to vesicles containing acid phospholipids, including PA

Table 4
Effect of PIP2 on affinity for Arf1•GTPγS

[PIP2] (μM)	K_d (μM)
22.5	12.9±2.2
45	9.6±0.8
90	5.6±0.2

Affinity for [L8K]Arf1•GTPγS was determined in a competition assay as described in "Experimental Procedures" in Triton X-100 micelles containing the 360 μM PA and the indicated concentration of PIP2.

(Fig. 5C). Specific activity of cameleon PZA2 was indistinguishable from that of PZA2 (Fig. 5D). Activity of both was stimulated by PI(4,5)P2 (Fig. 5E and F) but not PIP3 (Fig. 5E). The potency of PIP2 was increased by PA, but PA by itself had little effect on activity (Fig. 5F).

Cameleon PZA2 was used to test for a conformational change on binding phospholipids, assuming that the change would involve relative movement of the N-terminus of the PH domain and the C-terminus of the ANK repeat domain. A change in the distance between the ends could be detected as a change in resonance energy transfer (FRET) between CFP and Citrin. In Fig. 5G and H, typical fluorescence spectra are shown. The proteins were excited with light at 432 nm and emission spectra between 440 nm and 580 nm were recorded. The peak at 475 nm is due to emission from CFP. The peak at 525 nm is emission from citrin and is the consequence of FRET from CFP to citrin. We found that the effect of lipids on FRET correlated with binding to the hydrophobic surface rather than stimulation of enzymatic activity. The height of the peak at 525 nm decreased relative to the peak at 475 nm, which represents a decrease in FRET, on addition of PIP2 (compare solid black line to dotted red line in Fig. 5G) or PA (red line in Fig. 5H). PIP2 with PA (blue line in Fig. 5H) had the same effect as PA alone (red line). These results together with the protease inactivation results support the idea that the conformation of PZA changes on binding a vesicle. However, the conformational change is not sufficient for full activation, leading us to propose an additional conformational change on binding PI(4,5)P2, which results in activation.

To test this idea, we determined the effect of a water-soluble PIP2 analog on PZA GAP activity. We titrated

