The Association of ASAP1, an ADP Ribosylation Factor-GTPase Activating Protein, with Focal Adhesion Kinase Contributes to the Process of Focal Adhesion Assembly

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ASAP1 (ADP ribosylation factor [ARF]- GTPase-activating protein [GAP] containing SH3, ANK repeats, and PH domain) is a phospholipid-dependent ARF-GAP that binds to and is phosphorylated by pp60^{Src}. Using affinity chromatography and yeast two-hybrid interaction screens, we identified ASAP1 as a major binding partner of protein tyrosine kinase focal adhesion kinase (FAK). Glutathione S-transferase pull-down and coimmunoprecipitation assays showed the binding of ASAP1 to FAK is mediated by an interaction between the C-terminal SH3 domain of ASAP1 with the second proline-rich motif in the C-terminal region of FAK. Transient overexpression of wild-type ASAP1 significantly retarded the spreading of REF52 cells plated on fibronectin. In contrast, overexpression of a truncated variant of ASAP1 that failed to bind FAK or a catalytically inactive variant of ASAP1 lacking GAP activity resulted in a less pronounced inhibition of cell spreading. Transient overexpression of wild-type ASAP1 prevented the efficient organization of paxillin and FAK in focal adhesions during cell spreading, while failing to significantly alter vinculin localization and organization. We conclude from these studies that modulation of ARF activity by ASAP1 is important for the regulation of focal adhesion assembly and/or organization by influencing the mechanisms responsible for the recruitment and organization of selected focal adhesion proteins such as paxillin and FAK.

INTRODUCTION

Attachment of cells to the extracellular matrix (ECM) is primarily mediated by the integrin family receptors (Hynes, 1992). Engagement of heterodimeric integrin receptors leads

to the clustering of integrins and recruitment of numerous proteins to form multi-protein complexes on the cytoplasmic face of the plasma membrane termed focal adhesions (BurrIDGE *et al.*, 1988). Focal adhesions serve to anchor actin cytoskeleton to the plasma membrane and to provide a linkage between the extracellular environment and the cytoplasm (BurrIDGE and Chrzanowska-Wodnicka, 1996). The recruitment of cytoskeletal proteins and the assembly of focal adhesions are functionally important for a number of cellular processes, including cell migration, survival, and proliferation (Lauffenburger and Horwitz, 1996). In the case of migrating cells (or cells spreading on ECM proteins), there is a requirement for the coordinated reorganization of the actin cytoskeleton and the formation of new attachments with the substratum (Huttenlocher *et al.*, 1995; Bretscher, 1996). This process is temporally and spatially controlled, consistent with integrins functioning as both cell adhesion receptors and as initiators of signaling cascades that convey signals from ECM to actin cytoskeleton (Bretscher, 1996).

Because integrins are catalytically inactive, their signaling ability is dependent upon the recruitment and activation of

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Abbreviations used: ECM, extracellular matrix; ARF, ADP-ribosylation factor; ASAP, ARF-GAP containing SH3, ankyrin repeats and PH domain; Cas, Crk-associated substrate; FAK, focal adhesion kinase; FN, fibronectin; FRNK, FAK-related nonkinase; GAP, GTPase-activating protein; GIT, G protein-coupled receptor kinase-interacting target; GST, glutathione S-transferase; mAb, monoclonal antibody; Pyk2, proline-rich tyrosine kinase 2; PAP, Pyk2 C terminus-associated protein; PH, pleckstrin homology; PIX, PAK-interactive exchange factor; PKL, paxillin kinase linker; REF, rat embryo fibroblasts; SH2, Src homology 2; SH3, Src homology 3.

