The BAR domain of the Arf GTPase-activating protein ASAP1 directly binds actin filaments

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The BAR domain of the Arf GTPase-activating protein (Arf GAP) with SH3 domain, ankyrin repeat and PH domain 1 (ASAP1) establishes a connection between the cell membrane and the cortical actin cytoskeleton. The formation, maintenance, and turnover of actin filaments and bundles in the actin cortex are important for cell adhesion, invasion, and migration. Here, using actin cosedimentation, polymerization, and depolymerization assays, along with total internal reflection fluorescence (TIRF), confocal, and EM analyses, we show that the N-terminal N-BAR domain of ASAP1 directly binds to F-actin. We found that ASAP1 homodimerization aligns F-actin in predominantly unipolar bundles and stabilizes them against depolymerization. Furthermore, the ASAP1 N-BAR domain moderately reduced the spontaneous polymerization of G-actin. The overexpression of the ASAP1 BAR–PH tandem domain in fibroblasts induced the formation of actin-filled projections more effectively than did full-length ASAP1. An ASAP1 construct that lacked the N-BAR domain failed to induce cellular projections. Our results suggest that ASAP1 regulates the dynamics and the formation of higher-order actin structures, possibly through direct binding to F-actin via its N-BAR domain. We propose that ASAP1 is a hub protein for dynamic protein–protein interactions in mechanosensitive structures, such as focal adhesions, invadopodia, and podosomes, that are directly implicated in oncogenic events. The effect of ASAP1 on actin dynamics puts a spotlight on its function as a central signaling molecule that regulates the dynamics of the actin cytoskeleton by transmitting signals from the plasma membrane.

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The spatiotemporal reorganization of the actin cytoskeleton is often coupled with the deformation of membranes in cellular processes including migration, adhesion, and division (1–3). The actin cytoskeleton receives, integrates, and responds to intra- and extracellular signaling events by changing its organization. Changes in actin organization are driven by the regulated assembly of globular actin (G-actin) into filamentous actin (F-actin), the formation of distinct higher-order F-actin structures, such as bundles, and the coordinated disassembly of F-actin to G-actin (4). The cortical actin cytoskeleton is highly dynamic, and interactions between the actin cytoskeleton and the juxtaposed plasma membrane are often directly mediated by actin-binding motifs in membrane-associated molecules and lipid-dependent activation mechanisms (1, 5–8).

ASAP1, an Arf GTPase-activating protein (Arf GAP), is a direct mediator of the membrane-associated actin cytoskeleton and is critical for the regulation of circular dorsal ruffles, focal adhesions, invadopodia, and podosomes (5, 9–12). Unifying features of these cytoskeletal structures are thick actin bundles that assemble and disassemble in a strictly controlled manner. How ASAP1 regulates the actin cytoskeleton is not fully understood. ASAP1 is a multifunctional scaffolding and signaling protein (Fig. 1A) that interacts through its C-terminal Src homology 3 (SH3) domain with actin cytoskeleton effectors, such as focal adhesion kinase (13), and through its proline-rich domain with Src-kinases and CrkL (5, 10, 14). The N-terminal N-BAR domain mediates ASAP1 dimerization and interacts with the actin-based molecular motor nonmuscle myosin-2A (11). The central pleckstrin homology (PH) domain regulates the catalytic GAP domain, which converts Arf-GTP to Arf-GDP (5). PI(4,5)P2 binding to the PH domain activates ASAP1 GAP activity (15). Overexpression of ASAP1 is reported in several mammalian cancers and associated with enhanced cellular migration or invasion rates that promote metastasis (16–18).

We investigated the possibility that ASAP1 functions not only as a scaffolding hub protein for well-described regulators of the actin cytoskeleton at the cytoskeleton-membrane interface but also as an F-actin binding protein. The data presented here reveal the direct interaction between ASAP1 and F-actin: the ASAP1 N-BAR domain directly binds F-actin and aligns it into predominantly unipolar bundles in vitro. Binding of ASAP1 to F-actin protects actin bundles from depolymerization and reduces the spontaneous polymerization of G-actin in vitro. In vivo, overexpression of ASAP1BARPH induces long cellular projections in fibroblasts, underlining its essential role at the cytoskeleton–plasma membrane interface. In summary, ASAP1BARPH inhibits actin turnover likely by stabilizing F-actin bundles, as they are found in focal adhesions and invadopodia, structures that are of importance in cancer cell migration and progression (19).

Results

Considering the pivotal role of ASAP1 in F-actin-rich structures, such as circular dorsal ruffles, focal adhesions, invadopodia, and podosomes, we investigated whether ASAP1 could directly interact with F-actin.
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**ASAP1BARPH directly binds to F-actin**

To test for a potential interaction between ASAP1 and F-actin, we used high-speed centrifugation assays in which soluble binding proteins (Fig. 1, A–B and D–H) would cosediment with F-actin (Fig. 1C). As a control for a positive binding event, we used the well-characterized tail domain of vinculin (VTail) that binds to F-actin under the assay conditions (Fig. 1H; ~78% at 25 μM F-actin).

