Mislocalization or Reduced Expression of Arf GTPase-activating Protein ASAP1 Inhibits Cell Spreading and Migration by Influencing Arf1 GTPase Cycling*

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ADP-ribosylation factor (Arf) family of small GTP-binding proteins plays a central role in membrane trafficking and cytoskeletal remodeling. ASAP1 (Arf-GAP containing SH3, ankyrin repeats, and PH domain) is a phospholipid-dependent Arf GTPase-activating protein (Arf-GAP) that binds to protein-tyrosine kinases Src and focal adhesion kinase. Using affinity chromatography and mass spectrometry (MS), we identified the adaptor protein CD2-associated protein (CD2AP) as a candidate binding partner of ASAP1. Both co-immunoprecipitation and GST pull-down experiments confirmed that CD2AP stably interacts with ASAP1 through its N-terminal SH3 domains. Using a mislocalization strategy, we show that sequestration of endogenous ASAP1 to mitochondria with a CD2AP SH3-mito fusion protein (the three N-terminal SH3 domains of CD2AP fused to Listeria monocytogenes ActA mitochondria-targeting sequence) inhibited REF52 cell spreading and migration in response to fibronectin stimulation. Using an alternative strategy we show that suppressing ASAP1 expression with small interfering RNA duplexes also significantly retarded cell spreading and inhibited cell migration. Furthermore, abrogation of ASAP1 function using either small interfering RNAs or mislocalization approaches caused an increase of GTP loading on Arf1 and loss of paxillin from adhesions. These results taken together with our previous observations that overexpression of ASAP1 inhibits cell spreading and alters paxillin localization to adhesions (Liu, Y., Loijens, J. C., Martin, K. H., Karginov, A. V., and Parsons, J. T. (2002) Mol. Biol. Cell. 13, 2147–2156) suggest that the recruitment of certain adhesion components such as paxillin requires dynamic GTP/GDP turnover of Arf1 GTPase.

Cell migration is a cyclic process comprising of four basic steps: adhesion, attachment, translocation, and retraction of the rear (1, 2). Among the many events that are regulated during the migratory cycle, coordinated regulation of the actin cytoskeleton, formation, turnover and maturation of adhesion structures, coordinated translocation of intracellular vesicles have been studied as key events in the process. Of increasing importance is elucidating how membrane internalized from the cell surface is trafficked to the leading edge of migrating cells to fulfill the requirement of surface area increase as a result of cell front extension (3). An understanding of the coordination between membrane traffic, cell adhesion, and actin re-organization has been facilitated by the identification and characterization of ADP-ribosylation factors (Arfs), a family of small GTP-binding proteins. The Arf family members were originally identified as co-factors for cholera toxin-catalyzed ADP ribosylation of Gs (4). The primary physiological function of Arfs, however, is to regulate endocytosis and vesicle trafficking by controlling the interaction of coat proteins with intracellular organelle membranes (5–7). More recently, Arfs have also been implicated in the regulation of cytoskeletal remodeling (8–11), although the mechanisms by which they act are poorly understood. Arf1, the prototypic member of this family, is reported to mediate the recruitment of paxillin to focal adhesions and to facilitate Rho-stimulated stress fiber formation in Swiss 3T3 fibroblasts (9). Arf6, the least conserved member of the Arf family, cycles between plasma membrane and endocytic compartments, depending on its nucleotide status (12). Both the constitutively active and the dominant negative forms of Arf6 cause pronounced cell morphology changes when overexpressed in cells (8, 10). It has been shown that Arf6 functions primarily through the activation of lipid-modifying enzymes such as phosphatidilylinositol 4-phosphate 5-kinase and the modulation of actin cytoskeleton (11, 13). Arf6 also plays a role in clathrin-mediated endocytosis by recruiting Nm23-H1, a nucleoside diphosphate kinase, which provides a source of GTP for dynamin-dependent fission of coated vesicles (14). The dual functions of Arf GTPases in both vesicle trafficking and actin cytoskeletal remodeling point to these proteins being candidates for the coordinators that regulate critical steps during cell migration.

Evidence for Arfs as regulators of adhesion also comes from the identification of proteins with Arf-GAP homology that bind to focal adhesion components. Paxillin kinase linker, also known as GIT (G protein-coupled receptor interacting target) 2/CAT (cool-associated, tyrosine-phosphorylated) 2, binds di-

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1 The abbreviations used are: Arf, ADP-ribosylation factor; ASAP1, Arf-GAP containing SH3, ankyrin repeats and pleckstrin homology domain; CD2AP, CD2-associated protein; FAK, focal adhesion kinase; GAF, GTPase-activating protein; GST, glutathione S-transferase; mAb, monoclonal antibody; PH, pleckstrin homology; REF, rat embryo fibroblasts; SH3, Src homology 3; siRNA, small interfering RNA; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; GFP, green fluorescent protein; EGF, epidermal growth factor.

