Interaction of the N terminus of ADP-ribosylation factor with the PH domain of the GTPase-activating protein ASAP1 requires phosphatidylinositol 4,5-bisphosphate

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Edited by Alex Toker

Arf GAP with Src homology 3 domain, ankyrin repeat, and pleckstrin homology (PH) domain 1 (ASAP1) is a multidomain GTPase-activating protein (GAP) for ADP-ribosylation factor (ARF)-type GTPases. ASAP1 affects integrin adhesions, the actin cytoskeleton, and invasion and metastasis of cancer cells. ASAP1’s cellular function depends on its highly-regulated and robust ARF GAP activity, requiring both the PH and the ARF GAP domains of ASAP1, and is modulated by phosphatidylinositol 4,5-bisphosphate (PIP2). The mechanistic basis of PIP2-stimulated GAP activity is incompletely understood. Here, we investigated whether PIP2 controls binding of the N-terminal extension of ARF1 to ASAP1’s PH domain and thereby regulates its GAP activity. Using [Δ17]ARF1, lacking the N terminus, we found that PIP2 has little effect on ASAP1’s activity. A soluble PIP2 analog, dioctanoyl-PIP2 (diC8PIP2), stimulated GAP activity on an N terminus–containing variant, [L8K]ARF1, but only marginally affected activity on [Δ17]ARF1. A peptide comprising residues 2–17 of ARF1 (2–17)ARF1 inhibited GAP activity, and PIP2-dependently bound to a protein containing the PH domain and a 17-amino acid-long interdomain linker immediately N-terminal to the first β-strand of the PH domain. Point mutations in either the linker or the C-terminal α-helix of the PH domain decreased [2–17]ARF1 binding and GAP activity. Mutations that reduced ARF1 N-terminal binding to the PH domain also reduced the effect of ASAP1 on cellular actin remodeling. Mutations in the ARF N terminus that reduced binding also reduced GAP activity. We conclude that PIP2 regulates binding of ASAP1’s PH domain to the ARF1 N terminus, which may partially regulate GAP activity.

ASAP1 is a GAP-activating protein (GAP) for ADP-ribosylation factor (Arf) GTPases (1, 2). It is composed of BAR, PH, Arf GAP, ankyrin repeat, proline-rich, E/DLPPKP repeat, and SH3 domains. In cells, ASAP1 controls integrin adhesion complexes and actin remodeling (3–7). ASAP1 has been found to affect cell behaviors dependent on adhesions and actin, including proliferation, invasion, and metastasis of cancer cells (3, 8–13). The cellular function of ASAP1 depends on its highly-regulated and robust Arf GAP activity, which refers to the enzymatic activity of converting ArfGTP to ArfGDP. The phosphoinositide PIP2 increases enzymatic power from ~10^4 M^-1 s^-1 to greater than 10^8 M^-1 s^-1 (14, 15). PIP2-dependent activity of ASAP1 requires the PH domain that is immediately N-terminal to the catalytic Arf GAP domain (16, 17).

Over 300 proteins in humans contain PH domains (18–21), which are composed of a sandwich of seven β-strands capped at one end by an α-helix. Proteins with PH domains regulate signaling, membrane trafficking, and the actin cytoskeleton (22–24). Approximately 15% of PH domains bind to phosphoinositides (25). They can also bind to small GTPases (24), such as Arf (26–31) and other PH domains, as with DOK7 (docking protein 7) to mediate homodimerization (32). They have also been reported to bind to a phosphoinositide and protein simultaneously, functioning as a coincidence detector (27).

PH domains have been found to control protein function by one of three mechanisms. First, proteins can be recruited to surfaces by

This work was supported by NCI Intramural Program Project BC-007365 from the National Institutes of Health (to R. A. B., M. D. H., M. E. Y., and P. A. R.), an Alex’s Lemonade Stand Foundation Young Investigators Award, and by the NCI Rasopathies Initiative from the National Institutes of Health (to M. E. Y.). The authors declare that they have no conflicts of interest with the contents of this article.

This article contains Figs. S1–S3.

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17354 J. Biol. Chem. (2019) 294(46) 17354–17370

The abbreviations used are: GAP, GTPase-activating protein; Arf, ADP-ribosylation factor; Arl, Arf-like GTPase; BAR, Bin/amphiphysin/RVS; CDR, circular dorsal ruffles; DH, Dbl homology; diC8PIP2, dioctanoyl PIP2; EDANS, 5-(2-aminoethyl)amino)naphthalene-1-sulfonic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; HVR, hypervariable region; HMQC, heteronuclear multiple quantum correlation; LUV, large unilamellar vesicle; MSP, membrane scaffolding protein; PA, phosphatidic acid; PDB, Protein Data Bank; PDGF, platelet-derived growth factor; PH, pleckstrin homology; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PTB, phosphotyrosine binding; TAMRA, carboxylic acid of tetramethylrhodamine; ND, nanodisc.
their PH domains binding to membrane components, including phosphoinositides and GTPases (20, 24, 26, 27, 30, 33–35). Recruitment concentrates and orients the protein on a surface that contains a target molecule. In addition, PH domains can autoinhibit or position other structural elements of a protein to inhibit intramolecular catalytic domains, as described for kinases and guanine nucleotide exchange factors (36–41). For p63RhoGAP, Goa binding to a C-terminal extension of the PH domain relieves autoinhibition (36). In the case of the Arf exchange factors, cytohesins, cooperative binding of phosphoinositide and Arf6-GTP or Arf4-GTP to the PH domain relieves PH domain–mediated autoinhibition (29, 39, 42). Finally, as is observed in some exchange factors, PH domains or extensions of the PH domain can contribute directly to binding the substrate protein (43, 44).

The PH domain of ASAP1 might contribute to GAP activity by the third mechanism, through a direct interaction with the substrate. We have found recruitment of the Arf GAP domain to a hydrophobic surface containing the substrate Arf1-GTP is not sufficient for GAP activity, and the cognate PH domain is necessary (16). We have also found that GAP activity requires a unique structural feature of Arf family GAPase, the N-terminal extension from the GTP-binding domain, which has previously been found to bind to ASAP1 (although the binding site has not been determined) (45). Furthermore, the interaction might be regulated by PIP2. PIP2 binding to the PH domain is necessary for activity (14, 15, 17, 46). These observations, together with the precedent of cytohesin, in which a phosphoinositide, phosphatidylinositol 3,4,5-trisphosphate, regulates binding of Arf6-GTP to the PH domain (29), have led us to hypothesize that, rather than mediating recruitment to a lipid bilayer, PIP2 binding to the PH domain of ASAP1 regulates binding to the N terminus of Arf1 to control GAP activity.