We first addressed whether ASAP1FL would interact and cosediment with F-actin. Increasing concentrations of F-actin resulted in the sedimentation of ~20% ASAP1FL at 25 μM F-actin (Fig. 1). Next, we tested ASAP1BARPZA, which contains the three domains that support regulated catalysis and the N-BAR domain, in actin cosedimentation assays. Similar to ASAP1FL, ~20% ASAP1BARPZA sedimented at 25 μM F-actin (Fig. 1G). To test whether this low extent of binding would be indicative for weak F-actin binding properties of ASAP1FL and ASAP1BARPZA or may be caused by autoinhibition (20), we tested a fragment of ASAP1 lacking the N-BAR domain but containing all domains necessary for ASAP1 GTPase-activating (GAP) activity, ASAP1PZA. ASAP1PZA did not sediment with F-actin to an appreciable extent compared with ASAP1BARPZA (Fig. 1F). This observation led us to test ASAP1BARPH, a short and catalytically inactive ASAP1 fragment. In contrast to ASAP1BARPZA and ASAP1PZA, ASAP1BARPH strongly bound to F-actin and showed saturated binding at substoichiometric concentrations (Fig. 1D, ~86% at 25 μM F-actin). ASAP1PH, which lacks the N-BAR domain, did not bind F-actin to an appreciable extent under the same assay conditions (Fig. 1E). The isolated ASAP1 N-BAR domain is refractory to purification and could not be tested (11). The densitometric analysis of the sedimentation data is shown in Fig. 1 and reveals the extent of binding in the following order: ASAP1BARPH > ASAP1FL ≈ ASAP1BARPZA > ASAP1PH = ASAP1PZA.

To investigate the specificity and reversibility of the interaction between ASAP1BARPH and F-actin, we performed cosedimentation assays as a function of the ionic strength. Increasing [NaCl] led to a decreased extent of complex formation between ASAP1BARPH and F-actin (Fig. 1S A). A similar behavior was observed for the interaction between F-actin and VCFP,Tail (Fig. 1S B). ASAP1BARPH and VCFP,Tail did not show a salt dependence in their sedimentation behavior and remained soluble in the absence of F-actin (Fig. 1S C–D). Taken together, the cosedimentation data reveal a direct interaction between ASAP1 and F-actin that is mediated by the amino-terminal N-BAR domain of ASAP1.

**ASAP1BARPH aligns F-actin into thick bundles**

Our previous biochemical characterization showed that the ASAP1 N-BAR domain forms antiparallel homodimers (21). Concomitantly, a crescent-shaped structure was observed for ASAP1BARPH (Fig. 2A). EM micrographs showed that the ASAP1BARPH fragment is shown in Fig. S2. Homology modeling of ASAP1BARPH (Fig. 2C) and superimposition with the obtained electron micrographs showed that the ASAP1PH domains extend from both ends of the molecule. Homology modeling of ASAP1BARPH in negative-stain EM (Fig. 2A). Class averages revealed that the central BAR dimer is 4–5 nm wide and 15–16 nm long (Fig. 2B). The global average and variance images shown in Fig. 2B indicate that the PH domain is flexibly linked to the N-BAR domain. The length of the molecule extended to 26–27 nm in cases where the PH domain was directly in line with the N-BAR domains. A more systematic classification of the ASAP1BARPH fragment is shown in Fig. S2. Homology modeling of ASAP1BARPH (Fig. 2C) and superimposition with the obtained electron micrographs showed that the ASAP1PH domains extend from both ends of the molecule. Homology modeling of ASAP1BARPH in negative-stain EM (Fig. 2A). Class averages revealed that the central BAR dimer is 4–5 nm wide and 15–16 nm long (Fig. 2B). The global average and variance images shown in Fig. 2B indicate that the PH domain is flexibly linked to the N-BAR domain. The length of the molecule extended to 26–27 nm in cases where the PH domain was directly in line with the N-BAR domains. A more systematic classification of the ASAP1BARPH fragment is shown in Fig. S2. Homology modeling of ASAP1BARPH (Fig. 2C) and superimposition with the obtained electron micrographs showed that the ASAP1PH domains extend from both ends of the molecule. Homology
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modeling and sequence analysis of ASAP1BARPH further indicated that the concave surface formed by the N-BAR domains contains clusters of positively charged amino acids (Fig. 2C, bottom). Based on the salt dependence of the actoASAP1BARPH interaction (Fig. S1A) and the results from previous studies on the F-actin binding properties of the F-BAR protein pacsin2 and PICK1 (22, 23), we hypothesize that electrostatic interactions between positively charged clusters on the concave surface of the ASAP1 N-BAR domain mediate binding to the negatively charged surface of F-actin.