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rectly to the LD4 domain of paxillin and the guanine nucleotide exchange factor p21-activated kinase-interacting exchange factor and thus mediates the association of paxillin with a complex composed of p21-activated kinase, Nck, and p21-activated kinase-interacting exchange factor (15). A paxillin kinase linker-related Arf-GAP, GIT1, binds to paxillin and FAK and its overexpression causes a loss of paxillin from focal adhesions (16). The inhibition of paxillin localization to adhesions is also observed upon overexpressing another distantly related Arf-GAP ASAP1 (Arf-GAP containing SH3 domain, ankyrin repeat, and PH domain) (17). ASAP1 is found in perinuclear regions, membrane ruffles, and focal adhesions (17–19). ASAP1 binds to a proline-rich motif of FAK through its C-terminal SH3 domain (17) and overexpression of ASAP1 inhibits cell spreading and platelet-derived growth factor-induced dorsal ruffles (17, 19). An ASAP1-related protein PAPα (Pyk2 Cterminal minus associated protein α/KIAA0400) interacts with the FAKrelated protein tyrosine kinase Pyk2 (20) and paxillin (21). Overexpression of PAPα inhibits paxillin recruitment to focal contacts (21), a phenotype common to overexpression of GIT1 and ASAP1. The phenotypes caused by these Arf-GAPs are likely because of the down-regulation of Arfs activity. However, the multidomain nature of ASAP1-type Arf-GAPs suggests that they may possess other functions in addition to modulating Arf activity in vivo.

Here we show a requirement for ASAP1, a focal adhesion-enriched Arf-GAP, in integrin signaling. We identified CD2AP/CMS (Cas ligand with multiple SH3 domains), an SH3 domain-containing adaptor protein that interacts with ASAP1 via its N-terminal SH3 domains. Using a mislocalization strategy, we show that sequestration of endogenous ASAP1 to mitochondria with a CD2AP SH3-mito fusion protein (the three N-terminal SH3 domains of CD2AP fused to Listeria monocytogenes ActA mitochondria-targeting sequence) (22) inhibited REF52 cell spreading and migration in response to fibronectin stimulation. Using an alternative strategy we show that suppressing ASAP1 expression with siRNAs also significantly retarded cell spreading and inhibited cell migration. Furthermore, abrogation of ASAP1 function with either siRNAs or mislocalization approaches resulted in an increase of GTP loading on Arf1 and loss of paxillin from adhesions. These results taken together with our previous observations that overexpression of ASAP1 inhibits cell spreading and disturbs paxillin localization to adhesions (17) indicate that the recruitment of certain adhesion components, such as paxillin, requires dynamic turnover of Arf1 GTPase. In addition, under conditions in which paxillin recruitment to cell adhesions was compromised, tyrosine phosphorylation of paxillin was not substantially affected. These seemingly contradictory observations raise the possibility that they may possess other functions in addition to modulating Arf activity in vivo.

EXPERIMENTAL PROCEDURES

DNA Constructs—The mouse ASAP1 mammalian expression construct prokaryotic ASAP1 was a gift from Paul A. Randazzo (National Institutes of Health). This construct was generated by subcloning an N-terminal FLAG tag and the mouse ASAP1 cDNA into pcDNA3 (Invitrogen) (18). The mouse CD2AP sequence was generously provided by Andrey S. Shaw (Washington University, St. Louis, MO). To generate a FLAG-tagged full-length SH3ABC and ASH3 variants of CD2AP, standard PCR was carried out. The PCR products were digested with BamHI and NotI and subcloned into pFlag2AB (23). The glutathione S-transferase (GST)-CD2AP SH3 constructs were generated by subcloning the corresponding BamHI/NotI fragments into pGEX4T-1 (Amersham Biosciences). The Listeria ActA mitochondria-targeting sequence (mito) (22) was provided by Frank B. Gertler (MIT). AA protein 381–610 of ActA were amplified with PCR and subcloned into the Xbal and BclI sites of pEGFP-C1 (BD Biosciences) to create GFP-mito. To make the GFP-SH3ABC-mito construct, the three N-terminal SH3 domains of CD2AP were amplified and inserted into the Xhol and BamHI sites of GFP-mito. The hemagglutinin-tagged Arf1 variants (wild type, Q71L, and T31N) and the GST-GGA expression vector were provided by James E. Casanova (University of Virginia).