Results

Cognate PH domain of ASAP1 and the N terminus of Arf are necessary for PIP2-stimulated GAP activity

Previously, we found that PIP2-stimulated activity of a recombinant protein composed of the PH, Arf GAP, and ankyrin repeat domains of ASAP1, [325–724]ASAP1, referred to as PZA (for PH, Zinc binding, which comprises the Arf GAP catalytic domain, and Ankyrin repeat domains, see Fig. 1A) by more than 10,000-fold (14). In addition, we found that (i) the cognate PH domain of ASAP1 is required for activity (16); (ii) membrane recruitment is not sufficient for activity (16, 17); and (iii) Arf1 lacking its N-terminal 17 amino acids is a poor substrate (see Fig. 1A for sequence of the N terminus) (45). The N terminus of native Arf1 is myristoylated. To explore the role of the N terminus of Arf1 for PIP2-stimulated GAP activity of ASAP1, PZA was titrated into reaction mixtures containing myrArf1-GTP, nonmyrArf1, or [Δ17]Arf1-GTP and large unilamellar vesicles (LUVs) with or without incorporated phosphatidylyserine (PS), which reduces the concentration of PIP2 needed for maximum activation (14). GAP activity is reported as the C50, which is the concentration of PZA needed to induce 50% of the GTP bound to Arf1 to be hydrolyzed in 3 min and is inversely related to enzymatic power (Fig. 1B and Table 1) (47, 48). For myrArf1, as we have previously found, there was a greater than 10,000-fold difference between the C50 values measured in the absence and presence of PIP2. Results using nonmyristoylated Arf1 were similar. In contrast, the difference was 2-fold for [Δ17]Arf1. We then determined if the results extrapolated to full-length ASAP1. We were not able to achieve as high a concentration of the full-length protein as we were with PZA, which limited the titrations. Nevertheless, the data were sufficient to conclude that the PIP2 dependence of full-length ASAP1 and PZA was similar and that [Δ17]Arf1 was a poor substrate for full-length ASAP1 (Fig. 1B). Given the importance of an intact N terminus of Arf1 for PIP2-stimulated activity, we considered that a peptide derived from the N terminus of Arf1 (2–17)Arf1 might affect activity. We found that [2–17]Arf1 inhibited activity with an IC50 of 5.6 ± 0.6 μM (Fig. 1C). A peptide composed of the same amino acids but in a scrambled order ([2–17]scrambled) was more than 15-fold less efficient as an inhibitor. A peptide in which three lysines were replaced with asparagines ([K10D, K15D, K16D, 2–17]Arf1) was also an inefficient inhibitor of GAP activity. Full-length ASAP1 was similarly inhibited by [2–17]Arf1 but not [2–17]scrambled (Fig. 1C). Thus, PIP2-stimulated GAP activity required that Arf1 have an intact N terminus and was specifically inhibited by a peptide composed of the isolated Arf1 N terminus.

PIP2-dependent activity is independent of a lipid surface

The myristoylated N terminus of Arf1 mediates recruitment to surfaces (49–51) and binds to PIP2 (52, 53). Therefore, part of the difference in GAP activity against full-length myristoylated Arf1 and [Δ17]Arf1 might be due to lack of recruitment of [Δ17]Arf1 to the surface containing ASAP1. To determine whether there was a recruitment-independent component of PIP2 activation, we sought conditions that would allow us to measure activity without LUVs. When bound to GTP, full-length Arf1 is not stable without a hydrophobic surface, but we have previously identified a point mutant of Arf1, [L8K]Arf1, that is stable without a hydrophobic surface (45). [L8K]Arf1 is as efficient a substrate as WT Arf1 for ASAP1 when the PIP2 in the reaction is presented in mixed micelles of Triton X-100 (45, 54). [L8K]Arf1 is ~10-fold less efficient as a substrate than WT Arf1 when the PIP2 in the reaction is incorporated into LUVs. However, GAP activity on [L8K] Arf1, like that on WT Arf1, depends on PIP2 (Fig. 2A and Table 1). Importantly, the GAP activity of ASAP1 with [L8K]Arf1 as a substrate is inhibited by the [2–17]Arf1 peptide, with an IC50 of 0.8 ± 0.1 μM, but not by the [2–17]scrambled peptide (Fig. 2B). A second consideration for examining activity without a hydrophobic surface is the soluble PIP2 analog. In crystal structures of the ligand-bound and unliganded forms of the PH domain (14), PIP2 binds to the N terminus of Arf1 in the acyl group of PIP2 required to stabilize the loop and support GAP activity. We tested [L8K]Arf1 as a substrate with either no PIP2 or 200 μM diC4PIP2, diC6PIP2, or diC8PIP2, which all have critical micelle concentrations greater than 1 mM (55, 56). We

7 We determined the critical micelle concentration for diC8PIP2 to be >1 mM, using the method described in Ref. 57.
observed that PIP$_2$ analogs increased activity dependent on acyl length, with more than a 7,000-fold increase in activity with diC8PI$_2$ compared with no PIP$_2$ (Fig. 2C and Table 2). Titration of diC8PI$_2$ revealed a sigmoidal dependence for PIP$_2$-stimulated activity with a Hill coefficient of 1.9 ± 0.27 (S.E.) (Fig. 2D), consistent with previous studies identifying two PIP$_2$-

Figure 1. N-terminal extension of Arf1 is necessary for PIP$_2$-stimulated ASAP1 GAP activity. A, schematic of recombinant proteins used in this paper. The domain structure of ASAP1 is shown in the schematic at top. Abbreviations used are: BAR, Bin/amphiphysin/RVS; PH, pleckstrin homology; Arf GAP, Arf GTPase-activating protein; ANK, ankyrin repeat; Pro-Rich, proline-rich; (E/DLPPKP)$_8$, tandem repeats of E/DLPPKP; SH3, Src homology 3. Recombinant proteins used in the studies are shown below the schematic of full-length ASAP1. The acronyms for the proteins include “Z” for the Arf GAP domain, which is a Zinc-binding motif, and “Pd” for the PH domain of phospholipase C. Below the ASAP1-derived proteins, a representation of Arf1 and the amino acids comprising the N-terminal 2–17 residues, the same amino acids in a scrambled sequence, and the sequence with lysines changed to aspartates are shown. B, comparison of PIP$_2$-stimulated GAP activity of ASAP1 using full-length Arf1 and [Δ17]Arf1 as substrates. PZA (left panel) or full-length ASAP1 (right panel) was titrated into a GAP reaction containing 0.1 μM full-length Arf1 or [Δ17]Arf1 and LUVs at a total phospholipid concentration of 500 μM containing 15% PS, 5% PIP$_2$, or without PS or PIP$_2$, as indicated. The summary of three experiments is shown for PZA and two experiments for full-length ASAP1. Error bars are S.E. C, inhibition of Arf GAP activity by an N-terminal peptide of Arf1. A peptide comprising amino acids 2–17 of Arf1 ([2–17]Arf1), a peptide of the same amino acid composition but with a scrambled sequence ([2–17]scrambled), or [2–17]Arf1 with the indicated amino acid changes (see A for sequences) were titrated into a mixture containing 0.1 μM myrArf1, LUVs at a total phospholipid concentration of 500 μM containing 15% PS, 5% PIP$_2$, and either PZA (0.3 nM) (left panel) or full-length ASAP1 (1.4 nM) (right panel) sufficient to induce ~50% of the GTP bound to Arf to be hydrolyzed in a fixed time used for the assay in the absence of peptide. Activity in the absence of peptide for PZA was 0.4 and 0.44 min$^{-1}$ for full-length ASAP1. The activity in the absence of peptide was taken to be maximum GAP activity (100%). The summary of three experiments is shown for PZA and two for full-length ASAP1. Error bars are S.E.
PIP₂ regulates Arf1 binding to the ASAP1 PH domain