other signaling molecules, including focal adhesion kinase (FAK) (Schaller *et al.*, 1992), pp60^{Src} family kinases (Rohr-schneider, 1980), protein kinase C (Woods and Couchman, 1992), and the Rho family of small GTPases (Schwartz and Shattil, 2000). The attachment of cells on ECM proteins results in an increase in FAK phosphorylation on tyrosine residues and the concomitant activation of FAK kinase activity (Lipfert *et al.*, 1992; Schaller and Parsons, 1994). The phosphorylation of FAK on Tyr397 creates a docking site for the SH2 domain of pp60^{Src}. Binding of pp60^{Src} to Tyr397 activates pp60^{Src} catalytic activity by displacing phosphorylated Tyr527, a Tyr residue in the C terminus of pp60^{Src} whose phosphorylation and interaction with the SH2 domain negatively regulates pp60^{Src} kinase activity (Schaller *et al.*, 1994). The structure of FAK has also provided insight to how signals are transduced from integrin receptors to the actin cytoskeleton. The N-terminal domain of FAK binds directly *in vitro* to peptides corresponding to regions of the cytoplasmic domain of β integrin subunits (Schaller *et al.*, 1995), and has recently been shown to bind to certain growth factor receptors (Sieg *et al.*, 2000). The C-terminal domain of FAK contains binding sites for a variety of molecules, including the adapter protein Crk-associated substrate (Cas) (Harte *et al.*, 1996; Polte and Hanks, 1997), the GTPase-activating protein GTPase regulator associated with FAK (Graf) (Hildebrand *et al.*, 1996; Taylor *et al.*, 1998, 1999), and the cytoskeletal proteins paxillin (Hildebrand *et al.*, 1995) and talin (Chen *et al.*, 1995). The associations of FAK with these molecules are believed to provide linkages between integrins, small GTP binding proteins, and serine/threonine kinases (Burridge and Chrzanowska-Wodnicka, 1996; Parsons *et al.*, 2000).

Members of the ADP ribosylation factors (ARF) family of small GTPases were originally identified as cofactors required for the cholera toxin-catalyzed ADP ribosylation of Gs (Kahn and Gilman, 1986). One function of ARFs is to regulate endocytosis and vesicle trafficking by controlling the interaction of coat proteins with intracellular membranes (Donaldson *et al.*, 1992; Stamnes and Rothman, 1993; Donaldson and Klausner, 1994). In addition, ARFs have recently been implicated in the regulation of cytoskeletal remodeling (D'Souza-Schorey *et al.*, 1997; Radhakrishna and Donaldson, 1997; Norman *et al.*, 1998; Song *et al.*, 1998), although the exact mechanisms by which they act are poorly understood. ARF1 is reported to mediate the recruitment of paxillin to focal adhesions and to facilitate Rho-stimulated stress fiber formation in Swiss 3T3 fibroblasts (Norman *et al.*, 1998). ARF6, the least conserved member of the ARF family, cycles between plasma membrane and endocytic compartments, depending on its nucleotide status (Radhakrishna and Donaldson, 1997). Both constitutively active and dominant negative mutants of ARF6 have been shown to cause pronounced cell morphology changes when overexpressed in cells (D'Souza-Schorey *et al.*, 1997; Song *et al.*, 1998). A function of ARFs in integrin signaling has been suggested by virtue of the identification of proteins with ARF-GTPase-activating protein (GAP) homology that bind to focal adhesion components. ASAP1 (ARF-GAP containing SH3, ANK repeats, and PH domain) is a phospholipid-dependent ARF-GAP that binds to and is phosphorylated by pp60^{Src} (Brown *et al.*, 1998). ASAP1 is found in focal adhesions, and overexpression of ASAP1 is reported to block cell spreading

and platelet-derived growth factor-induced cell ruffling (Randazzo *et al.*, 2000). The ASAP1-related protein proline-rich tyrosine kinase 2 (Pyk2) C terminus-associated protein (PAP) α /PAG3/KIAA0400 interacts with the FAK-related protein tyrosine kinase Pyk2 and paxillin (Andreev *et al.*, 1999; Kondo *et al.*, 2000). A second family of ARF-GAPs includes G-protein-coupled receptor kinase-interacting target 1 (GIT1)/CAT1/APP1 and GIT2/CAT2/paxillin kinase linker (PKL). PKL binds directly to the LD4 domain of paxillin and the guanine nucleotide exchange factor PAK-interactive exchange factor (PIX) and thus mediates the association of paxillin with a complex composed of PAK, Nck, and PIX (Turner *et al.*, 1999). The association of several ARF-GAPs with focal adhesion proteins suggests that ARF GTPases and associated GAPs are important regulators of integrin signaling pathways during cell attachment and migration.

We have used several approaches to identify proteins that stably interact with the C-terminal region of FAK. We report here the characterization of ASAP1, an ARF GTPase-activating protein that interacts via its C-terminal SH3 domain with a proline-rich motif in the C-terminal region of FAK. Endogenous ASAP1 colocalizes with FAK and can be coimmunoprecipitated with FAK from extracts of both adherent and suspended cells. Transient overexpression of wild-type ASAP1 in rat embryo fibroblasts (REF52 cells) inhibited cell spreading and focal adhesion localization of paxillin and FAK. In contrast, overexpression of a catalytically inactive form of ASAP1 or a truncated form of ASAP1 bearing a deletion of the C-terminal SH3 domain failed to substantially inhibit cell spreading and paxillin/FAK localization to focal adhesions. We suggest that the association of ASAP1 with FAK as well as its GAP activity are functionally important in the organization and/or trafficking of paxillin/FAK-containing adhesion complexes in adherent cells.