Homodimerization of ASAP1BARPH by means of its N-terminal BAR domain creates a protein complex with potentially two F-actin binding sites that may cross-link F-actin into bundles. The cosedimentation assays shown in Fig. 1, D–H do not distinguish between F-actin binding and bundling activity, as both single actin filaments and bundles sediment at high speeds. To test the hypothesis that ASAP1BARPH bundles F-actin, we incubated F-actin with a series of ASAP1 fragments and used EM to visualize the protein complexes. The electron micrograph in Fig. 2D shows the filamentous structure of actin alone as a reference. In contrast, the reconstituted actoASAP1BARPH complex formed straight, axially aligned dense bundles (Fig. 2E) that resemble actoVTail bundles in their ultrastructure (Fig. 2F). F-actin bundling was also observed for ASAP1F1 (Fig. 2G) and ASAP1BARPZA (Fig. 2H) but to a reduced extent compared with that of ASAP1BARPH (Fig. 2E), in agreement with the reduced binding properties observed in F-actin cosedimentation assays (Fig. 1, I and G). Unlike ASAP1BARPH and ASAP1BARPZA (Fig. 2, E and J), ASAP1PH (Fig. 2F) and ASAP1PZA (Fig. 2H) did not significantly bind and bundle F-actin. Notably, the dense, three-dimensional higher-order F-actin bundles did not allow the determination of the binding mode of ASAP1 fragments in the bundle.

ASAP1BARPH preferentially aligns F-actin into unipolar bundles

To explore the actoASAP1BARPH bundles and their polarity in more detail, we used total internal reflection fluorescence (TIRF) microscopy. First, we observed the ASAP1BARPH-induced bundling of F-actin in an endpoint assay in which ATTO488-labeled G-actin was polymerized in the presence of increasing concentrations of ASAP1BARPH. The resulting actin structures were analyzed after 10 min (Fig. 3, A and B). As shown in Fig. 3A, increasing concentrations of ASAP1BARPH aligned actin filaments in dense bundles that contain up to a maximum ~6 actin filaments under the experimental conditions (Fig. 3B).

To determine whether the actoASAP1BARPH bundles are of mixed or uniform polarity, the polymerization of ATTO488-labeled G-actin into filaments in the presence of ASAP1BARPH was observed with TIRF microscopy. Bundling events of two polymerizing filaments were identified and the polarity of the bundle determined. As shown in Fig. 3, C and D, unipolar and bipolar fusion events were observed. We also observed that in the presence of ASAP1BARPH, polymerizing actin filaments often originated from small bundles with unipolar polarity (Fig. 3E), leading to the hypothesis that ASAP1BARPH preferentially aligns actin filaments into unipolar bundles.

The same trend was observed in EM, where the orientation of individual actin filaments in short, actoASAP1BARPH bundles was determined by labeling the actin barbed end with a gelsolin cap prior to bundle formation (Fig. 3F).

Gelsolin is an F-actin-severing protein that remains attached to the barbed end of the actin filament as a cap after severing (24). The presence of a gelsolin cap was used to determine the polarity of the shortened actin filaments. Quantification of n = 77 filaments showed that 92.2% (n = 71) of the filaments in actin bundles are parallel and 7.8% antiparallel (n = 6). The observed, predominantly unipolar polarity of actoASAP1BARPH bundles agrees with the polarity of actin found in focal adhesions and cellular structures where ASAP1 and F-actin colocalize (25). In the absence of gelsolin, ASAP1BARPH paired actin filaments and promoted the formation of thick bundles. The presence of fully bundled and individual actin filaments indicates a cooperative binding mode (Fig. 3G) that agrees with the small actin bundles observed in TIRF microscopy (Fig. 3, A and E). Under saturating conditions, ASAP1BARPH completely aligned actin into thick unipolar bundles that can associate laterally into higher-order bipolar arrangements (Fig. 3H).

ASAP1BARPH stabilizes F-actin bundles against depolymerization

ASAP1 localizes to thick F-actin bundles in circular dorsal ruffles and focal adhesions (11) in vivo and induces F-actin bundling in vitro (Fig. 2E). To test whether ASAP1BARPH would stabilize F-actin within these bundles, we investigated its effect on the dilution-induced depolymerization of F-actin. Increasing concentrations of ASAP1BARPH inhibited the depolymerization of F-actin in a concentration-dependent manner (Fig. 4A). The same behavior was observed for the VTail but not the isolated ASAP1PH domain (Fig. 4, B and C), which were used as positive and negative controls, respectively. In summary, the data show that substoichiometric concentrations of ASAP1BARPH stabilize F-actin bundles against depolymerization in vitro.