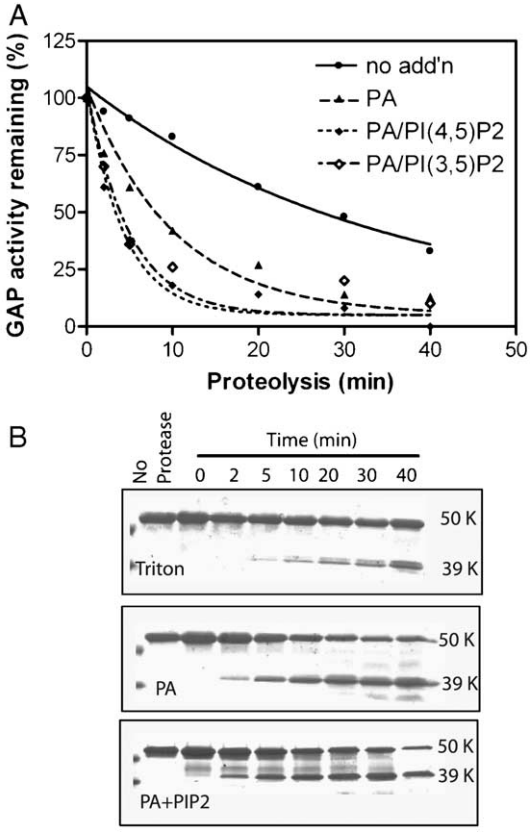


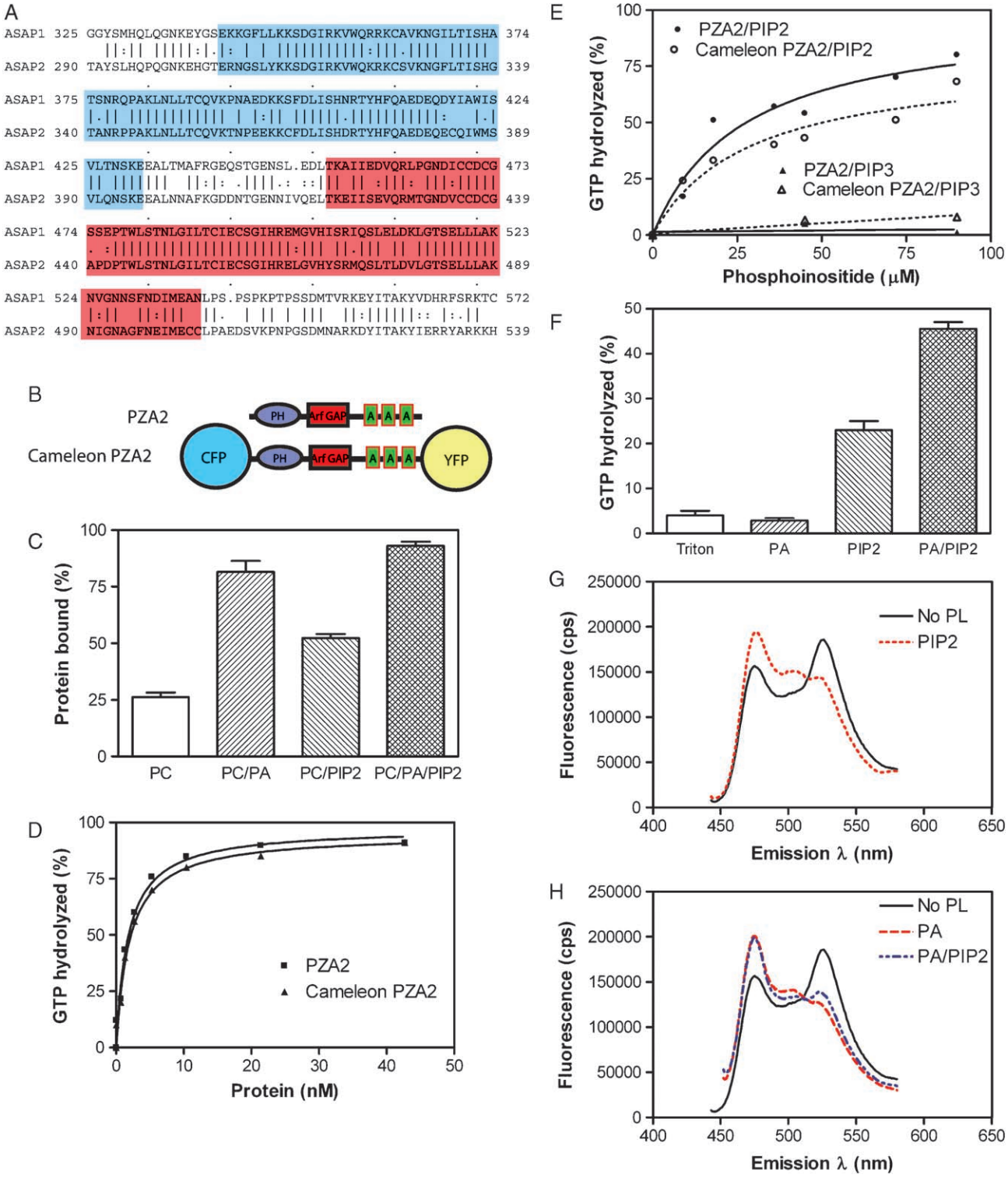
Fig. 4. Limited proteolysis as a test for lipid-dependent conformational changes in ASAP1. A. Protease sensitivity of activity. PZA (8 μg) was incubated with 50 ng of trypsin in a total volume of 75 μl at 30 °C with: (i) 0.1% Triton X-100; (ii) 360 μM PA; (iii) 360 μM PA and 90 μM PI(4,5)P2 or; (iv) 360 μM PA and 90 μM PI(3,5)P2. Ten μl aliquots were quenched with 200 ng trypsin inhibitor from egg after the indicated period of incubation. GAP activity was determined for material from each time point. B. Examination of proteolytic degradation products. Reactions were scaled up 4-fold and products resolved by SDS-PAGE on 4–20% gels.