MATERIALS AND METHODS

DNA Constructs

Hexahistidine-tagged FAK-related nonkinase (FRNK; His-FRNK) has been described previously (Ma, *et al.*, 2001). For yeast two-hybrid analysis, sequences encoding the C-terminal 201 amino acids of chicken FAK 853-1053 were amplified by polymerase chain reaction (PCR) using primers FAT1 (5'-GCGGATCCCTCCAGGGCCAGCT-3') and FAT2 (5'-GCGAATTCCTAGTGGGGCCTGGACTG-3'). The resultant PCR product was cloned into the *Bam*HI and *Eco*RI sites of pGBT10, which was derived from pGBT9 (CLONTECH, Palo Alto, CA) by insertion of a multiple cloning site (5'-*Bam*HI-AatII-*Eco*RI-*Sal*I-*Pst*I-3').

The glutathione S-transferase (GST)-ASAP1 SH3 and CasL SH3 constructs were generated by subcloning the *Bam*HI/*Eco*RI fragments from clone FV38, 23, 22 (Figure 1), respectively, into pGEX3X (Amersham Pharmacia, Piscataway, NJ). The construction of GST-FRNK, GST-P2FAT, and FRNK constructs containing proline to alanine mutations has been described elsewhere (Harte *et al.*, 1996).

The mouse ASAP1 mammalian expression construct pFlagASAP1 was a generous 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, Carlsbad, CA) (Brown *et al.*, 1998). To generate the ASAP1 Δ SH3 mutant, ASAP1 cDNA was amplified by PCR using primers A5Full (5'-ATAAGCTTCGATGAGATCTTCAGCCTCCCG-3') and A3SH3 (5'-CGGAATTCACCCCGTATTGATTTTCTC-3'). The resultant PCR product was digested with *Hind*III and *Eco*RI and was sub-

Figure 1. FAK C-terminal interacting proteins. (A) FAK binding proteins and the documented sites of interaction are indicated. The amino-terminal domain, the central catalytic domain, two proline-rich motifs, and the focal adhesion targeting (FAT) domain of FAK are denoted for full-length FAK, the autonomously expressed C-terminal domain protein, FRNK, and the yeast two hybrid "bait" P2FAT. The individual domains of ASAP1 are indicated: unique region, PH domain, GAP domain, ankyrin repeats, proline-rich motif, and SH3 domain. ASAP1 SH3 clones identified in the two-hybrid screen are aligned with the full-length ASAP1 protein. (B) A FRNK affinity column was made by coupling His-tagged, bacterially expressed FRNK to Sepharose beads. Murine brain lysate was mixed with either Tris-Sepharose (lane 1) or recombinant His-tagged-FRNK-Sepharose (lane 3). As an additional control (lane 2), His-tagged-FRNK-Sepharose beads were eluted without the addition of brain lysate. Proteins recovered by incubation of the beads with elution buffer were separated by SDS-PAGE and visualized by Coomassie Blue staining as described previously (Weed *et al.*, 2000). Bands representing proteins selectively associated with FRNK (lane 3) were subjected to liquid chromatography-mass spectrometry analysis as described in "Materials and Methods." Positions of identified proteins are indicated. For ASAP1 and PAP α , 23 and 18 peptides were sequenced, providing 24 and 16% coverage of the respective proteins. (C) Yeast two-hybrid analysis of the interaction of P2FAT bait with FAK-interacting proteins. CG1945 cells were cotransformed with P2FAT bait plasmid and clones identified from two-hybrid screen. The resultant transformants were grown on Leu⁻Trp⁻His⁻ plates supplemented with 5 mM 3-aminotriazole to test *HIS3* reporter activity as described in "Materials and Methods."

cloned into pcDNA3Flag3AB (Devarajan *et al.*, 1997). The R497K mutant was created from pFlagASAP1 with primers R497K1 (5'-GTTCCGGAATCCATAAGGAAATGGGGGTTTC-3') and R497K2 (5'-GAACCCCATTTCTTATGGATTCCGGAAC-3') using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All constructs were confirmed by direct DNA sequencing.