Identification of ASAP1 Binding Partners by Affinity Chromatography—HER293 cells were grown to 80% confluence and transfected with FLAG-tagged ASAP1. 24 h after transfection, cells were lysed with lysis buffer (20 mM HEPES-KOH, pH 7.8, 50 mM KCl, 150 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40, 10 mg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.05 units/ml of aprotinin, 1 mM sodium vanadate). Affinity purification, sequencing, and MS analysis of ASAP1-binding proteins were performed as described previously (24).

In Vitro Binding Assays and GST-GGA Pull-down Assays—GST fusion proteins were expressed in BL21 Escherichia coli and purified using glutathione-Sepharose (Amersham Biosciences). Equal amounts of GST fusion proteins or GST alone (5 mg) were incubated with 500 ml of 1 mg/ml cell lysates in modified radiomimune precipitation assay buffer (RIPA) (50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM EDTA, 5% glycerol, 0.5% Triton X-100, 10 mg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.05 units/ml of aprotinin, 1 mM sodium vanadate) at 4 °C for 1.5 h. The beads were washed twice with modified RIPA buffer and once with Tris-buffered saline. Associated proteins were subjected to SDS-PAGE and Western blotting using an anti-ASAP1 polyclonal antibody. To measure intracellular levels of Arf1-GTP, GST-GGA pull-down assays were performed as described previously (25).

Antibodies and Co-immunoprecipitation Assay—The anti-ASAP1 rabbit polyclonal antibody was a generous gift from Paul A. Randazzo (National Institutes of Health). The anti-ASAP1 mouse monoclonal antibody (mAb) was purchased from BD Biosciences. Anti-CD2AP rabbit polyclonal antibody was purchased from Santa Cruz. Anti-FLAG antibody was purchased from Sigma. Anti-paxillin mAb anti-FAK mAb, and anti-GFP mAb were purchased from BD Biosciences. Anti-paxillin phospho-Tyr31 and phospho-Tyr118 were purchased from BioSource Inc. Erk1 mAb was a gift from M. J. Weber (University of Virginia). Anti-hemagglutinin and anti-Arf1 polyclonal antibodies were provided by James E. Casanova (University of Virginia). Voltage-dependent anion channel polyclonal antibody was a product from GeneTex (Orange, CA).

To immunoprecipitate endogenous ASAP1, 5 mg of anti-ASAP1 mAb was incubated with 500 ml of phosphate-buffered saline (PBS) containing 50 ml of anti-mouse IgG-coupled agarose beads (Sigma) at 4 °C for 1 h. The beads were washed three times with cold PBS and incubated with 750 ml of clarified cell lysates at 4 °C for 2 h. Immune complexes were collected by centrifugation, washed three times with 1.0 ml of lysis buffer and resuspended by SDS-PAGE, and Western blotted with anti-ASAP1 antibody. Antibody binding was detected using horseradish peroxidase conjugated sheep anti-rabbit IgG followed by enhanced chemiluminescence (ECL, Amersham Biosciences). To immunoprecipitate endogenous CD2AP, a similar procedure was followed except that polyclonal anti-CD2AP antibody was coupled onto 50 ml of Protein A-conjugated Sepharose beads (Sigma).

To immunoprecipitate endogenous CD2AP variants, 20 ml of M2-agarose (Sigma) was incubated with 500 ml of clarified cell lysates in lysis buffer at 4 °C for 2 h and the immune complexes were subsequently subjected to the washing steps as described above.

Cell Culture, Transfection, and Immunofluorescence Microscopy—HER293 and REF52 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen), 10 mg/ml penicillin, and 0.25 mg/ml streptomycin (Invitrogen). For transient transfection experiments, cells were grown to ~70% confluence in 100-mm dishes and transfected with 4 mg of DNA using PolyFect™ (Qiagen). Twenty-four hours after transfection, REF52 cells were trypsinized and replated on fibronectin in DMEM, fixed with 4% paraformaldehyde, and immunostained as described previously (26).

Mitochondria Staining, Fractionation, and Cell Spreading Assay—To visualize the subcellular localization of mitochondria, prewarmed DMEM, 10% fetal bovine serum containing 125 mM MitoTracker Red CMXRos (Molecular Probes) was added onto REF52 cells previously plated on fibronectin. After 40 min incubation at 37 °C, the medium was removed and the cells were washed and fixed with 4% paraformaldehyde above. The mitochondrial GFP fusion proteins carrying mito-targeting sequence were detected with fluorescence microscopy. The mitochondria fractions from whole cell lysates were prepared using the procedure of Eskes et al. (27) with a modified mitochondria homogenization buffer (0.2 mM mannitol, 10 mM HEPES-NaOH, pH 7.4, 50 mM sucrose, 1 mM EDTA supplemented with proteases inhibitors). For cell spreading assays, REF52 cells were trypsinized and re-plated on fibronectin for the indicated 24 h
after transfection. The quantitation of cell spreading was performed as described previously (17, 28).