dibutanoyl PI(4,5)P2, dibutanoyl PI(3,5)P2 or P_i into the GAP reaction (Fig. 6A). Activity of wild type PZA was stimulated 3.5-fold by 1 mM dibutanoylPI(4,5)P2. Neither dibutanoyl PI(3,5)P2 nor P_i affected activity of PZA. The effect of dibutanoyl P(4,5)P2 required an intact binding site in the PH domain (Fig. 6B). GAP activity of [360Q]PZA was not affected by this lipid analog. This result is

Fig. 5. FRET to test for conformational changes. A. Alignment of ASAP1 and ASAP2/PAPα PH and Arf GAP domains. The PH domain is highlighted blue. The Arf GAP domain is highlighted red. B. Schematic of protein used for FRET studies. A protein comprised of the PH, Arf GAP and ANK repeat domains of ASAP2/PAP is called PZA2. ASAP2/PAPα was used for the construction of a fusion protein we call cameleon-PZA2. CFP=cyan fluorescent protein; YFP=citrin, a modified yellow fluorescent protein [46]. C. Characterization of protein used for FRET studies: Binding to phospholipids vesicles. Cameleon PZA2 binding to vesicles of the indicated concentrations was determined as described in Fig. 1 and under "Experimental Procedures." D. Characterization of protein used for FRET studies: Relative activity. PZA2 or cameleon PZA2 was titrated into a GAP reaction containing 360 μM PA and 90 μM PIP2. E. Characterization of protein used for FRET studies: Lipid dependence. GAP activity in 1 nM of the indicated proteins was determined in the presence of 360 μM PA and the indicated concentration of PI(4,5)P2 or PI(3,5)P2. F. Characterization of protein used for FRET study: Effect of PA and PIP2 on cameleon PZA2 GAP activity. Activity was determined with 3 nM cameleon-PZA2 in 0.1% Triton X-100 or Triton containing 360 μM PA and 90 μM PI(4,5)P2 as indicated. G. Effect of PIP2 on FRET between fluors in cameleon-PZA2. Fluorescence spectra of cameleon PZA2 with 90 μM PIP2 was determined. H. Combined effect of PA and PIP2 on FRET. The spectrum was determined with 360 μM PA and 90 μM PIP2. The spectrum in the presence of PA is shown with a red line, in the presence of PA and PIP2 with a blue line.

consistent with induction of an activating conformational change since there is no surface on which to concentrate reactants in this case.

To further test the hypothesis that phosphoinositide binding to the PH domain induces a conformational change distinct from the change that occurs on binding a surface, we determined the effect of water-soluble PIP2 analogs on the spectral properties of the PH domain (Fig. 6C). The PH domain has 2 tryptophans. We found that water-soluble PI(4,5)P2 increased the fluorescence emission at 340 nm, consistent with a change in environment of a tryptophan and, by inference, a conformational change in



the PH domain. Water-soluble PI(3,5)P2, which does not efficiently stimulate GAP activity, did not affect tryptophan fluorescence.

4. Discussion

The PH domain is a common structural motif. In many cases, it mediates recruitment to a site of action by binding either lipid or protein [1,2,17,18]. Recently, the PH domains of DH domain proteins have been proposed to function as part of a substrate binding site [19,20]. Here, we have tested the idea that the PH domain of ASAP1 folds with the Arf GAP domain to form a substrate binding site that is regulated by lipid binding.

We first proposed that the PH domain of ASAP1, like that of DH-PH domain proteins [19], forms part of the catalytic interface with the substrate protein, based on our finding that a protein consisting of the Arf GAP and ANK repeat domains of ASAP1, but without the PH domain, completely lacked activity. Consistent with that conclusion, here we found that a protein comprised of the PH domain of PLC δ1 [45,53,55] and the Arf GAP and ANK repeat domains of ASAP1 had no GAP activity. A corollary of our proposal is that the PH and Arf GAP domains form an integrally folded structure. The results of protection, mutational analysis and hydrodynamic experiments, with computer analysis of possible structures, were consistent with this conclusion.