Identification of FRNK Binding Partners by Affinity Chromatography

Recombinant FRNK (8 mg) was coupled to cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia) according to the manufacturer's instructions. Resins were suspended in 1 ml of column buffer (20 mM HEPES-KOH, pH 7.8, 50 mM KCl, 1 mM EDTA, and 1% NP40) and stored at 4°C. Coupling efficiency was ~90%. Affinity purification, sequencing, and analysis of FRNK binding proteins from murine brain extracts were done according to previously described procedure (Weed *et al.*, 2000).

Yeast Two-Hybrid Analysis

Transformation of yeast strain CG1945 was performed by the lithium acetate method (Gietz and Schiestl, 1991). β -Galactosidase activity was measured by the filter lift assay (Bartel and Fields, 1995). Yeast transformants carrying GAL4BD-P2FAT bait were transformed with a day 9.5 mouse embryonic cDNA library fused to VP16 activation domain (Joberty *et al.*, 2000) and selected for growth on Leu⁻Trp⁻His⁻ plates supplemented with 5 mM 3-aminotriazole. The positive clones were subsequently subjected to β -galactosidase

assay. Plasmid DNA was isolated from yeast by phenol extraction and was recovered in the KC8 strain of *Escherichia coli* by selection on minimal medium plates without Leu.

In Vitro Binding Assays

GST fusion proteins were expressed in *E. coli* and were purified (Smith and Johnson, 1988) using glutathione-Sepharose (Amersham Pharmacia). Equal amounts of GST fusion proteins or GST alone (5 μ g) were incubated with 500 μ l of 1 mg/ml cell lysates in modified radioimmunoprecipitation assay buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM EDTA, 5% glycerol, 0.5% Triton X-100, 10 μ g/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.05U/ml aprotinin, and 1 mM sodium vanadate) at 4°C for 2 h. The beads were washed twice with modified radioimmunoprecipitation assay buffer and once with Tris-buffered saline. Associated proteins were subjected to SDS-PAGE analysis and western blotting with FAK specific monoclonal antibody (mAb) 2A7 (Wu *et al.*, 1991) or anti-Flag mAb M5 (Sigma, St. Louis, MO).

Antibodies and Coimmunoprecipitation Assay

ASAP1-specific rabbit polyclonal antiserum 642 was a generous gift from Paul A. Randazzo. Mouse anti-FAK mAb 2A7 was described previously (Wu *et al.*, 1991). Anti-Flag mAb M5 and anti-vinculin mAb were purchased from Sigma. Anti-paxillin mAb, anti-FAK mAb, and anti-phosphotyrosine mAb RC20 were purchased from BD Transduction Laboratories (Lexington, KY). Anti-FAK phospho-Tyr397 antibody was purchased from BioSource Inc. (Camarillo,

CA). Anti-Cas was a generous gift from Amy Bouton (University of Virginia).

To immunoprecipitate endogenous FAK, 5 μ g of anti-FAK mAb 2A7 was incubated with 500 μ l of phosphate-buffered saline containing 50 μ l of Protein A-Sepharose (Sigma) complexed to rabbit anti-mouse immunoglobulin G (IgG) at 4°C for 1 h. The beads were washed three times with cold phosphate-buffered saline and were incubated with 500 μ g of clarified cell lysates in lysis buffer (20 mM HEPES, pH 7.8, 50 mM KCl, 1 mM EDTA, 1% NP-40, 10 μ g/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.05U/ml aprotinin, and 1 mM sodium vanadate) at 4°C for 2 h. Immune complexes were collected by centrifugation, washed twice with 1.0 ml of lysis buffer, separated by SDS-PAGE, and western blotted with anti-ASAP1 antibody 642. Antibody binding was detected using horseradish peroxidase-conjugated sheep anti-mouse IgG or horseradish peroxidase-conjugated protein A, followed by enhanced chemiluminescence (Amersham Pharmacia).

Cell Culture, Transfection, and Immunofluorescence Microscopy

REF52 and C3H10T1/2 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 10 μ g/ml penicillin, and 0.25 μ g/ml streptomycin (Invitrogen). For transient transfection experiments, cells were grown to 80% confluency in 100-mm dishes and were transfected with 10 μ g of epitope-tagged ASAP1 construct using SuperFect (Qiagen, Valencia, CA). Twenty-four hours after transfection, REF52 cells were trypsinized and replated on fibronectin in DMEM, fixed with 4% paraformaldehyde, and immunostained as described previously (Weed *et al.*, 1998). Endogenous ASAP1 was detected with antibody 642 (Randazzo *et al.*, 2000), and endogenous FAK was detected with anti-FAK mAb 77 (BD Transduction Laboratories). In cell spreading assays, epitope-tagged ASAP1 was detected with anti-Flag mAb. To examine the localization and organization of actin and focal adhesion proteins paxillin, vinculin, and FAK, cells were stained with Texas-Red phalloidin (to detect polymerized actin), anti-ASAP1 642 and anti-paxillin mAb, or anti-vinculin mAb or anti-FAK mAb. Rabbit IgG was detected with fluorescein isothiocyanate-labeled goat anti-rabbit, and mouse IgG was detected with Cy5-labeled goat anti-mouse (Jackson Laboratories, West Grove, PA). The transfected and nontransfected cells were distinguished by the level of ASAP1 staining.