Cell Migration Assays—Cell migration was assessed using modified Transwell chambers (8 μm pores, BD Biosciences) as described previously (29). To assess fibroactin-stimulated haptotaxis, the underside of the membrane was coated with 1.5 μg/ml fibronectin suspended in PBS in the lower chamber and PBS was placed in the upper chamber. After incubating the chambers overnight at 4 °C, the fibronectin solution was removed and DMEM was added to each chamber. For EGF-mediated chemotaxis, DMEM supplemented with 10 ng/ml EGF was added to the lower chamber and unsupplemented DMEM was added to the upper chamber. REF52 cells (1 x 10⁵) were suspended in the upper chamber and allowed to migrate for 6 h at 37 °C. Nonmigrating cells were removed with a cotton swab. Cells migrating through the membrane were washed twice with PBS, fixed in 4% paraformaldehyde at room temperature for 20 min, and counted under a Zeiss Axiovert 135TV inverted microscope. Nontransfected cells were stained with crystal violet prior to counting to aid in visualization.

Preparation of siRNAs and Transfection—Target specific siRNA duplexes with symmetric 3′ dTdT overhangs were synthesized by Dharmacon. Two siRNA sequences were selected from position 902–920 (5′-CAGCUAACUGCCUCUCCGAG-3′) and position 1642–1660 (5′-UGAUUAUGGAAAGCAAU-3′) of mouse ASAP1b (GenBankTM accession number AF075462). Both siRNA duplexes reduced ASAP1 expression to a similar extent and induced similar cellular phenotypes. Transient transfection of REF52 cells with siRNAs was performed using a calcium phosphate protocol (30).

Statistical Analysis—Each experimental group was analyzed using single factor analysis of variance. p values were obtained by performing two-tailed Student’s t test using Microsoft Excel software. Statistical significance was defined as p < 0.05.

RESULTS
Identification of Arf-GAP ASAP1 Binding Partners—To better understand the role of ASAP1 in cellular processes regulating dynamic actin organization, we exploited affinity chromatography and MS to identify proteins that interact with full-length ASAP1 (Fig. 1). Using FLAG-tagged ASAP1 coupled to agarose beads, several proteins were identified as ASAP1 binding partners. MS analysis revealed sequence matches with known binding partners for ASAP1 such as FAK, as well as a novel binding protein, an 80-kDa adaptor protein CD2AP/CMS, hereafter referred to as CD2AP (Fig. 1B). Peptides identified by MS represent 42% coverage of full-length CD2AP. To further validate this interaction, FLAG-CD2AP was immunoprecipitated from extracts of HEK293 cells and ASAP1 was recovered by elution with two sequential applications of FLAG peptide followed by elution with 1% SDS. The individual fractions were analyzed with SDS-PAGE and Western blotting along with lysate control (Fig. 1C). As shown in Fig. 1C, the profile of CD2AP elution paralleled that of FLAG-tagged ASAP1, indicating CD2AP stably associates with ASAP1.

CD2AP Associates with ASAP1 in Vivo—To demonstrate that CD2AP and ASAP1 form stable complexes within the cell, we performed co-immunoprecipitation experiments. Endogenous ASAP1 was immunoprecipitated from HEK293 cell lysates using an ASAP1-specific antibody. Endogenous CD2AP was readily detected in ASAP1 immune complexes as revealed by blotting with a CD2AP-specific antibody (Fig. 2A, lane 2). The association of ASAP1 with both FAK (17) and CD2AP raises the possibility that localization of ASAP1 within the cell is spatially controlled by its binding partners. As shown in Fig. 2B, when endogenous ASAP1 was immunoprecipitated from HEK293 cell lysates, both FAK and CD2AP were found in the immune complexes. However, in a reciprocal experiment, only ASAP1, not FAK was detected in CD2AP immunoprecipitation complexes (Fig. 2C). These results indicate that intracellular ASAP1 is present in at least two pools, one associated with FAK, the other with CD2AP.