Having obtained evidence that the PH and Arf GAP domains were integrally folded, we considered possible consequences of PIP2 binding to the PH domain for Arf GAP activity. PIP2 binding has recently been found to induce a conformational change in the isolated PH domain of PKB [42] and previously, has been proposed to induce a conformational change in full-length PKB opening a phosphorylation site [57]. Because of the intimate association of the PH and Arf GAP domains, we considered the possibility that a similar conformational change could affect Arf GAP activity, either affecting the K_m or k_{cat} of the interaction. In the prevailing paradigm, PIP2, by binding to the PH domain, recruits ASAP1 to the surface containing Arf1•GTP, thereby increasing the relative concentrations of reactants. This mechanism would affect the apparent K_m for the GAP reaction. As a first attempt to discriminate among these possibilities, we examined the effect of PIP2 binding to ASAP1 on substrate saturation kinetics and did indeed find an effect on K_m and on $K_{d,Arf1\bullet GTP\gamma S}$. However, based on this analysis with the only identified effect being a change in affinity, we were unable to distinguish between the recruitment and conformational change models.

Using several strategies, we obtained evidence for two distinct phospholipid-induced conformational changes in ASAP1. One conformational change was related to binding the vesicle surface and was not tightly linked to changes in enzymatic activity. From this result, we inferred that there was a second conformational change associated with enzymatic activation. The finding that water soluble-analogs of PIP2 were able to stimulate GAP activity supported the conclusion that PIP2 induced an activating conformational change. In these experiments, we used a mutant of Arf1, [L8K]Arf1, that bound GTP independently of hydrophobic surfaces. All reactants were in solution and there was no lipid–water interface on which to concentrate reactants. The hypothesis that PIP2 binding induces an activating conformational change was further supported by changes in tryptophan fluorescence of the PH domain induced by the same water-soluble analog of PIP2.

We used computer modeling to consider the specific structural basis of our hypotheses. The compact model of the PZA structure presented here, based on the protection data, is consistent with the hydrodynamic experiments. This model reflects the structure in the absence of interaction with a lipid vesicle and PIP2. The location of the interface is found to greatly limit the available conformations for the linker region; it must be extended and packed tightly against the Arf GAP/ankyrin repeat domain. The trypsin digest results show increased cleavage of the linker in the presence of phospholipids, suggesting the linker is more exposed upon interaction with a hydrophobic surface. It would appear to be necessary for the PH domain to move towards the linker region to release the linker from its tightly packed, extended structure.

The N and C termini are relatively close in the compact model structure of PZA (~14 Å). In the FRET experiment, interaction with phospholipids vesicles caused the energy transfer between the termini-attached fluorophores to be reduced. If lipid interaction simply translates the PH domain nearer the linker region with no significant rotation, the termini should get even closer, disagreeing with the FRET result. Thus, a significant rotation of the PH domain relative to the compact model structure would seem to be required.

In our analysis, we found that lysine 349 within the PH domain was protected from biotinoylation in PZA. This residue could not be accommodated in the interface given geometric constraints. Furthermore, lysine 349 is within the PIP2 binding site, which is unlikely to be buried within the interface. The difference in biotinoylation may arise from a structural change in the PH domain on binding to the Arf GAP domain. This explanation seems reasonable given the model in which a PIP2-induced conformational change in the PH domain is transmitted to the ZA domain. We would therefore expect that the converse is true: the ZA domain influences the folding of the PH domain.

Regulation of ASAP1 is multifaceted. We found that the stimulation by the water-soluble PIP2 analog was much less than that observed with lipids. This result is