ASAP1BARPH reduces the spontaneous polymerization of G-actin

Several F-actin binding and bundling proteins alter actin polymerization (26). To test whether ASAP1BARPH affects the polymerization of G-actin to F-actin, we studied its influence on the spontaneous polymerization of G-actin in vitro. As shown in Fig. 4D, increasing concentrations of ASAP1BARPH decreased the polymerization kinetics of pyrene-labeled G-actin in a concentration-dependent manner. No significant effect on G-actin polymerization was observed for ASAP1PH, whereas the presence of VTail slightly decreased the actin polymerization kinetics (Fig. 4, E and F). In agreement with the bulk kinetic assays, increasing concentrations of ASAP1BARPH also decreased actin polymerization rates in TIRF microscopy assays in which the elongation of actin filaments was observed in real time (Fig. 4G). The presence of 500 nM ASAP1BARPH decreased the actin elongation rate around 50% compared with the control (Fig. 4H). We also observed that individual polymerizing actin filaments are straighter and less dynamic in the presence of ASAP1BARPH, indicating a stabilizing effect on the actin filament.
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A

0 nM ASAP1BARPH
10 nM ASAP1BARPH
50 nM ASAP1BARPH
75 nM ASAP1BARPH
100 nM ASAP1BARPH
500 nM ASAP1BARPH

B

Number Filaments/Bundle

[ASAP1BARPH] (nM)

C

0 min
2 min
4 min
6 min
8 min

D

0 min
1 min
2 min
3 min
4 min

E

0 min
0.5 min
1 min
1.5 min
2 min

F

G

H
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**F-actin does not alter the enzymatic activity of ASAP1BARPH**

After we examined the effect of ASAP1BARPH on actin dynamics in vitro, we tested whether F-actin regulates the ASAP1 GAP activity, which is important for the hydrolysis of GTP bound to the small GTPase Arf1 during cytoskeletal reorganization (27). The N-BAR domain of ASAP1 has been shown to autoinhibit its GAP activity, whereas other N-BAR domain-binding proteins, such as FIP3 and nonmuscle myosin-2A, stimulate the GAP activity of recombinant ASAP1 proteins containing the N-BAR domain by 10- to 20-fold (11, 28). Unlike FIP3 and nonmuscle myosin-2A, F-actin had no detectable effect on the GAP activity of ASAP1BARPH (Fig. 4).

**ASAP1BARPH induces the formation of cellular projections in fibroblasts**

Some actin-binding proteins and BAR domain-containing proteins were previously shown to induce the formation of cellular projections (29–32).

To examine the effect of ASAP1 on actin structures in cells, we transfected NIH3T3 fibroblasts with various Flag-tagged ASAP1 constructs. Overexpression of ASAP1FL, ASAP1BARPH, or ASAP1BARPZA was sufficient to induce substantial actin remodeling in cells. The most noticeable change was the formation of long actin-filled cellular projections (Fig. 5A). In contrast, ASAP1BARPH, a construct that lacks the BAR-PH tandem, failed to induce these structures (Fig. 5, A and B). We quantified the potency of ASAP1FL, ASAP1BARPZA, ASAP1BARPH, and ASAP1BARPH to induce the formation of actin-filled projections by scoring the percentage of cells exhibiting at least 10 actin projections longer than 5 μm (Fig. 5B). We found that the extent to which these projections were induced correlates with the extent to which the ASAP1 fragments bind F-actin in vitro (Fig. 1). This observation suggests that ASAP1 affects actin remodeling in cells, possibly through a direct interaction between the N-BAR domain and F-actin.

We further characterized the cellular projections induced by the overexpression of ASAP1FL and ASAP1BARPH. We found that the overexpression of ASAP1FL and ASAP1BARPH induced the formation of longer actin-filled projections than a vector control (Fig. 5C). Further, cells overexpressing ASAP1BARPH had a higher total number of projections than cells overexpressing ASAP1FL or a vector control (Fig. 5D). We noticed that the projections induced by the overexpression of ASAP1BARPH displayed vesicular structures at the tip where ASAP1BARPH was concentrated. In contrast, vesicular structures were less frequent in projections induced by the overexpression of ASAP1FL (Fig. 5A). We further measured the length of the projection induced by ASAP1FL or ASAP1BARPH by anti-Flag immunostaining and found that projections induced by ASAP1BARPH were longer than those induced by ASAP1FL (Fig. 5E). As a result, the extent to which actin filled these projections was lower in ASAP1BARPH than ASAP1FL projections (Fig. 5F).

Taken together, the data suggest that the N-BAR domain of ASAP1 is involved in the organization of the actin cytoskeleton in fibroblasts.

**Discussion**

The results presented here collectively demonstrate that the ASAP1 N-BAR domain directly interacts with F-actin. Dimerization of ASAP1BARPH creates a molecule that connects and aligns actin filaments into emerging higher-order, tightly packed bundles with predominantly unipolar polarity (Fig. 2E and 3, E and G). This feature qualifies ASAP1 as an actin-binding protein that organizes the eukaryotic actin cytoskeleton in a spatiotemporal manner. Although the F-actin binding properties of several BAR domain proteins have been explored in vitro, only pacsin2, PICK1, Bain1, Bin1, Gas7, PSTPIP2, MIM, and IRSp53 were described to interact with F-actin both in vitro and in vivo via their BAR domains (22, 23, 33–37). Moreover, ASAP1 and the recently described Bin1 (37) are the only N-BAR domain proteins reported to directly interact with F-actin so far.