The N-terminal SH3 Domains of CD2AP Stably Associates with ASAP1 Both in Vivo and in Vitro—Both ASAP1 and CD2AP are multidomain proteins containing SH3 domains and proline-rich motifs (Fig. 1A). To map the ASAP1-binding site on CD2AP, three FLAG-tagged CD2AP variants encompassing the full-length, the N-terminal region, or the C-terminal moiety of CD2AP were expressed in HEK293 cells, immunoprecipitated with M2-agarose beads and subsequently eluted with FLAG peptide as described under “Experimental Procedures.” Lysate input (input) and bound proteins (immunoprecipitate, IP) were analyzed with SDS-PAGE and visualized by silver staining. Bands present in the ASAP1 IP lane but not present in control lanes (24) were excised and subjected to MS analysis. Positions of CD2AP and FLAG-ASAP1 are indicated. C, FLAG-tagged ASAP1 was immunoprecipitated as in B and subjected to two sequential elutions with FLAG peptide (lanes 2 and 3) and a final elution with SDS (lane 4). The recovered proteins and lysate control (lane 1) were separated by SDS-PAGE and Western immunoblotted with antibodies specific for FLAG, FAK, and CD2AP, respectively.

FIG. 1. Identification of CD2AP as an ASAP1 binding partner. A, schematic representation of the structural domains of ASAP1, CD2AP, and CD2AP variants used. The individual domains of ASAP1 are indicated: unique region, PH domain, GAP domain, ankyrin repeat, proline-rich motif, and SH3 domain. The three SH3 domains, proline-rich motif, and coiled-coil domain of CD2AP are denoted for CD2AP, full-length and the N-terminal variants of CD2AP in approximately equal amounts (lanes 3 and 6), whereas the C-terminal variant of CD2AP failed to associate with ASAP1 (lane 9). These data show that CD2AP interacts with ASAP1 through its N-terminal SH3 domains.
localization to focal adhesions, membrane ruffles, or both. Because the subcellular localization of ASAP1 may be spatially controlled during cell spreading and migration, we speculated that the depletion of endogenous ASAP1 from its correct destination would compromise its function and lead to corresponding phenotypes.

The C terminus of *Listeria monocytogenes* ActA is targeted to mitochondrial membranes when exogenously expressed in eukaryotic cells (22). As shown in Fig. 4A, expression of a fusion protein containing GFP fused to amino acids 361–610 of ActA efficiently targeted GFP to mitochondria as revealed by co-staining of mitochondria markers Mito-Tracker (Fig. 4A) and cytochrome c (data not shown). We exploited the strong association between CD2AP and ASAP1, making a GFP-expression construct in which GFP was fused to the N-terminal SH3 domains of CD2AP that in turn was fused to the mitochondria-targeting sequence of ActA (hereafter referred to as mito). We hypothesized that this fusion protein (hereafter referred to as SH3ABC-mito) would serve as a molecular sink to trap ASAP1 onto mitochondria membranes. When expressed in REF52 cells, SH3ABC-mito efficiently sequestered endogenous ASAP1 onto mitochondria as evidenced by the co-staining of mitochondria and ASAP1 (Fig. 4B, panels c and f). In parallel cell fractionation experiments, the mitochondrial fraction from SH3ABC-mito expressing cells was significantly enriched for ASAP1 compared with GFP-mito expressing cells (Fig. 4C, compare lanes 4 and 6).

To assess the consequences of mislocalization of ASAP1 on cell spreading, cells expressing SH3ABC-mito were plated on fibronectin and the rate of cell spreading was measured (Fig. 4D). SH3ABC-mito-transfected cells exhibited a significant retardation of cell spreading compared with GFP-transfected control cells after plating on fibronectin for 1 h. Four hours after the initial plating, ~50% of SH3ABC-mito-transfected cells still displayed rounded phenotypes, indicating that mislocalization of ASAP1 prevents efficient cell spreading on fibronectin.

The importance of ASAP1 in the cellular response to fibronectin stimulation was further addressed using siRNAs targeted to ASAP1 to reduce intracellular levels of ASAP1. Two different siRNAs specific for ASAP1 were transfected into REF52 cells. Each oligonucleotide was efficient at reducing ASAP1 expression (Fig. 5, panels A and B). A pool of such siRNAs reduced ASAP1 levels in REF52 cells by 75%. To assess the effects of reducing ASAP1 levels on cell spreading, cells treated with siRNAs for ASAP1 or control siRNA (for luciferase, a gene not expressed in eukaryotic cells) were plated on fibronectin and the rate of cell spreading was determined.