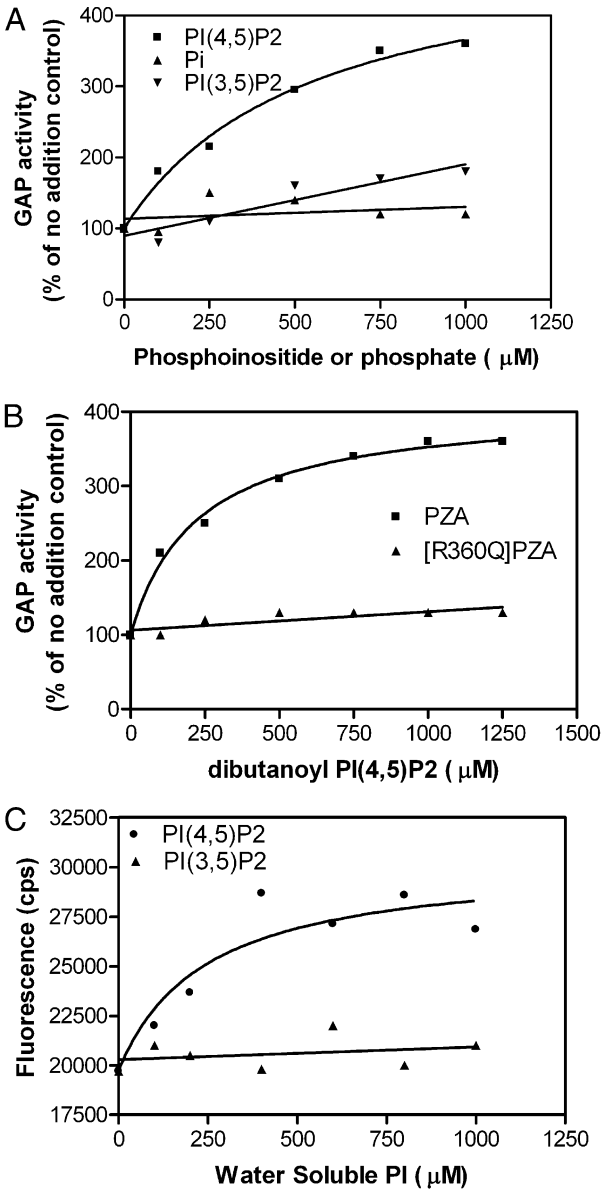


Fig. 6. Surface-independent PIP2-induced changes in ASAP1. A. Effect of water-soluble phosphoinositide analogs on GAP activity. The indicated water soluble analog of phosphoinositide or ortho-phosphate was titrated into a GAP reaction using [L8K]Arf1 as a substrate and PZA. No detergent was present. B. Effect of mutation in PH domain on water-soluble phosphoinositide-stimulated Arf GAP activity. Dibutanoyl PI(4,5)P2 was titrated into a GAP reaction using either 0.1 nM PZA or 2 nM [R360Q]PZA as an enzyme. C. Effect of water-soluble analogs of phosphoinositides on fluorescence spectra of the PH domain from ASAP1. Fluorescence spectra for PH in a buffer containing the indicated inositide was determined. 280 nm was used for excitation and emission at 340 nm was measured.

most likely attributable to three factors: 1) surface concentration does have a role in the regulation of the protein and this effect is lost when using the water-soluble analogs; 2) binding to the surface probably contributes to the total conformational change necessary for maximal activity; and 3) ASAP1 effectively has two acid lipid binding sites [40,39] which, because the ligands are restricted to a surface, function cooperatively [58]. These considerations together with the results reported here have led us to propose a model in which concentrating proteins on a surface as well as two conformational changes contribute to increasing reaction velocity. By coupling conformational change with recruitment, PIP2 binding to the PH domain could provide a high degree of regulation, accounting for the 10,000-fold stimulation of activity observed. Other regulatory mechanisms are probably also involved. For instance, phosphorylation of ASAP1 by Pyk2 and Src family kinases inhibits activity by lowering the affinity for PIP2 [59]. Recently, ASAP1 has been found to have a BAR domain that affects GAP activity (Nie et al., manuscript in preparation). Additional protein partners, including FAK [60] and Crk [61], could further contribute to temporal and spatial control of ASAP1 GAP activity.

With the PH domain forming part of the catalytic site, the interface of ASAP1 with Arf must be different than either GIT or Arf GAP1 type Arf GAPs. These Arf GAPs lack a PH domain N-terminal to the Arf GAP domain. Recent work comparing the structural requirements of Arf for interaction with three Arf GAPs revealed the N-terminus of Arf had a greater role for interacting with ASAP1 and AGAP1 than with Arf GAP1 [44]. Such differences would be anticipated if the catalytic interface differed among the Arf GAPs. The results in this paper help explain the basis of these differences. Further studies are necessary to better define variations in the Arf–Arf GAP interface and for understanding how the structural features of the Arf GAPs contribute to their specific biologic activities.

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