The data presented in this study favor a model in which the ASAP1BARPH dimer binds along the lateral surface of actin filaments. The putative actin-binding site or interface is solvent exposed and presumably located in the concave surface of the ASAP1BARPH dimer. Based on our experimental finding that ASAP1PH does not bind and bundle F-actin in vitro (Fig. 1E and 2F), we conclude the actin-binding site/interface lies within the N-BAR domain of ASAP1BARPH. The maximum distance between the two putative actin-binding sites of the ASAP1BARPH dimer is ~15–16 nm (Fig. 2 and Fig. S2), spanning ~5–6 actin subunits (2.7 nm) in an actin filament. The ASAP1 dimer may not only stabilize the filament but also cross-link actin filaments into predominantly unipolar bundles as they are found in focal adhesions and filopodia (25, 38). Bundling may also be facilitated by BAR domain tetramerization, as has been shown for the actin-bundling protein pacsin1 and PICK1 (39, 40). Intra- and interfilamental ASAP1BARPH cross-links effectively protect actin filaments from the dilution-induced depolymerization (Fig. 4A). The concentration dependence of the reaction indicates that several actin subunits can depolymerize at normal rates at low ASAP1BARPH concentrations until the subsequent cross-linked subunit is reached, which delays depolymerization. Therefore, increasing ASAP1BARPH concentrations would increase the number of cross-linked F-actin bundles and delay

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**Figure 3. ASAP1BARPH aligns F-actin in predominantly unipolar bundles.** A, actin polymerization endpoint assay. 500 nM ATTO488-labeled G-actin was polymerized in the absence or presence of increasing concentrations ASAP1BARPH and the resulting actin structures imaged after 10 min. The dimensions of the panels are 30 μm by 30 μm. B, increasing concentrations of ASAP1BARPH increase the number of actin filaments per bundle (n = 31–63). **,**, p < 0.0001, and *, p < 0.05, using one-way ANOVA with Dunnett’s multiple-comparison test. C–D, montage of images showing the time-dependent elongation of individual actin filaments in the presence of ASAP1BARPH. White arrows indicate parallel growth/bundling, and white asterisks indicate antiparallel growth/bundling events. C, Polymerization of 500 nM ATTO488-labeled G-actin in the presence of 500 nM ASAP1BARPH. Time interval, 2 min; scale bar, 1 μm. D, polymerization of 500 nM ATTO488-labeled G-actin in the presence of 50 nM ASAP1BARPH. Time interval, 1 min; scale bar, 1 μm. E, polymerization of 500 nM ATTO488-labeled G-actin in the presence of 25 nM ASAP1BARPH. Time interval, 30 s; scale bar, 2 μm. F–H, electron micrographs of 1000 nM F-actin capped with 20 nM gelsolin and incubated with 80 nM ASAP1BARPH (F), 80 nM ASAP1BARPH (G), or 800 nM ASAP1BARPH (H). The white arrows indicate parallel, the black arrows indicate likely antiparallel F-actin bundles. The plus sign indicates the barbed end of the actin filament. Scale bar, 100 nm.
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depolymerization (41, 42). We detected a small decrease in actin polymerization kinetics at low stoichiometric molar ratios of ASAP1BARPH (Fig. 4D), as they are likely found in eukaryotic cells (43). Higher actin:ASAP1BARPH ratios result in an ~50% decrease in actin polymerization rates, likely caused by actin bundling under the in vitro conditions. In a cellular context, we expect that the effect of ASAP1 on actin polymerization is minor compared with its effects on actin bundling and the protection of bundles from depolymerization.

The observation that the lipid-binding ASAP1PH domain, the catalytic GAP domain, the proline-rich domain, and the SH3 domain are not required for F-actin binding in vitro suggests that ASAP1 interacts with phospholipids, its substrate Arf, and other known binding partners of these domains simultaneously with F-actin. Consistent with this idea, we found that F-actin does not significantly change the enzymatic GAP activity of ASAP1BARPZA (Fig. 4I), underlining that F-actin binding and other biochemical functions of ASAP1 are not mutually exclusive.

While this work was under revision, an independent study was published that described the interaction between ASAP1 and F-actin (44). Corroborating our results, this study found that the N-BAR domain of ASAP1 directly binds to and bundles actin filaments in vitro. This study showed a correlation between ASAP1 expression and F-actin levels in cells. Further, it showed that reduced ASAP1 expression results in the depletion of ventral stress fibers, whereas increased ASAP1 expression results in more pronounced stress fibers and the appearance of cellular projections. Our cellular studies focus on cellular projections extending from the cell periphery in more detail, as the effect of ASAP1 expression on this type of structure has not been characterized extensively (5, 12). Moreover, the results from both studies suggest that the ASAP1 N-BAR domain is under autoinhibitory regulation, as has previously been described for other BAR domain proteins, such as PICK1, syndapin, and FCHSD2 (45, 46). Both studies show that the region C terminal to the BAR-PH tandem of ASAP1 negatively affects its F-actin binding properties in vitro and the induction of actin-filled projections in cells. Future studies will test if protein–protein interactions and/or posttranslational modifications in the C-terminal region of ASAP1 relieve the autoinhibition to regulate the N-BAR–F-actin interaction in cells.