**FIG. 2.** Co-immunoprecipitation (IP) of CD2AP and ASAP1. A, endogenous ASAP1 was immunoprecipitated from 500 μg of HEK293 whole cell lysate using an anti-ASAP1 antibody. The presence of endogenous CD2AP in the immune complexes was assessed using a rabbit anti-CD2AP antibody. The blot was stripped and the efficiency of ASAP1 immunoprecipitation was determined by blotting with anti-ASAP1 antibody. In lane 1, 25 μg of whole cell lysates were analyzed for protein expression. B, endogenous ASAP1 was immunoprecipitated as in A and the presence of endogenous CD2AP and FAK in the immune complexes was determined using CD2AP-specific and FAK-specific antibodies, respectively. C, endogenous CD2AP was immunoprecipitated from extracts of HEK293 cells using an anti-CD2AP antibody. The presence of endogenous ASAP1 and FAK in the immune complexes was determined using ASAP1-specific and FAK-specific antibodies, respectively. IB, immunoblot.

**FIG. 3.** The N-terminal SH3 domains of CD2AP interact with ASAP1. A, FLAG-tagged full-length CD2AP and N-terminal SH3ABC variant and the C-terminal ΔSH3 variant (Fig. 1A), were transfected into HEK293 cells. The exogenously expressed CD2AP variants were immunoprecipitated from cell lysates using anti-FLAG M2-agarose beads. Bound proteins were recovered with protein sample buffer and separated by SDS-PAGE. The presence of endogenous ASAP1 in the immune complexes was analyzed using ASAP1-specific antibody (IP; lanes 3, 6, and 9). Aliquots (25 μg) of cell lysate (L, lanes 1, 4, and 7) and flow-through (FL, lanes 2, 5, and 8) were analyzed to assess the efficiency of the immunoprecipitation. B, to map the targeting site of ASAP1, variants of CD2AP-SH3 domains (SH3A, lane 3; SH3B, lane 4; SH3C, lane 5; SH3ABC, lane 6) were fused to GST. The GST fusion proteins were incubated with 500 μg of HEK293 cell lysates as described under "Experimental Procedures." The associated proteins were subjected to SDS-PAGE and Western blotting using an anti-ASAP1 antibody.
As shown in Fig. 5C, attenuation of ASAP1 levels resulted in a significant delay of cell spreading on fibronectin. These data support the observations shown in Fig. 4D, indicating that ASAP1 function is necessary for efficient signaling in response to fibronectin stimulation.

**Mislocalization or Reduced Expression of ASAP1 Inhibits Cell Migration**—Given the importance of focal adhesion turnover on cell motility and our previous report that ASAP1 overexpression disturbs focal adhesion architecture (17), the effects of ASAP1 depletion on cell motility were tested using a Transwell migration assay. Fibronectin and EGF were used as haptotaxis- and chemotaxis-promoting agents, respectively. As shown in Fig. 6A, in cells treated with siRNAs for luciferase, both fibronectin and EGF stimulated cell migration by 2–3-fold. On the other hand, siRNA-mediated reduction of ASAP1 resulted in a 50% decrease in the basal level of chemokinesis (black column). In addition, attenuation of ASAP1 expression significantly inhibited the haptotactic response to fibronectin (white column) and the chemotactic response to EGF (gray column). Of note, the ASAP1-depleted cells appeared to retain the ability to respond to EGF as evidenced by a ~2-fold increase over basal chemokinesis. A similar phenotype was obtained with ASAP1-mislocalized cells (Fig. 6B), indicating that ASAP1 function is indispensable for fibronectin-stimulated haptotaxis.

**Abrogation of ASAP1 Increases GTP Loading on Arf1 and Alters Paxillin Localization to Adhesions**—ASAP1 exhibits strong GAP activity toward Arf1 (18). To investigate the mechanism by which ASAP1 contributes to fibronectin-dependent cell spreading and migration, we took advantage of the GTP-dependent interaction of Arf proteins with GGAs (Golgi-localized, ear-containing, Arf-binding proteins), a family of Arf-dependent adaptors (31). As shown in Fig. 7A, GST-GGA fusion proteins selectively precipitated the constitutively ac-
tive, GTP-bound mutant Arf1Q71L, whereas the GDP-bound dominant negative mutant Arf1T31N was undetectable in the GST-GGA beads. The GST-GGA pull-down assay was utilized to assess the change in the GTP loading on endogenous Arf1 in ASAP1-depleted cells (Fig. 7B). As shown in Fig. 7, B and C, the suppression of ASAP1 levels led to a 40% increase of GTP loading on Arf1. Mislocalization of endogenous ASAP1 also increased Arf1-GTP to a similar extent (Fig. 7, D and E). In a parallel analysis, siRNA-mediated inhibition of ASAP1 expression failed to increase the GTP loading on Arf6 (data not shown). Thus, these data are consistent with ASAP1 function-