The N terminus of Arf1 and the PH domain of ASAP1 are critical for PIP₂-stimulated activity by a mechanism that may not only depend on recruitment to a surface. We hypothesized that GAP activity is controlled by PIP₂-dependent binding of the N terminus of Arf1 to the PH domain of ASAP1 (schematic of the hypothesis is shown in Fig. 3). As a test of the hypothesis, we used three complementary assays (Figs. 4–6) to determine whether there is direct association between the ASAP1 PH domain and [2–17]Arf1. First, we measured interaction by Förster resonance energy transfer (FRET, see Fig. 4A for schematic of assay). The fluorophore 5’-(2-amino)naphthalene-1-sulfonic acid (EDANS, peak excitation wavelength 336 nm, emission peak 490 nm) was covalently linked to the C terminus of [2–17]Arf1. EDANS is an acceptor for FRET from tryptophan, which absorbs light at 280 nm and emits at a peak of ~340 nm. PH₁₈₅₅ contains two tryptophans (Trp-357 and Trp-422, see Fig. 4B for structure of PH domain). Given the dimensions of the PH domain, either tryptophan could act as a donor for EDANS if the modified peptide bound to the surface of the PH domain. PH₁₈₅₅ was incubated with 200 μM diC₈PIP₂ and the indicated concentrations of [2–17]Arf₁-EDANS. The solution was excited with 280 nm light, and an emission spectrum was measured. Representative spectra are shown in Fig. 4C, and a summary of three experiments with calculated FRET efficiencies is shown in Fig. 4D. In the absence of [2–17]Arf₁-EDANS, a single peak is observed with a maximum of ~340 nm (Fig. 4C), as expected for fluorescence emission from tryptophan. Addition of [2–17]Arf1-EDANS induced a concentration-dependent decrease in emission at 340 nm and a concomitant increase in emission at 490 nm, indicating FRET. Titrating peptide, we used quenching of tryptophan fluorescence to quantify FRET. We found the FRET signal was saturable with a half-maximum effect at 2.3 ± 0.14 μM [2–17]Arf₁-EDANS (Fig. 4D), similar to the concentration dependence observed for inhibition of GAP activity. Similar results were obtained with EDANS linked to the N terminus of [2–17]Arf1 (not shown). No FRET signal was observed with [2–17]scrambled EDANS (Fig. 4D). One would predict that [2–17]Arf1 without EDANS would compete with [2–17]Arf₁-EDANS for binding to PH₁₈₅₅, reducing FRET. [2–17]Arf1 or [2–17]scrambled without fluorophore were titrated into a reaction containing 1 μM PH₁₈₅₅, 4 μM [2–17]Arf₁-EDANS, and 200 μM diC₈PIP₂ (Fig. 4E). We found [2–17]Arf₁, but not [2–17]scrambled, competed with [2–17]Arf₁-EDANS to reduce FRET. Like the stimulation in GAP activity, binding, as determined by FRET, depended on diC₈PIP₂ (Fig. 4F) with a Hill coefficient of 1.4 ± 0.15. [R₃₆₀Q]PZA, a mutant with reduced affinity for PIP₂, had a lower affinity for [2–17]Arf₁-EDANS than PZA (Fig. 4G). For experiments with full-length ASAP1, quenching of tryptophan fluorescence was not a sensitive measure of FRET because there are six tryptophans in ASAP1 outside of the PH domain, the putative binding site for the peptide. Therefore, we titrated ASAP1 into the reaction and measured FRET as an increase in emission from the acceptor, EDANS, at 493 nm. FRET was observed when titrating full-length ASAP1 into a solution containing [2–17]Arf₁-EDANS but not with [2–17]scrambled-EDANS (Fig. 4H). FRET was not observed between PH₁₈₅₅ and [2–17]Arf₁-EDANS (Fig. 4D). Taken together, these data are interpreted as a specific PIP₂-dependent association between PH₁₈₅₅ and [2–17]Arf1.

Fluorescence anisotropy (59) was used as a complementary assay to detect binding between [2–17]Arf1 and PH₁₈₅₅. The principle of the assay is shown in Fig. 5A. A molecule with a fluorophore is incubated with a binding partner. The sample is excited with plane polarized light, and emission is measured along axes parallel and perpendicular to the plane of excitation. If the fluorophore does not tumble between the time of excitation and emission, emission is polarized with a consequent difference in emission intensity between the perpendicular planes,

binding sites on the ASAP1 PH domain (14) and at least one on Arf1 (52, 53, 58) that are necessary to form the active complex. Eight We also observed binding of diC₈PIP₂ to [2–17]Arf1 by circular dichroism spectroscopy (not shown).

The differences between GAP activity in LUVs and with PIP₂ analogs are described in more detail in a manuscript currently in preparation (N. S. Roy, X. Jian, R. Luo, P. A. Randazzo, and M. E. Yohe).
or anisotropy. Random tumbling of the fluorophore between the time of excitation and emission reduces anisotropy, while binding of a small fluorophore-containing molecule to a larger molecule would slow rotation, increasing anisotropy. [2–17]Arf1 covalently linked through a lysine added to the C-terminus to the fluorophore, tetramethylrhodamine (TAMRA), was incubated with PZA, ZA, Ph₅ASAP1, or PdZA and 200 μM diC₈PIP₂. The samples were excited with polarized light, and anisotropy was measured (Fig. 5B). When Ph₅ASAP1 or PZA were titrated into the solution, a saturable increase in anisotropy was observed with a half-maximal effect at 6.5 ± 2.6 and 3.2 ± 0.9 μM, respectively, consistent with the Kᵦ value measured by FRET. ZA and PdZA had no detectable effect on anisotropy of [2–17]Arf1-TAMRA.