The mechanism by which ASAP1 drives the formation of actin-filled cellular projections remains elusive. Our in vitro and in vivo results, together with the known biophysical properties of ASAP1 (20), suggest that ASAP1 induces the formation of actin-filled cellular projections by the actin-binding and -bundling activity of its N-BAR domain. In contrast, other BAR domain proteins, including IRSp53, IRTKS, FCHSD2, and srGAPs, likely induce the formation of cellular projections, such as filopodia, microvilli, or stereocilia, by interacting via their BAR domain with the membrane and via their C terminus with regulators of the actin cytoskeleton, including WASP/N-WASP, EPS8, or FMNL1 (34, 47–53).

Only IRSp53 and IRTKS directly bind and bundle actin filaments in vitro, although there is controversy in the literature (34, 53, 54). For IRSp53, which binds to lipids and actin via its I-BAR domain, membrane binding rather than actin binding has been shown to drive filopodia formation (51). In contrast to the actin-free cellular projections induced by the overexpression of the IRSp53 I-BAR domain (51, 55, 56), we show that the overexpression of ASAP1BARPH promotes the formation of actin-filled cellular projections (Fig. 5). This suggests that ASAP1BARPH is sufficient to couple actin to the membrane within these projections. ASAP1 contains an N-BAR domain, which binds to and induces convex membrane curvature (20, 21). Therefore, unlike the I-BAR-containing proteins IRSp53 and IRTKS, which bind to concave membrane curvature, or the F-BAR proteins FCHSDs and srGAPs, which bind to flatter membrane surfaces, it is unlikely that ASAP1BARPH induces cellular projections solely by membrane deformation. This does not exclude that membrane binding or bending can contribute to the formation of ASAP1-dependent cellular projections, which can be facilitated by the PH domain and Arf GAP domain, which binds to membrane-associated Arf-GTP, or simultaneous binding of lipids and F-actin by the N-BAR domain. To this end, it remains elusive whether and how the ASAP1 N-BAR domain discriminates between actin and membrane binding in cells. We speculate that the interaction with binding partners and changes in local concentration of actin and lipids plays a role in this process.

ASAP1 localizes to several distinct actin structures, including focal adhesions, invadopodia, podosomes, and circular dorsal ruffles (12). Based on the data presented in this study, we suggest that ASAP1 bundles and transiently stabilizes actin filaments in these structures. In this context, ASAP1 may control the assembly and/or resolution of the cellular structures by linking the actin cytoskeleton to the plasma membrane and functioning as a scaffolding hub for regulators of the actin cytoskeleton, such as FAK, Src, and the molecular motor nonmuscle myosin-2A (11). Consistent with this idea, the N-BAR and SH3 domains and Src-mediated phosphorylation of ASAP1 contribute to the formation of invadopodia and podosomes (57).

Moreover, the finding that ASAP1 preferentially aligns F-actin into unipolar bundles is in line with the known unipolarity of actin filaments in invadopodia and podosomes and at the ends of stress fibers (19, 58). ASAP1 colocalizes with F-actin only at the junction between stress fibers and focal adhesions instead of the whole length of the stress fibers (11). The ends of
stress fibers emanating from focal adhesions contain unipolar actin bundles, whereas the central region of stress fibers contains bipolar actin filaments.

The stabilizing effect of ASAP1 on F-actin bundles and turnover may also be of importance in cancer cell migration and invasion from the signaling perspective. The actin cytoskeleton supports cellular signaling events, and altered actin-bundling dynamics modulate signaling pathways that result in cancer initiation, progression, and metastasis (19), processes that are associated with ASAP1 overexpression (16–18). Therefore, the actin-bundling and stabilizing properties of ASAP1 might be part of its function in cancer cell migration and metastasis that
are often associated with the alteration in the activities of F-actin bundling proteins (19).

Experimental procedures

**Protein overproduction and preparation**

Flag-tagged full-length mouse ASAP1b (accession number AAC98350.1), ASAP1FL (amino acids 1–1090), was expressed in the baculovirus/Sf9 insect cell system, and polyhistidine-tagged mouse ASAP1b fragments ASAP1BARPH (amino acids 1–724), ASAP1PZA (amino acids 325–724), ASAP1BARPH (amino acids 1–431), ASAP1PH (amino acids 325–441), the tail domain of human vinculin (V_tail, amino acids 891–1066, accession number AAH39174.1), and CFP-tagged V_tail (V<sub>CFP</sub>Tail, amino acids 891–1066) were recombinantly overproduced in Escherichia coli and purified to electrophoretic homogeneity as described previously (7, 20, 59). G-actin was prepared from rabbit skeletal muscle acetone powder (Pel-Freez Biologicals) (60) or purchased as ATTO488- or ATTO565-labeled G-actin (Hypermol). Pyrene-labeled G-actin was from Hypermol or Cytoskeleton. N-ethylmaleimide (NEM)-modified rabbit skeletal muscle myosin-2 was from Hypermol, and human gelsolin was purchased from Cytoskeleton.