Arf1 GTPase has been implicated in the translocation of paxillin to cell adhesions in Swiss 3T3 fibroblasts (9). To investigate if the regulation of Arf1 by ASAP1 plays a role in paxillin localization to adhesions in vivo, we examined paxillin localization in both ASAP1-mislocalized cells and cells treated with ASAP1 siRNAs. As shown in Fig. 8A, paxillin was poorly organized in adhesions (e.g. showing an increase in diffuse cytoplasmic staining) when ASAP1 was mistargeted to mitochondria. The redistribution of paxillin was quantitated by assessing paxillin localization in transfected cells. As shown in Fig. 8B, about 40% of ASAP1-mislocalized cells exhibited a “minus cell” phenotype (e.g. poorly organized paxillin), compared with only 15% of the cells expressing GFP-mito. In parallel experiments we assessed the distribution of paxillin in cells treated with ASAP1 siRNAs and control luciferase siRNAs. As shown in Fig. 9, the reduction in ASAP1 expression resulted in a 35% increase in the number of cells exhibiting poorly organized paxillin. In contrast, treatment of cells with ASAP1 siRNAs as a GAP for Arf1 and the effects on cell adhesion being mediated, at least in part, by alteration in the level of GTP-Arf1.
Arf1-bound endogenous Arf1 was measured using an antibody specific for GST-GGA pull-down assays were performed as in Lucerase (Luc) or ASAP1. 72 h after transfection, cells were lysed and antibody. Arf was measured by immunoblotting with an anti-hemagglutinin antibody. Variants. Cell lysates were incubated with -T31N, or wild type (WT) variants. REF52 cells were transfected with hemagglutinin-tagged Arf1Q71L, Tyr118 is driven by adhesion formation, we speculated that three independent experiments (*, p value < 0.05). In each experiment ~100 cells were analyzed. had no effect on the localization of vinculin to cellular adhesions (data not shown). We and others have previously reported that overexpression of ASAP1 inhibits cell spreading and causes loss of paxillin from adhesions (17, 19). These observations taken together with the observations described above raise the possibility that the recruitment of certain adhesion components such as paxillin requires the dynamic turnover of Arf1 small GTPase rather than the translocation of paxillin to adhesions rather than the translocation of paxillin to adhesions. Thus, compromising ASAP1 function and Arf1 turnover in ASAP1-depleted cells may result in the enhanced release of tyrosine-phosphorylated paxillin from adhesions.

FIG. 7. Depletion of ASAP1 induces an increase of Arf1-GTP. A, REF52 cells were transfected with hemagglutinin-tagged Arf1Q71L, T31N WT, or wild type (WT) variants. Cell lysates were incubated with GST-GGA resins and bound proteins were resolved by SDS-PAGE as described under "Experimental Procedures." The amount of GTP-bound Arf was measured using an antibody specific for Arf1-GTP/total Arf1 were determined by quantitation of the Arf1 protein detected in the immunoblot. The data represent mean ± S.D. for three independent experiments (*, p value < 0.05). D, REF52 cells were transfected with GFP, GFP-mito, or GFP-SH3ABC-mito. 24 h after transfection, cells were trypsinized, replated on fibronectin-coated coverslips for 2 h, and stained for ASAP1 or paxillin. Representative images show GFP (panels a–c), ASAP1 (panels d–f), and paxillin (panels g–i). B, to quantitate the loss of paxillin from adhesions, the transfected cells were scored as "plus" if the cells exhibited well organized paxillin-containing adhesions or "minus" if paxillin staining was diffuse and poorly organized. The data represent mean ± S.D. for three independent experiments (*, p value < 0.05). In each experiment ~100 cells were analyzed.

FIG. 8. Mislocalization of endogenous ASAP1 to mitochondria inhibits paxillin localization to adhesions. A, REF52 cells were transfected with GFP (panels a, d, and g), GFP-mito (panels b, e, and h), or GFP-SH3ABC-mito (panels c, f, and i). 24 h after transfection, cells were trypsinized, replated on fibronectin-coated coverslips for 2 h, and stained for ASAP1 or paxillin. Representative images show GFP (panels a–c), ASAP1 (panels d–f), and paxillin (panels g–i). B, to quantitate the loss of paxillin from adhesions, the transfected cells were scored as "plus" if the cells exhibited well organized paxillin-containing adhesions or "minus" if paxillin staining was diffuse and poorly organized. The data represent mean ± S.D. for three independent experiments (*, p value < 0.05). In each experiment ~100 cells were analyzed.