Figure 2. PIP₂-stimulated activity does not depend on a hydrophobic surface. A, [L8K]Arf1 is a substrate for PIP₂-dependent ASAP1 GAP activity. PZA was titrated into a reaction containing [L8K]Arf1-GTP and LUVs with and without PS and PIP₂ as described in Fig. 1B. [2–17]Arf1 inhibits ASAP1 GAP activity against [L8K]Arf1. The effect of [2–17]Arf1 and [2–17]scrambled on GAP activity was determined as described in Fig. 1C. The concentration of PZA in the reactions was 3 nM, and the activity in the absence of peptide was 0.32 min⁻¹. C, dependence of Arf GAP activity on acyl chain length of PIP₂ analogs. PZA was titrated into a reaction containing 0.1 μM [L8K]Arf1-GTP and 200 μM PIP₂ analogs with the indicated acyl groups. GTP hydrolysis in 3 min was determined. D, diC₈PIP₂ dependence of GAP activity. diC₈PIP₂ was titrated into a reaction containing 0.5 nM PZA and 0.1 μM [L8K]Arf1. GTP hydrolysis in 3 min was determined. E, comparison of diC₈PIP₂-stimulated GAP activity using [L8K]Arf1 and [Δ17]Arf1 as substrates. PZA was titrated into a reaction containing either 0.1 μM [L8K]Arf1-GTP or [Δ17]Arf1-GTP and either no PIP₂ or 200 μM diC₈PIP₂. F and G, cognate PH domain of ASAP1 is required for activity with PIP₂ in LUVs and diC₈PIP₂. PZA, ZA, or PdZA were titrated into a mixture containing either diC₈PIP₂ and 0.1 μM [L8K]Arf1-GTP (F) or LUVs with PS and PIP₂ and 0.1 μM myrArf1-GTP (G). H, inhibition of diC₈PIP₂-stimulated GAP activity by [2–17]Arf1. The experiment was performed as described in Fig. 1C with [2–17]Arf1 or [2–17]scrambled using diC₈PIP₂ to stimulate activity and 0.1 μM [L8K]Arf1-GTP as substrate. The concentration of PZA was 0.5 nM. Activity with no peptide was 0.32 min⁻¹. Results shown are the summary of three experiments. Error bars are S.E.
substrates [2–17]Arf1 and PHASAP1. The resulting signal was proportional to the concentration of PZA, plotted against PZA concentration and fit to a hyperbola to estimate C50 values. The values are the average of three experiments. The raw data are shown in Fig. S2.

| Lipid analog | C50 (nM) |
|--------------|----------|
| No addition  | 2200 ± 1208 |
| diC8PIP2     | 1200 ± 730  |
| diC8PIP2H   | 160 ± 35    |
| diC8PIP2L   | 0.3 ± 0.03  |
| diC8PS      | 2.1 ± 0.51  |
| diC8FA      | 6.0 ± 2.8   |

Table 2 Dependence of GAP activity on acyl chain length and headgroup of phospholipid analogs

PZA was titrated into a reaction using 0.1 μM [L8K]Arf1-GTP as substrate and 200 μM of the indicated lipid analog. GTP hydrolysis in 3 min was determined. The percent of GTP hydrolyzed was plotted against PZA concentration and fit to a hyperbola to estimate C50 values. The values are the average of three experiments. The raw data are shown in Fig. S2.

Table 3 diC8 PIP2-stimulated GAP activity using [L8K]Arf1 and [Δ17]Arf as substrates

PZA was titrated into a GAP reaction using either 0.1 μM [L8K]Arf1-GTP or [Δ17]Arf1-GTP as substrate and 200 μM diC8 PIP2 where indicated. GTP hydrolysis in 3 min was determined. The percent of GAP on Arf that was hydrolyzed was plotted against PZA concentration and fit to a hyperbola to estimate C50 values. The data for [L8K]Arf1 are from Table 2. The values are the average of three experiments. The raw data are shown in Fig. S3.

| Substrate | −PIP2 | +diC8PIP2 |
|-----------|-------|-----------|
| [L8K]Arf1 | 2200 ± 1208 | 0.3 ± 0.03 |
| [Δ17]Arf1 | 29000 ± 6100 | 9500 ± 3400 |

To corroborate the FRET and anisotropy results and to establish a suitable assay for high-throughput screening, binding was evaluated by an AlphaScreen® proximity assay (PerkinElmer Life Sciences) (Fig. 6A). With this approach, biotinylated [2–17]Arf1 binds streptavidin-conjugated donor beads, whereas polyhistidine-tagged PHASAP1 binds nickel chelate acceptor beads. Upon excitation with 680 nm light, the donor beads generate singlet oxygen. If [2–17]Arf1-biotin binds PHASAP1, the acceptor bead is proximal to the donor bead and able to receive the singlet oxygen, resulting in a chemiluminescent signal at 615 nm (60). The reactions were performed in the presence of diC8PIP2 to facilitate binding between [2–17]Arf1 and PHASAP1. The resulting signal was proportional to the concentration of PHASAP1 in solution (Fig. 6B). Titrating nonbiotinylated [2–17]Arf1 to compete with [2–17]Arf1-biotin for binding to the PH domain reduced the signal with a IC50 of 5.4 μM [2–17]Arf1 (Fig. 6C), consistent with the Kd value measured by FRET and anisotropy.10

PIP2-dependent binding of N terminus of Arf1 to the PH domain of ASAP1 correlates with PIP2-dependent GAP activity

As an initial effort to identify the binding determinants between PHASAP1 and [2–17]Arf1, [2–17]Arf1-TAMRA was incubated with the PH domain of ASAP1 ([325–451]ASAP1, diC8PIP2H, and the cross-linker EDC. A cross-linked product was identified by SDS-PAGE (data not shown). MS analysis of the product revealed cross-linking between Glu-337 of ASAP1 and Lys-10 of Arf1. The crystal structure revealed that the ASAP1 PH domain has a 17-amino acid N-terminal extension, containing Glu-337, prior to the first β-strand in the canonical

10 High throughput screens for inhibitors using this assay are ongoing.
**PIP₂ regulates Arf1 binding to the ASAP1 PH domain**

![Figure 3. Hypothesized mechanism for PIP₂ regulation of GAP activity.](image)

Y419A mutation did not affect [2–17]Arf1 binding, PIP₂ binding, or GAP activity. The other mutations had less than 2-fold effects on PIP₂ binding. Effects on [2–17]Arf1 binding correlated with changes in GAP activity. The L423A mutation affected both [2–17]Arf1 binding and GAP activity by 4-fold, the Y419E mutation affected binding by 4.5-fold, and activity by 3-fold and the G339I mutation affected binding by 3-fold and activity by 15-fold. The results are consistent with the hypothesis that GAP activity depends on binding of the N terminus of Arf1 to the PH domain.

Another prediction of the hypothesis is that the introduction of mutations into the N terminus of Arf1 that reduced binding of the N terminus to the PH domain would render Arf1 a less efficient substrate. In contrast to [2–17]Arf1-TAMRA, [K10D,K15D,K16D,2–17]Arf1-TAMRA did not bind to the PH domain, detected as a change in anisotropy (Fig. 8G). [L8K,K10D,K15D,K16D]Arf1 was ~1000-fold less efficient a substrate than [L8K]Arf1 (Fig. 8H). The correlation of reduced binding with reduced GAP activity is consistent with our hypothesis, although we cannot exclude that the lack of activity was because the mutant Arf1 did not bind PIP₂ (52).