**F-actin cosedimentation assay**

For cosedimentation assays shown in Fig. 1, 3 μM ASAP1, ASAP1 fragment, or V<sub>Tail</sub> was incubated with increasing concentrations of F-actin (0–25 μM) in PBS supplemented with 1 mM MgCl2 incubated for 10 min at 22 °C, and sedimented for 15 min at 100,000 × g at 4 °C in a TLA-100 rotor in a Beckman Ultima MAX-XP ultracentrifuge. Following sedimentation, the supernatant was removed and the pellet resuspended in an equal volume of assay buffer. Supernatant and pellet fractions were resolved on a 4–12% Bis-Tris gel (Thermo Fisher Scientific) or purchased as ATTO488- or ATTO565-labeled G-actin (Hypermol). Pyrene-labeled G-actin was from Hypermol or Cytoskeleton.

**Actin polymerization and depolymerization assays**

Polymerization of 3.5 μM MgATP–G-actin (10% pyrene labeled) was induced by the addition of 1/10 10X KMEI buffer (50 mM KCl, 100 mM imidazole, pH 7.0, 10 mM MgCl2, 10 mM EGTA) in the presence and absence of various concentrations (0.075–1.5 μM) of ASAP1BARPH, ASAP1PH, or V<sub>Tail</sub>. The time-dependent change in pyrene fluorescence was recorded in a QuantaMaster fluorescence spectrophotometer (Photon Technology International; excitation wavelength, 365 nm; emission wavelength, 407 nm) at 20 °C. For polymerization data analysis, the dead time was added back to the data, the baseline fluorescence at the start of the reaction was subtracted, and the data were normalized by division by the plateau fluorescence (62). For dilution-induced F-actin depolymerization assays, 10 μl of 4 μM F-actin (25% pyrene labeled) was incubated with an equal volume of substoichiometric concentrations (0.075–1.5 μM) of ASAP1BARPH, ASAP1PH, and V<sub>Tail</sub> for 30 min and diluted 20-fold with 1× KMEI buffer (50 mM KCl, 1 mM MgCl2, 1 mM EGTA, 10 mM imidazole, pH 7.0). The time-dependent decrease in pyrene fluorescence was measured in a QuantaMaster fluorescence spectrophotometer (Photon Technology International; excitation wavelength, 365 nm; emission wavelength, 407 nm) at 20 °C. For actin polymerization and depolymerization assays, the storage buffer of ASAP1 and vinculin fragments was exchanged to 1× KMEI with a Zeba Spin desalting column (Thermo Fisher Scientific) or dialysis prior to the assay.

**TIRF microscopy**

15–20-μl flow cells were constructed with double-sided tape. The chamber was coated with 5% BSA in 20 mM imidazole, pH 7.0, 300 mM KCl, 0.5 mM DTT and functionalized with 0.1 mg/ml NEM. The time-dependent polymerization of 0.5 μM ATTO488-labeled (degree of labeling, ~0.3) G- to F-actin was monitored in the presence or absence of ASAP1 fragments in TIRF buffer (1× KMEI buffer supplemented with 0.17% methylcellulose, 0.2 mM ATP, 3 mg/ml glucose, 0.1 mg/ml glucose oxidase, 0.2 mg/ml catalase, and 10 mM DTT) on a Nikon Ti-Eclipse H-TIRF microscope with a 100× 1.49 numeric aperture (NA) objective at ambient temperature over 10 min. The time-dependent growth of the actin barbed end was manually tracked after a 30-pixel rolling ball radius background subtraction, image registration, and brightness adjustment, if necessary, in Fiji (61). The polarity of F-actin bundles was determined as described previously (63). To determine the number of actin filaments in a bundle, images were processed as described above, and the maximum fluorescence across an
individual filament was measured for 15 filaments per flow cell and the average intensity calculated. The number of actin filaments in a bundle was determined by dividing the fluorescence intensity of the bundle by the average intensity of a single actin filament from the same experiment. Data corresponding to the mean ± S.D. of a single filament was not included in the analysis of bundles (Fig. 3B). Data analysis was performed with Fiji (61). Data were plotted with Origin (OriginLab), and statistical analysis was performed with Prism (GraphPad).