DISCUSSION

In this report, we show that CD2AP, a ubiquitously expressed adaptor protein, stably associates with the Arf GTPase-activating protein ASAP1 through its N-terminal SH3 domains. Mislocalization of endogenous ASAP1 to mitochondria with a CD2AP SH3-mito fusion protein inhibits fibroconnectin-mediated cell spreading and cell migration. Using a totally different approach, we show that suppressing ASAP1 expression with siRNAs retards cell spreading and inhibits cell migration. Abrogation of ASAP1 function and cell migration. Abrogation of ASAP1 function using either mislocal-
Arf1 Cycling Regulates Adhesion Signals

Different domains of ASAP1 have been implicated in the targeting of ASAP1 to its correct intracellular localizations. ASAP1 is found in perinuclear regions, focal adhesions, and membrane ruffles (17, 18). We have reported in an earlier study (17) that ASAP1 binds to FAK through its C-terminal SH3 domain, which accounts for its localization to cell adhesions. In addition, ASAP1 possesses a phosphatidylinositol 4,5-bisphosphate-interacting PH domain (18) that could potentially target ASAP1 to plasma membrane. In this study we show that ASAP1 forms stable complexes with the adaptor protein CD2AP. As shown previously (36–39), CD2AP localizes to membrane ruffles in different cell types. Cin85, a close relative of CD2AP, is recruited to receptor tyrosine kinase-Cbl complexes upon growth factor stimulation (40). Therefore, the interaction of ASAP1 with CD2AP provides a possible mechanism by which ASAP1 localization on the plasma membrane is further refined upon growth factor receptor engagement. The observed localization of ASAP1 to the plasma membrane challenges the dogma that ASAP1 functions solely as an Arf1 GAP in that Arf6, not Arf1, is found at the cell periphery. The failure to see an increase in GTP loading of Arf6 upon inhibition of ASAP1 expression suggests that at least in REF52 cells, ASAP1 is not an efficient GAP for Arf6. Thus, a small functional pool of Arf1 is likely present at peripheral membrane regions.

Increasing evidence indicates that GTP/GDP turnover is important for the function of the Arf family of GTPases. A conceptually traditional role of Arf-GAPs is to promote the GTP hydrolysis on Arfs and thus is predicted to counter the phenotypes caused by Arf-guanine nucleotide exchange factors or catalytically active Arfs. However, as demonstrated in this report, abrogation of ASAP1 function with different approaches caused similar phenotypes to those observed with ASAP1 over-expression, suggesting an important role for the dynamic cycling of Arf1 GAPase in adhesion assembly rather than its active GTP-bound form. In the case of Arf6, the expression of a constitutively active mutant Arf6Q67L generally blocks the actin-dependent protrusive activity caused by the Arf6 guanine nucleotide exchange factor (41, 42), consistent with Arf6 cycling between active and inactive forms to function properly. In
In a recent study, Hashimoto et al. (43) showed that both Arf6Q67L and Arf6T27N inhibited the invasiveness of the breast cancer cell MDA-MB-231, indicating that a similar requirement for dynamic GTP/GDP turnover exists for Arf6 function as well.

As demonstrated in this study, both fibronectin-dependent haptotaxis and EGF-dependent chemotaxis were impaired under conditions that attenuate ASAP1 function. However, cells with significantly reduced levels of ASAP1 retained the ability to respond to EGF, albeit at reduced levels. This contrasts to the relatively complete inhibition of chemokinesis and fibronectin-stimulated haptotaxis. These results indicate that ASAP1 depletion leads to an inhibition of common pathways required for haptotaxis and chemotaxis. However, EGF may activate other signaling pathways that partially compensate for the motility defects induced by the loss of ASAP1 function. It is possible that other compensatory Arf GAPs are exploited by EGF under ASAP1-depleted conditions.

The role of ASAP1 in chemotaxis appears complex. In an earlier study, Furman et al. (44) showed that overexpression of ASAP1 enhances cell migration toward platelet-derived growth factor. In contrast, ASAP1 overexpression was shown to inhibit the formation of platelet-derived growth factor-induced membrane ruffles in a GAP-dependent manner (19). We have observed an inhibitory effect of ASAP1 overexpression on EGF-dependent chemotaxis. However, it is unclear to what extent platelet-derived growth factor and EGF signal to common migration machinery.

Arf1 is reported to mediate paxillin recruitment from a perinuclear region to adhesions (9). Both overexpression of ASAP1 (17) and abrogation of ASAP1 function alter the localization of paxillin but not vinculin to cellular adhesions. Surprisingly, tyrosine phosphorylation of paxillin was not significantly reduced under conditions in which paxillin localization was affected. Because it is generally assumed that tyrosine phosphorylation of paxillin takes place in response to FAK activation and complex formation with paxillin (45), these observations indicate that paxillin stability in adhesions may be compromised in the absence of Arf1 turnover. We suggest that Arf1 may be an important regulator of adhesion dynamics in response to integrin engagement and activation.

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