**Mutations in the PH domain that decrease binding of the N terminus of Arf1 also decrease the effect of the full-length ASAP1 on actin remodeling**

ASAP1 regulates actin remodeling in cells. One GAP-dependent function is reduction of the formation of circular dorsal ruffles (CDRs) in fibroblasts treated with platelet-derived growth factor (PDGF) (4). If binding of the N terminus of Arf to the PH domain of ASAP1 were important to function, we would predict mutants with reduced binding would be less active in cells. We determined the effect of ectopic expression of WT ASAP1, [R497K]ASAP1 (a mutant with the catalytic arginine changed to lysine, which has less than 1/10,000th the activity of WT ASAP1 (4, 15)), [G339I]ASAP1 and [L423A]ASAP1. As reported previously, WT ASAP1 reduced the number of cells with CDRs. [R497K]ASAP1 did not decrease the number of cells with CDRs. [G339I]ASAP1 and [L423A]ASAP1 had an intermediate effect on CDR formation, which correlated with reduced binding to the N terminus of Arf1 and a reduction in GAP activity (Fig. 9). These results indicate the interaction between the PH domain of ASAP1 and the N terminus of Arf1 is critical for the cellular functions of ASAP1.

**Discussion**

We examined the mechanism by which PIP₂ binding to the PH domain of ASAP1 modulates activity of the GAP domain. In previous studies, we found that both the cognate PH domain of ASAP1 and the N-terminal 16 amino acids of Arf were necessary for PIP₂-stimulated GAP activity. Here, we report that (i) one mechanism by which the PH domain regulates GAP activity is independent of recruitment to membranes, (ii) PIP₂ binds to the PH domain to regulate binding to the N terminus of the substrate Arf1-GTP, and (iii) disrupting the Arf1 N terminus–PH domain association reduces GAP activity. Mutations that reduced binding of the N terminus of Arf1 to ASAP1 also reduced the activity of ASAP1 in cells.

The results are consistent with our hypothesis that PIP₂ regulates binding of the N terminus of Arf1 to the PH domain of ASAP1, which might underlie the high degree of regulation of the catalytic activity.

The PH domain of ASAP1 contributes to enzymatic activity primarily by one of the three distinct mechanisms that have been described for PH domains. The first mechanism we had investigated was recruitment to a membrane containing a target protein (20). Our previous studies had established that recruitment was not sufficient (16, 17), and our results examining the effect of diC₈PIP₂ and comparing the activity of [325–724]ASAP1 with [339–724]ASAP1 reported here are additional evidence that recruitment is not the primary mechanism by which ASAP1 is activated by PIP₂.

PH domains have also been found to position autoinhibitory motifs in Grp1 and Akt such that deletion of the PH domain increases activity (37, 39). Similarly, the PH domain in SOS1 occludes the DH domain (41), the PH domain of p63RhoGEF autoinhibits the DH domain (36), and two PH domains occlude the active site in the nucleotide exchange factor Farp2 (40). Autoinhibition is relieved by Gα₅ binding to a C-terminal extension of the PH domain of p63RhoGEF (36) and Arf6 binding to the PH domain and N- and C-terminal linkers on the PH domain of cytohesin (29, 39, 42). For ASAP1, deletion of the PH domain results in loss of activity (17), leading us to conclude that autoinhibition by the PH domain is not a feature of the regulation of ASAP1. Our results indicate that the PH domain of ASAP1 contributes by a third mechanism by which PH domains can affect an adjacent enzymatic domain directly interacting with the substrate of the catalytic domain. PIP₂ binding to two sites within the PH domain of ASAP1 (14) is necessary for activa-
Figure 4. PIP$_2$-dependent binding of the N-terminal 16 amino acids of Arf1 to the PH domain of ASAP1 detected by FRET. 

**A**, principle of FRET. Nonemissive transfer of energy occurs between fluorophores with overlapping emission and excitation spectra dependent on $1/d^6$, where $d$ is the distance between the fluorophores. Consequently, FRET occurs over short distances, such as those achieved with intermolecular association. The FRET pair used in these experiments was the tryptophans in the PH domain, with an emission peak of ~340, and EDANS, a fluorophore with an excitation peak at about 336 nm and emission at 493 nm. 

**B**, structure of the ASAP1 PH domain. The ribbon structure of the ASAP1 PH domain is shown. Side chains of tryptophans 357 and 422 are shown. Lysine 342, the first residue of the β1 strand of the PH domain, is indicated as well as residues that were changed for experiments presented in Fig. 5. The image was rendered with Chimera (90), PDB code 5C6R. 

**C**, representative emission spectra of PHASAP1 with [2–17]Arf1-EDANS. Emission spectra of 1 μM PHASAP1 in 200 μM diC8PIP2 and the indicated concentration of [2–17]Arf1-EDANS after excitation with 280 nm light. 

**D**, [2–17]Arf1-EDANS dependence of FRET efficiency. 1 μM PHASAP1 or PHPLC1 was mixed with 200 μM diC8PIP2 and the indicated concentrations of [2–17]Arf1-EDANS or [2–17]scrambled-EDANS. Emission spectra were measured as in C, and FRET efficiency was calculated from quenching of tryptophan fluorescence as described under “Experimental procedures.” FRET was quantified from quenching of tryptophan fluorescence. The results are the summary of three independent experiments and plotted as the mean ± S.E. 

**E**, unlabeled [2–17]Arf1 reduces FRET signal of [2–17]Arf1-EDANS. FRET was determined for 1 μM PHASAP1, 4 μM [2–17]Arf1-EDANS, and the indicated concentration of [2–17]Arf1 without EDANS. FRET was quantified from quenching of tryptophan fluorescence. Summary of three experiments is shown. Data are plotted as the mean ± S.E. F, PIP$_2$ dependence of [2–17]Arf1-1PH domain binding measured by FRET. FRET was determined for a mixture of 1 μM PHASAP1, 15 μM [2–17]Arf1-EDANS, and the indicated concentrations of diC8PIP2. Summary of three experiments is shown. G, FRET signal requires an intact PIP$_2$-binding site. FRET, calculated as described under “Experimental procedures,” was determined for a solution containing 1 μM PZA or [R360Q]PZA and the indicated concentrations of [2–17]Arf1-EDANS. Summary of three experiments is shown. H, [2–17]Arf1 binding to full-length ASAP1. Full-length ASAP1 was titrated into a mixture containing 2 μM [2–17]Arf1-EDANS or [2–17]scrambled-EDANS, as indicated, and 200 μM diC8PIP2. Samples were excited with 280 nm light. Emission at 493 nm is shown. Summary of two experiments is shown. Data are plotted as the mean ± S.E.


**PIP₂ regulates Arf1 binding to the ASAP1 PH domain**

A molecule with a linked fluorophore is excited with plane polarized light. If the molecule tumbles rapidly, relative to the lifetime of the excited fluorophore such that the fluorophore randomly changes its orientation before emission, the emission is depolarized and intensity in perpendicular planes is similar. If the molecule tumbles slowly, e.g. as would occur if it binds to a larger molecule, the emission will be partly polarized, with unequal intensities in perpendicular planes, which is a measure of anisotropy.