RESULTS

Identification of FAK C-Terminal Binding Partners

To better understand the role of FAK in integrin signaling, we utilized both the yeast two-hybrid screen and affinity chromatography to identify proteins that interact with the C-terminal amino acids of FAK (Figure 1). Using an affinity matrix consisting of purified His-tagged FRNK (Figure 1A) coupled to Sepharose beads, several proteins (Figure 1B) were identified as strong FRNK binding partners. Mass spectrometry analysis of the individual bands revealed sequence matches with the ARF GTPase-activating protein, ASAP1, and a related family member, PAP α /KIAA0400 (Figure 1B). Also identified was insulin degrading enzyme (IDE), a protein implicated in the degradation of intracellular insulin (Duckworth *et al.*, 1998).

The C-terminal region of FAK contains a proline-rich PXXP motif (referred to as Site II) that interacts with the SH3 domain of Graf (Hildebrand *et al.*, 1996; Taylor *et al.*, 1998, 1999), a GAP for the Rho family of GTPases. In a parallel screen using a GAL4 fusion protein encompassing Site II and the FAT domain (P2FAT) as the bait (Figure 1A), we

identified two independent clones encoding the SH3 domain of ASAP1 (Figure 1B) from a 9.5-d mouse embryo cDNA library (Joberty *et al.*, 2000). In addition, cDNAs encoding proteins previously shown to bind to this region of FAK were also identified, including paxillin, CasL, and the paxillin homolog Hic-5. All clones expressing both GAL4BD-P2FAT and the identified mouse cDNAs were positive for cell growth on His⁻ plates (Figure 1C) and β -galactosidase expression (unpublished results), indicating strong protein-protein interactions. Because two independent interaction screens identified ASAP1 as a FAK binding protein, we proceeded to characterize the function of this interaction more carefully. Further analysis of PAP α and insulin degrading enzyme was not carried out.

ASAP1 SH3 Domain Stably Associates with Site II of FAK In Vitro

To verify the interaction of the ASAP1 SH3 domain with FAK, GST fusion proteins were produced that contained GST fused to each of the two ASAP1 SH3 sequences identified from the two-hybrid screen. In addition, GST-CasL SH3 was also generated to serve as a positive control in the pull-down assay. Lysates from mouse 10T1/2 cells were incubated with 5 μ g of each GST fusion protein, or GST alone, immobilized on glutathione beads, and the associated proteins were analyzed by SDS-PAGE and western blotting using anti-FAK mAb 2A7. As shown in Figure 2A, both GST fusion proteins containing the ASAP1 SH3 domains readily bound endogenous FAK from cell lysates (Figure 2, lanes 3 and 4). A GST fusion protein containing the CasL SH3 domain (Figure 2, lane 5) also bound efficiently to FAK, whereas GST alone failed to bind FAK (Figure 2, lane 2). These data clearly show that the SH3 domain of ASAP1 forms a stable complex with FAK in vitro.

The C terminus of FAK contains two proline-rich sequences, ... P⁷¹²PKPSRPGYPSP... and ... P⁸⁷⁴PKKPPRPGAP... , which function as binding sites for SH3 domain-containing proteins. Because the P2FAT bait used in the two-hybrid screen encompassed the second but not the first proline-rich motif of FAK, we speculated that the major binding site of the ASAP1 SH3 was the second proline-rich motif. To test this, Pro $\rightarrow$ Ala point mutations (P715A and P878A) were generated to disrupt the first and the second PXXP motifs, respectively. These point mutations were constructed in the context of FRNK, which comprises the two proline-rich sequences and the FAT domain (Figure 1A). GST-FRNK fusion proteins were generated and assayed for their ability to associate with full-length ASAP1 transiently overexpressed in 10T1/2 cells. GST-FRNK P878A, which contains a mutation within the proline-rich region proximal to the FAT domain of FAK (Site II), displayed decreased association with ASAP1, whereas P715A mutation (Site I) had no impact on this interaction (Figure 2B, lanes 4 and 5). The association of ASAP1 with FRNK was not dependent upon paxillin binding because another mutation, L1034S, which blocked paxillin binding to FAK/FRNK (Tachibana *et al.*, 1995), did not affect ASAP1 association with FRNK (Figure 2B, lane 7).