EM and image processing

Proteins were diluted to 100 nM in buffer containing 10 mM MOPS, pH 7.0, 150 mM NaCl, 0.1 mM EGTA, 2 mM MgCl₂. In the presence of gelsolin, the buffer was supplemented with 0.2 mM CaCl₂. Samples were applied to the grid immediately after dilution and stained with 1% uranyl acetate. Micrographs were recorded on a JEOL 1200EX II microscope using an AMT XR-60 charge-coupled device camera, operating at 80.0 kV at room temperature. Catalase crystals were used as a size calibration standard. Image processing was carried out using SPIDER as described previously (64). Initially, 4723 particles were aligned and classified into 200 classes using a variance threshold mask that incorporated the whole molecule. Classes predominated by misaligned particles were discarded, resulting in a data set with 4441 particles. This data set was classified into 5 distinct N-BAR classes using a mask drawn manually around the central N-BAR domain dimer region. The 4441-particle data set was also classified into 5 PH classes based on a mask drawn manually around one of the PH domain regions at the end of the molecule (PH_top). This process was repeated for the opposite PH domain (PH_bottom). Therefore, each particle was assigned to 1 of 5 N-BAR classes (Fig. S2, B and C), 1 of 5 PH_top classes, and 1 of 5 PH_bottom classes. This resulted in 125 distinct classes. All particles within a given class were recombined and averaged, resulting in the matrix shown in Fig. S2A. To quantify parallel and antiparallel interactions from EM images, the polarity of actin filaments running into paired or sparsely bundled regions was determined using the presence of gelsolin caps at the barbed ends as well as visual inspection of the actin. Only regions in which actin filaments were tightly connected over more than 50 nm were quantified.

Homology modeling

The structure of the N-BAR–PH tandem domain (amino acids 49–431) of mouse ASAP1 was modeled using the template structures of the human BAR domain of APPL1 (PDB entry 2Q13), the human BAR domain of ACAP1 (PDB entry 4NSW), and the human PH domain of ASAP1 (PDB entry 5C79) using Modeller 9v19 (65). The final N-BAR–PH model was subjected to the steepest descent energy minimization method using the molecular modeling toolkit in the Chimera molecular visualization package (66).

Immunofluorescence staining, confocal microscopy, and image analysis

NIH3T3 fibroblasts (ATCC) were grown at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific). Fibroblasts were transiently transfected with plasmids pCI, pCI-Flag-ASAP1<sup>FL</sup> (amino acids 1–1090) (10), pCI-Flag-ASAP1<sup>BARPZ</sup> (amino acids 1–431), pCI-Flag-ASAP1<sup>BARPZ</sup> (amino acids 431–1090), or pCI-Flag-ASAP1<sup>BARPZ</sup> (amino acids 1–724) using Lipofectamine 2000 reagent (Invitrogen). The latter plasmids were generated by PCR amplification and subsequent cloning into the pCI vector backbone. 24 h after transfection, cells were plated on 10-µg/ml fibronectin-coated coverslips and fixed in 4% paraformaldehyde after 5.5 h of adherence. Fixed cells were incubated in 15 mM glycine for 10 min, 50 mM NH₄Cl twice for 10 min each time and then permeabilized and blocked with 0.2% saponin, 0.5% BSA, and 1% FBS in PBS for 20 min. Cells were stained with anti-Flag antibody (Cell Signaling Technology) and rhodamine-phalloidin (Invitrogen) for 1 h, followed by secondary antibodies for 1 h, and mounted in DakoCytomation fluorescent mounting medium (Dako). Images for fixed cells were taken at the focal plane where cells adhere to the substrate on a Zeiss LSM 510 attached to a Zeiss Axiovert 100M with a 63×, 1.4 NA Plan Neofluar oil immersion lens (Carl Zeiss) or on a Nikon Ti2 inverted microscope and A1R live-cell resonant dual-scanner confocal system using a 60×, 1.49 NA CF160 Apochromat TIRF oil immersion objective. Statistical analysis was performed with Prism (GraphPad).

Cellular projections were quantified by using the following criteria. Cells were scored as cells with long projections when they had at least 10 actin-containing projections longer than 5 µm. For the total number of actin projections per cell, all actin projections (stained by rhodamine-phalloidin) extending ≥0.2 µm from the cell periphery were counted. The projection length was measured by manually line-tracking projections based on rhodamine-phalloidin or anti-Flag antibody staining in Image J. The percent length filled with actin was calculated by dividing the actin projection length by the Flag projection length for each projection.

GAP assay

The GAP activity of ASAP1<sup>BARPZ</sup> was determined, as described earlier (11, 59), in the presence of up to 5 µM F-actin and myrArf1-GTP as substrate. The reaction was stopped after 3 min and protein-bound nucleotide trapped on nitrocellulose, eluted with formic acid, and analyzed by TLC.

Data availability

All data are contained within the article.

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Abbreviations—The abbreviations used are: G-actin, globular actin; GAP, GTPase-activating protein; BAR, Bin/Amphiphysin/Rvs; PH, pleckstrin homology; TIRF, total internal reflection fluorescence; F-actin, filamentous actin; NEM, N-ethylmaleimide; NA, numeric aperture.

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