Fluorescence anisotropy of [2–17]Arf1-TAMRA linked to PIP₂ is increased by incubation with the ASAP1 PH domain. Fluorescence anisotropy of 1 μM [2–17]Arf1-TAMRA in a mixture containing 200 μM diC₈PIP₂ and the indicated protein at the indicated concentration was determined. Summary of three experiments is shown and plotted as the mean ± S.E.

The association of the ASAP1 PH domain with the substrate for the GAP domain, Arf1, has some features in common with other proteins with PH domains that regulate small GTP-binding proteins. A PH domain binding a substrate to contribute to catalysis was first reported for DH–PH domain proteins. The PH domain of Dbs binds to switch 2 and helix α₃β of Rac1 to promote exchange of nucleotides (62). For Brag2, an Arf-exchange factor, switch 1 of Arf binds to an expansion of the PH domain, formed by the linker between sec7 and the PH domain (43). ASAP1 is distinct from these in that the PH domain binds to the N-terminus of Arf1.

Like the PH domains of cytohesins, the ASAP1 PH domain binds to Arf under the control of PIP₂ (29, 39, 42), but the mechanism is distinct. Switch 1, the interswitch domain, and switch 2 contribute to binding to the PH domain of the cytohesin Grp1. In contrast, the N terminus of Arf binds to the PH domain of ASAP1, although we cannot exclude some involvement of switch 1 or 2. Another difference is that binding to the cytohesin PH domain is driven by binding a single phosphoinositide molecule. For ASAP1, simultaneous binding of two phospholipids to a single PH domain is necessary. A third difference is that Arf binding to the PH domain of cytohesin 3 (Grp1) involves linkers on both the N- and C-terminal regions of the PH domain. Although there is some contribution of an interdomain linker, the linker is not required for binding between the ASAP1 PH domain and Arf1.

We investigated the role of a 17-amino acid extension of the PH domain based on the precedent of cytohesins (29, 39, 42)
and p63RhoGEF (36). For cytohesins, linkers N- and C-terminal to the PH domain are critical for binding Arf6. For p63RhoGEF, a C-terminal extension of the PH domain mediates binding to G/H9251q. We found that the 17-amino acid N-terminal extension of the PH domain had a minimal contribution to binding the N terminus of Arf1 but was nevertheless critical for GAP activity, independent of both PIP2 binding and peptide binding. This result provides additional support for the notion that the PH domain is not simply a recruitment mechanism as the PH domain without the linker was recruited to LUVs by PIP2 nearly as well as the PH domain with the linker, but PZA without the linker had a fraction of the activity of PZA containing the linker. In addition, the results indicate that there is a difference in regulation of GAP activity by PIP2 in a membrane compared with a soluble analog of PIP2, with a larger differential in activity with and without the linker observed with LUVs than with diC8PIP2. Possible mechanisms by which the N-terminal extension of the PH domain affects the GAP domain and how this is influenced by a lipid bilayer are currently being explored.

Other PH domains bind to protein and phospholipids simultaneously but cooperativity similar to that seen in cytohesin and ASAP1 has not been reported. One example is the phosphotyrosine binding (PTB) domain11 of disabled-1 (Dab1), which binds simultaneously to PIP2 and peptides containing Asn–Pro–Xaa–Tyr found in, for example, amyloid precursor protein and the apolipoprotein E receptor 2 (64, 65). Dab1 binds to PIP2 through a site that aligns with the PIP2-binding site of the PH domain of PLC/H92541. The peptide-binding site is separate and involves the β-strand and the C-terminal α-helix. Other than by restricting the protein to the cellular site with the target peptide, PIP2 does not influence peptide binding (65). Another example of a PH domain that binds simultaneously to a phosphoinositide and protein is the PH domain of FAPP1 (Four-phosphate-adaptor protein 1), which binds to Arf1 and phosphatidylinositol 4-phosphate simultaneously (26, 27). Both ligands are on the same surface, which allows coincidence detection; however, we are not aware of evidence for cooperative binding.

The proposed function of the ASAP1 PH domain is similar to models for GTPases (66, 67). For both, ligand binding (phosphoinositides or guanine nucleotides) controls the affinity for a target protein. The important difference between PH domains and GTPases is related to the mechanisms for switching between active and inactive conformations. For GTPases, high-affinity binding of nucleotide rapidly activates the protein, whereas rapid inactivation is achieved by a catalytic event, GTP hydrolysis. PH domains do not have a catalytic activity to rapidly convert to the inactive form. The conversion from inactive to active and back is

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11 PH, PTB, EVH1, and RanBD have a common fold, referred to as the PH domain superfold (63).
controlled by ligand binding and dissociation. Cooperative binding of phospholipids, as reported for ASAP1 (14), may provide rapid allosteric regulation of activity. Our results highlight the possible importance of extensions from the nucleotide-binding domain for signaling in Ras-superfamily proteins. A role of an extension from the nucleotide-binding domain for protein function was described as early as 1991 for Arf family proteins. In Arf, the N-terminal extension from the nucleotide-binding fold (68) is myristoylated and associates with membranes when Arf is bound to GTP. Arf1 without the extension was found to be inactive in vitro and in cell-based functional assays (69, 70). A peptide composed of these 16 amino acids of Arf1 blocked in vitro assays of Arf activity (69, 71). Several years later, the finding of relative movement of the Arf1 N terminus on GTP binding supported the idea that it functioned as a third switch motif in Arf (50, 72). Here, we observe that it binds directly to the GAP, which promotes GAP activity. Efficient interaction of Arf with the exchange factor Brag2 might also involve the N terminus of Arf (43, 73). Extensions of other G proteins from the GTPase domain have been found to bind effector proteins. Most relevant for Arf is Arl2 in which the N-terminal helix, switch 1, and switch 2 are all part of the interface with Binder of Arl Two (BART) (74). The C-terminal hypervariable region (HVR), an extension of the nucleotide-binding fold, of other Ras superfamily members might be similarly important. In molecular dynamic simulations coupled with FRET measurements in living cells, the accessibility of the HVR of Ras was determined by the bound nucleotide (75–77). In other studies, Rheb and KRas4b were reported to bind phosphodiesterase through the HVR with no contact with switch 1 or 2 (78, 79).