ASAP1 Associates with FAK In Vivo and Localizes to Focal Adhesions

To demonstrate that ASAP1 and FAK form stable complexes within the cell, coimmunoprecipitation experiments were

Figure 2. The SH3 domain of ASAP1 interacts with the second proline-rich motif of FAK. (A) Constructs encoding GST fusion proteins were derived from sequences identified by yeast two-hybrid analysis, including two independent ASAP1 SH3-containing clones (lanes 3 and 4) and one CasL SH3 clone (lane 5). These GST fusions or GST alone were incubated with 10T1/2 mouse fibroblast cell lysate (500 μ g) as described in "Materials and Methods," and associated proteins were analyzed by Western blotting using 2A7 anti-FAK mAb. CasL SH3 serves as positive control. Equal amounts of GST fusion proteins were used in the pull-down assay (unpublished results). (B) To map the targeting site of ASAP1, variants of GST-FRNK or GST-P2FAT were generated encoding proteins mutated in Site I (P715A, lane 4), Site II (P878A, lane 5), or in the paxillin binding domain (L1034S, lane 7). The GST fusion proteins were incubated with lysate (500 μ g) from 10T1/2 cells transiently overexpressing Flag-tagged ASAP1, and the associated proteins were eluted and subjected to SDS-PAGE and Western blotting using anti-Flag mAb. The blot was subsequently stripped and reprobed with anti-paxillin to assess paxillin binding. In A and B, 25 μ g of whole cell lysate was loaded as a control (lane 1).

carried out. REF52 or 10T1/2 cells were grown to 90% confluency, and endogenous FAK was immunoprecipitated from the whole cell lysates using a FAK-specific mAb 2A7. As shown in Figure 3A, endogenous ASAP1 was readily detected in FAK immune complexes as revealed by blotting with an ASAP1-specific polyclonal antiserum 642 (Figure 3, lanes 3 and 6). A control antibody, rabbit anti-mouse IgG (R α M), failed to immunoprecipitate FAK-ASAP1 complex (Figure 3, lanes 2 and 5). To determine if the interaction between FAK and ASAP1 is adhesion dependent, the ability of ASAP1 to coprecipitate with FAK immune complexes was compared in the lysates of continuously adherent versus

Figure 3. Coimmunoprecipitation of ASAP1 and FAK. (A) REF52 and 10T1/2 cells were grown to 90% confluency. Endogenous FAK was immunoprecipitated from 500 μ g of whole cell lysate using an anti-FAK mAb 2A7. The presence of endogenous ASAP1 in the immune complexes was assessed using ASAP1-specific antiserum 642. The blot was subsequently stripped and the efficiency of immunoprecipitation was assessed by blotting with anti-FAK mAb 2A7. Immunoprecipitation with rabbit anti-mouse IgG served as a control (lanes 2 and 5). Whole cell lysates (25 μ g) were analyzed for FAK and ASAP1 expression (lanes 1 and 4). (B) Endogenous FAK was immunoprecipitated as in A from lysates of 10T1/2 cells cultured continuously on plastic (lane 1, Ad), detached, and held in suspension for 1 h (lane 2, S), detached, held in suspension for 1 h, and replated on fibronectin (2.5 μ g/cm²) for 30 min (lane 3) or 60 min (lane 4). ASAP1 and Cas present in FAK immune complexes were detected with specific antibodies 642 and CasB, respectively. Approximately 1–5% of endogenous ASAP1 was found associated with FAK in immune complexes.

suspended or replated 10T1/2 fibroblasts. As shown in Figure 3B, ASAP1 was efficiently coprecipitated with FAK from suspended cells as well as from adherent cells. Another FAK associating protein, Cas, was also found in the FAK immune complexes in the lysate from suspended cells (Figure 3B), suggesting that a multi-protein complex containing FAK and its binding partners may exist during the process of focal adhesion turnover.

The association of ASAP1 with FAK both in vitro and in vivo points to ASAP1 being a focal adhesion protein. To confirm the subcellular localization of ASAP1, indirect immunofluorescence labeling of REF52 cells was carried out using ASAP1 antiserum 642. As shown in Figure 4, this antibody displayed strong staining of focal adhesions, (Figure 4, B and E), giving a pattern of staining identical to that observed with antibodies to paxillin or FAK, two well-characterized focal adhesion proteins (Figure 4, C and F).