Figure 7. Effects of the N-terminal extension (residues 325–339) of the PH domain on peptide and PIP2 binding and GAP activity. A, binding of the PH domain to PIP2 in LUVs. Sucrose-LUVs, with a total phospholipid concentration of 500 μM containing 75 μM PS and the indicated concentration of PIP2, were incubated with the indicated recombinant proteins and removed from bulk solution by centrifugation. Protein that precipitated with the LUVs was fractionated by SDS-PAGE, visualized with GEL-CODE Blue stain (Thermo Fisher Scientific), and quantified by scanning densitometry. The results presented are the summary of three independent experiments plotted as means ± S.E. B, PH domain binding to [2–17]Arf1. Binding of the indicated recombinant PH domain of ASAP1 to [2–17]Arf1 was measured by FRET as described in Fig. 3. The summary of three independent experiments is presented. C, GAP activity with diC8PIP2. The indicated proteins were titrated into a reaction mixture containing 200 μM diC8P2 and 0.1 μM [L8K]Arf1-GTP. GAP activity was measured as described in Fig. 1 and under “Experimental procedures.” Results shown are the summary of three experiments. D, GAP activity with LUVs. The indicated proteins were titrated into reaction mixtures with LUVs containing 15% PS, 5% PIP2, a total phospholipid concentration of 500 and 0.1 μM myr-Arf1-GTP. GAP activity was determined as in C. The summary of three experiments is shown. E, evaluation of binding of the ASAP1 PH domain to nanodiscs by NMR. Nanodiscs were titrated into a solution containing the indicated [13C]methyl-labeled PH domain, and chemical shift perturbations were followed. Inset, 2D 1H–13C HMQC spectra of Ile-371 with increasing concentrations of nanodiscs. F, GAP activity in nanodiscs. The indicated proteins were titrated into a reaction containing 5 μM myr-Arf1-GTP as a substrate. The total concentration of exposed lipids was 500 μM. The percentage of GTP bound to myr-Arf1 hydrolyzed in 3 min is plotted against protein concentration.
In summary, our results support the hypothesis that PIP$_2$-dependent binding of the N terminus of Arf1 to the PH domain of ASAP1 regulates GAP activity. In ongoing studies, we will continue to test the hypothesis with full-length ASAP1 in cells and will identify the binding interface and the mechanism by which PIP$_2$ binding to the PH domain facilitates the interaction.
**Materials**

The following peptides were purchased from Lifetecn, Hillsborough, NJ: 1) [2–17]Arf1, sequence GNIFANLFKGLFGKKE; 2) [2–17]scrambled, sequence AKLGKGLGFNFNGIFE; 3) [2–17]Arf1-TAMRA, sequence GNIFANLFKGLFGKKE-K(TAMRA); 4) [K10D, K15D, K16D, 2–17]; 5) [2–17]Arf1-EDANS, sequence GNIFANLFKGLFGKKE-E(EDANS); 6) [2–17]scrambled-EDANS, sequence AKLGKGLGFNFNGIFE-E(EDANS); 7) [2–17]Arf1-biotin, sequence GNIFANLFKGLFGKKE(Biotin). [α32P]GTP was purchased from PerkinElmer Life Sciences; PEI-cellulose plates were from Selecto Scientific; nitrocellulose filters, 25-mm circles, BA85, were from GE Healthcare; Nucleopore Track-Etch membranes were from Whatman. The following lipids were from Avanti Polar Lipids: phosphatidylcholine; phosphatidylethanolamine; PS; cholestrol, phosphatidylinositol, phosphatidylinositol 4,5-bisphosphate; 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; and 1-oleoyl-2-oleoyl-sn-phosphatidylinositol 4,5-bisphosphate. The following lipid analogs were from Echelon Biosciences and Cayman Chemicals: 1,2-dibutanoyl-PIP2; sodium salt (diC6PIP2); 1,2-dioxanoyl-PIP2; sodium salt (diC8PIP2); 1,2-dioctanoyl-PIP2; sodium salt (diC8PIP2).

Lipofectamine LTX with Plus reagent were purchased from Thermo Fisher Scientific. Fibronectin and recombinant human PDGF BB were purchased from Sigma.

Alexa Fluor 594 phalloidin (A12381), GFP rabbit polyclonal antibody (A6455), and anti-rabbit secondary antibody Alexa Fluor 488 (A12106) were from Invitrogen. Dako fluorescence antibody (A6455), and anti-rabbit secondary antibody Alexa Fluor 488 (A12106) were from Invitrogen. Dako fluorescence mounting medium was purchased from Agilent.

**Experimental procedures**

**Materials**

The following peptides were purchased from Lifetecn, Hillsborough, NJ: 1) [2–17]Arf1, sequence GNIFANLFKGLFGKKE; 2) [2–17]scrambled, sequence AKLGKGLGFNFNGIFE; 3) [2–17]Arf1-TAMRA, sequence GNIFANLFKGLFGKKE-K(TAMRA); 4) [K10D, K15D, K16D, 2–17]; 5) [2–17]Arf1-EDANS, sequence GNIFANLFKGLFGKKE-E(EDANS); 6) [2–17]scrambled-EDANS, sequence AKLGKGLGFNFNGIFE-E(EDANS); 7) [2–17]Arf1-biotin, sequence GNIFANLFKGLFGKKE(Biotin). [α32P]GTP was purchased from PerkinElmer Life Sciences; PEI-cellulose plates were from Selecto Scientific; nitrocellulose filters, 25-mm circles, BA85, were from GE Healthcare; Nucleopore Track-Etch membranes were from Whatman. The following lipids were from Avanti Polar Lipids: phosphatidylcholine; phosphatidylethanolamine; PS; cholesterol, phosphatidylinositol, phosphatidylinositol 4,5-bisphosphate; 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; and 1-oleoyl-2-oleoyl-sn-phosphatidylinositol 4,5-bisphosphate. The following lipid analogs were from Echelon Biosciences and Cayman Chemicals: 1,2-dibutanoyl-PIP2; sodium salt (diC6PIP2); 1,2-dioxanoyl-PIP2; sodium salt (diC8PIP2); 1,2-dioctanoyl-PIP2; sodium salt (diC8PIP2).

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Alexa Fluor 594 phalloidin (A12381), GFP rabbit polyclonal antibody (A6455), and anti-rabbit secondary antibody Alexa Fluor 488 (A12106) were from Invitrogen. Dako fluorescence mounting medium was purchased from Agilent.

**Protein expression and purification**

Bacterial expression vectors for polyhistidine-tagged [325–724]ASAP1 (ASAP1 PZA), polyhistidine-tagged [325–451]ASAP1 (PH_ASAP1), polyhistidine-tagged [441–724]ASAP1 (Z), [1–134]PLCδ1 (PH_PLCδ1), the chimeric protein composed of [1–134]PLCδ1 fused to [441–724]ASAP1 (PdZA), Arf1, [L8K]Arf1, and [Δ17]Arf1 have been previously described (15–17). Expression vectors for polyhistidine-tagged [334–451]ASAP1, [339–451]ASAP1, [339–724]ASAP1, and [339–724]ASAP1 were prepared by PCR amplification of the ORF and inserting the ORF into pET19 in the Nde/EcoRI sites. Expression vectors for polyhistidine-tagged [Y419A,325–451]ASAP1, [Y419E,325–451]ASAP1, [I423A,325–451]ASAP1, [Q331A,L332A,Q333A,325–451]ASAP1, [G339I,325–451]ASAP1, [Y419E,325–451]ASAP1, [I423A,325–451]ASAP1, [Q331A,L332A,Q333A,325–451]ASAP1, [G339I,325–724]ASAP1, and [G339I,325–724]ASAP1 were prepared using the QuickChange site-directed mutagenesis kit (Agilent). Recombinant proteins were expressed and purified from bacteria as described (17, 80, 81). Full-length ASAP1 with a C-terminal hexahistidine tag was expressed in Sf9 cells using a baculovirus expression system and purified on a His trap column followed by size exclusion on Sephacryl S-300. The plasmid for expressing ASAP1b-GFP in mammalian cells, which included a 37-amino acid linker (NLSSDSSLSSPSALNSLSSP), was expressed in Baculovirus insect cells, presented as a percentage, was used to summarize the data for four experiments. The number of transfected cells containing CDRs and the total number of transfected cells were determined by manual counting. Representative images for each of the conditions are shown. Arrows point to cells with CDRs. The ratio of cells forming CDRs under each condition to the control cells, presented as a percentage, was used to summarize the data for four experiments with all conditions except [R497K]ASAP1, which was examined in two experiments (46 ± 10% of control cells (expressing GFP) had CDRs). Error bars are S.E.; *p < 0.05; **p < 0.01 compared with WT ASAP1; &p < 0.05 compared with GFP-transfected control, based on paired t tests calculated using GraphPad Prism.

**Preparation of LUVs**

LUVs were prepared by lipid extrusion. Lipids in chloroform solution were mixed in a siliconized glass 12 × 17-mm tube at the indicated molar ratios with a total lipid mass of 0.5 to 2.5.
μmol. Chloroform was removed by streaming nitrogen over the solution for 1 h and then placing the tubes in a lyophilizer for an additional hour. Lipids were hydrated with 0.1 to 0.5 ml (volume for final lipid concentration of 5 mM total lipid) of 25 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM DTT, with or without 10% sucrose (w/v) as indicated, and then subjected to five freeze-thaw cycles followed by extrusion through Whatman Nucleapore Track-etched membranes, with 1-μm pores, in an Avanti Polar Lipids lipid extruder (82).

**GAP assays**

GAP activity was determined by measuring the conversion of [α-32P]GTP bound to Arf1 to [α-32P]GDP as described (80). For experiments in which the GAP was titrated into a reaction, the concentration of GAP required to induce hydrolysis of 50% of the bound GTP in 3 min is referred to as the C50. For experiments with a fixed concentration of GAP, activity was determined by measuring the conversion of 680 nm and emission of 615 nm). After a 60-min incubation at room temperature, 1 μl containing 80 μg/ml nickel chelate acceptor beads (20 μg/ml, final) and 80 μg/ml streptavidin donor beads (20 μg/ml, final) (AlphaScreen® histidine (nickel chelate) detection kit, catalog no. 6760619R, PerkinElmer Life Sciences) was dispensed into each well and allowed to incubate for 60 min at room temperature in the dark. The AlphaScreen® signal was read by a PerkinElmer Life Sciences EnVision plate reader with an ultrasensitive luminescence detector and 1,536 plate HTS AlphaScreen® aperture (excitation of 680 nm and emission of 615 nm).

**Analysis of chemical cross-linking of [2–17]Arf1 to PHASAP1**

20 μM [2–17]Arf1-TAMRA and 20 μM PHASAP1 were mixed in the presence of 500 μM sucrose-loaded LUV with 2.5% PIP2 and 15% PS in 100 mM Mes, pH 6.0, 150 mM NaCl. After 20 min, EDC was added to a final concentration of 600 μM. The mixture was incubated for 2 h at 25 °C with shaking. The reaction was quenched with 4 mM β-mercaptoethanol. The pH of the reaction was adjusted to 7 by addition of Tris, pH 8. The sample was treated with trypsin followed by centrifugation to precipitate the LUVs. Mass spectra of the trypsin-treated supernatants were collected with an Orbitrap Fusion mass spectrometer, and data were analyzed with pLink software (87, 88).

**Preparation of membrane scaffolding protein (MSP) belt proteins**

The plasmids for MSPA H5 were the generous gift of Drs. Franz Hagn and Gerhard Wagner (Harvard Medical School) to Dr. Byrd. The proteins were expressed and purified as described previously (89).

**Preparation of NDs**

Lipids were mixed in chloroform solutions, then air-dried with nitrogen flow, and re-solubilized with cholate in aqueous buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 75 mM sodium cholate). NDs were prepared using 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 1-oleoyl-2-oleoyl-sn-phosphatidylcholine 4,5-bisphosphate. NDs were assembled by mixing MSPΔH5 with solubilized lipids at a 1:45 ratio, followed by removal of sodium cholate from the mixture with Bio-Beads.
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SM-2 (Bio-Rad). Assembled NDs were then purified via a Superdex-200 size-exclusion column (GE Healthcare).

**NMR measurements and chemical shift perturbation analysis**

All NMR spectra were collected at 25 °C on a Bruker Avance III 850 MHz spectrometer equipped with TCI triple-resonance cryoprob. Chemical shift perturbations were analyzed by Equation 3,

$$\Delta \delta_{obs} = \Delta \delta_{max} \cdot \left[ \frac{(n \cdot \delta_{P}) + L + k_{d}}{(n \cdot \delta_{P}) + L + k_{d}} - 1 \right] - 4 \cdot \left( \frac{n \cdot \delta_{P}}{L} \right)^{1/2} \cdot \frac{2 \cdot \eta \cdot \delta_{P}}{L} \tag{Eq. 3}$$

where \(\Delta \delta_{obs}\), \(\Delta \delta_{max}\), \(n\), \(P\), \(L\), and \(k_d\) values are the change in the observed chemical shift from the free state, the maximum change in chemical shift, the total PH domain concentration, the concentration of ND, and the dissociation constant, respectively. Data converged to \(n\), the number of sites, equal to 2, indicating that a nanodisc can bind one PH domain on each of its sides.

**Cell biology**

NIH 3T3 clone 7, kindly provided by Dr. Douglas Lowy, was maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1× penicillin/streptomycin. Cells were transfected with 2.5 μg/ml plasmids for expression of GFP or the indicated ASAP1 protein fused to GFP with Lipofectamine LTX Plus reagent and used 24 h later. CDRs were induced in cells plated on 10 μg/ml fibronectin-coated coverslips that were maintained for 5 h in Opti-MEM with no serum by treating with 20 ng/ml PDGF for 5 min. Cells were fixed with 4% paraformaldehyde. Before staining for immunofluorescence, cells were permeabilized with 0.2% saponin, 0.5% BSA, and 1% fetal bovine serum in PBS. Actin staining for immunofluorescence, cells were permeabilized with 0.2% saponin, 0.5% BSA, and 1% fetal bovine serum in PBS. Actin in the CDRs was stained with Alexa 594 phalloidin, and ASAP1–GFP or GFP was visualized using primary GFP rabbit polyclonal antibody followed by the anti-rabbit secondary antibody conjugated to Alexa Fluor 488. Mounting was done using Dako fluorescein isothiocyanate (FITC) containing proteins.

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**Author contributions**—We thank Kate Hebron for insightful discussions, Douglas Lowy for Clone 7 NIH 3T3 fibroblasts, and Valerie Barr for help with microscopy. Molecular graphics were performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from National Institutes of Health Grant P41-GM103311.
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