Figure 4. ASAP1 is localized to focal adhesions. REF52 cells were plated on a fibronectin-coated coverslip ($2.5 \mu\text{g}/\text{cm}^2$) for 4 h without serum. Cells were fixed, permeabilized, and immunostained to detect actin (Texas Red-phalloidin, A and D), ASAP1 (anti-ASAP1 antibody 642, B and E), paxillin (anti-paxillin, C), or FAK (anti-FAK, F) as described in "Materials and Methods."

Overexpression of ASAP1 Inhibits Cell Spreading and the Localization of Paxillin and FAK to Focal Adhesions

The association of ASAP1 with FAK, coupled with its localization to focal adhesions, indicates that ASAP1 may play a role in regulating events linked to integrin signaling pathways such as the cytoskeletal changes associated with cell adhesion and spreading. To test whether the interaction between FAK and ASAP1 and/or the GAP activity of ASAP1 was functionally important for integrin-mediated cell adhesion and spreading, two ASAP1 mutants were generated (Figure 5A). ASAP1 Δ SH3 is a truncated variant that lacks the C-terminal SH3 domain and thus fails to stably bind FAK (Figure 5B, lane 3). ASAP1R497K bears an Arg $\rightarrow$ Lys mutation at a conserved position in the GAP domain and is defective for GAP activity (Randazzo *et al.*, 2000); however, it still binds to FAK (Figure 5B, lane 4). Each of the variant forms of ASAP1, along with wild-type ASAP1, were tested for their ability to perturb cell spreading on fibronectin upon expression in REF52 cells. As shown in Figure 5, ASAP1wt-transfected cells exhibited a significant inhibition of cell spreading compared with nontransfected cells at 1 h (Figure 5, C and D). Four hours after initial plating, $\sim 40\%$ ASAP1wt-expressing cells still displayed rounded phenotype (Figure 5D). Cells transfected with the GAP-deficient mutant, ASAP1R497K, showed a modest inhibition of cell spreading compared with the control cells (Figure 5D). Finally, cells expressing the Δ SH3 variant exhibited a clear inhibition of cell spreading, although the inhibitory effects were not as pronounced as that observed in cells expressing wild-type ASAP1. In an independent set of experiments, we determined that expression of GFP fusions to wild-type ASAP1 and GAP-deficient ASAP1 inhibited cell spreading to virtually the same extent as Flag-tagged wild-type ASAP1 and GAP-deficient ASAP1, respectively (unpublished results). We speculate that the inhibition of cell spreading observed in cells expressing Δ SH3 variant may reflect, in part, the negative regulation of ARF activity by this enzymatically active variant protein. The modest inhibition of spreading observed with the GAP-deficient variant

Figure 5. Transient overexpression of ASAP1 inhibits spreading of REF52 cells. (A) Diagram of ASAP1 constructs. wt denotes wild-type ASAP1; Δ SH3 denotes a variant of ASAP1 lacking the SH3 domain, and R497K denotes a variant of ASAP1 bearing a mutation in the GAP domain that inhibits GAP activity. (B) REF52 cells were transiently transfected with ASAP1wt, ASAP1 Δ SH3, and ASAP1R497K constructs. Twenty-four hours after transfection, cell lysates were prepared and GST pull-down assays were carried out using GST (lane 1) or GST-FRANK fusion proteins as described in Figure 2B. In lanes 5–7, 25 μg of cell lysates were analyzed to assess the expression level of three ASAP1 constructs. (C) A representative field of ASAP1wt-transfected cells plated on fibronectin (FN) for 1 h. Cells were trypsinized and plated on FN 24 h after transfection. Actin and ASAP1 were visualized using Texas Red phalloidin or anti-Flag epitope tag antibodies as described in MATERIALS AND METHODS. (D) REF52 cells were transiently transfected with ASAP1wt, ASAP1 Δ SH3, and ASAP1R497K, respectively. Twenty-four hours after transfection, cells were trypsinized and replated on FN for the indicated times and were immunostained for actin and ASAP1 as described in C. Cells were scored with respect to the extent of cell spreading as described previously (Randazzo *et al.*, 2000; Richardson and Parsons, 1996). The data represent mean $\pm$ SD for two independent experiments.

likely reflects interactions mediated by other domains of ASAP1.

ARFs have been implicated in membrane trafficking (Donaldson *et al.*, 1992; Stamnes and Rothman, 1993; Donaldson and Klausner, 1994) and cytoskeletal remodeling (D'Souza-Schorey *et al.*, 1997; Radhakrishna and Donaldson, 1997; Norman *et al.*, 1998; Song *et al.*, 1998). To investigate the role of ASAP1 in the regulation of the subcellular local-

Figure 6. Transient overexpression of ASAP1 alters the focal adhesion localization of paxillin. REF52 cells were transiently transfected with ASAP1wt, ASAP1ΔSH3, and ASAP1R497K. Twenty-four hours after transfection, cells were trypsinized and replated on FN-coated coverslip (2.5 $\mu\text{g}/\text{cm}^2$) for 4 h in the absence of serum. Cells were then fixed, permeabilized, and costained for actin (A, D, and G), ASAP1 (B, E, and H), or paxillin (C, F, and I). (A) The white arrow denotes the observed decrease in the overall number of actin stress fibers present in ASAP1 expressing cells. (C, F, and I) The “white stars” denote the transfected cells present in B, E, and H.

izations of paxillin and other focal adhesion components, REF52 cells were transiently transfected with ASAP1wt, ASAP1ΔSH3, or GAP-deficient ASAP1R497K, and paxillin localization was visualized as described in “Materials and Methods.” As shown in Figure 6, in ASAP1wt-transfected cells plated on fibronectin for 4 h, the distribution of paxillin was predominantly cytosolic, and paxillin localization to focal adhesions was significantly attenuated. In contrast, in cell expressing ASAP1ΔSH3 and ASAP1R497K, paxillin was almost exclusively localized to focal adhesions. To provide a quantitative measurement of these observations, paxillin localization was assessed in a population of transfected cells 4 h after initial plating. Cells exhibiting significant number (>20) of paxillin-positive focal adhesions were scored as “+ cells.” Cells that exhibited <10 paxillin-positive focal adhesions were scored as “– cells.” The designation “± cells” indicates those cells that had less than normal but more than 10 focal adhesions and/or cells that had focal adhesions with significantly smaller size. As shown in Figure 7, ~50% of ASAP1wt-transfected cells displayed a “– cells” phenotype, whereas most ($>60\%$) ASAP1ΔSH3 and ASAP1R497K-transfected cells showed a typical “+ cells” phenotype.

Although “– cells” exhibited reduced focal adhesion staining by anti-paxillin mAb, most cells still displayed some filamentous actin structures (Figure 5A, arrowhead). We suspected that other focal adhesion proteins such as vinculin could potentially drive formation of focal adhesion-like structures in the absence of paxillin in ASAP1-transfected cells. Therefore, vinculin localization was examined in cells transiently overexpressing ASAP1 constructs. As shown in Figure 8, unlike paxillin, vinculin localization was not affected by overexpression of wild-type ASAP1 (Figure

Figure 7. Quantitation of paxillin-positive focal adhesions in cells expressing wt and variant forms of ASAP1. Cells were scored for the presence of paxillin containing focal adhesions as described in “Materials and Methods” and “Results.” For each variant construct, three independent experiments were performed and 100 cells were scored for each experiment. The data represent mean $\pm$ SD of the three independent experiments.

8, d–f). Quantitation of vinculin-positive focal adhesions in transfected cells showed that $>50\%$ of ASAP1wt-transfected cells exhibited a “+ cells” phenotype (unpublished results). The effects of ASAP1 variants on FAK localization were also examined. Similar to paxillin, FAK localization was perturbed by the expression of wild-type ASAP1, but not the ASAP1ΔSH3 or the GAP-deficient mutant (Figure 8, j–l). These results, coupled with the data from above, clearly indicate that overexpression of wild-type ASAP1 inhibits cell spreading and reduces the stable association of paxillin and FAK, but not vinculin, with focal adhesion structures. These observations are consistent with paxillin/FAK and vinculin being recruited to focal adhesions by different pathways.

DISCUSSION

In this report, we show that ASAP1, an ARF GTPase-activating protein localized to focal adhesions, stably associates with FAK. The interaction of ASAP1 and FAK was initially identified using both yeast two-hybrid screen and protein affinity purification analysis. The C-terminal SH3 domain of ASAP1 selectively interacts with the second proline-rich motif in the C-terminal region of FAK. This interaction was verified by the pull-down of FAK with purified GST-ASAP1-SH3 domains, the pull-down of ASAP1 with purified GST-C-terminal FAK fusion proteins, and by coimmunoprecipitation of endogenous FAK and ASAP1 proteins. ASAP1 and FAK colocalize in focal adhesions of REF52 fibroblasts. Transient overexpression of wild-type ASAP1 significantly retarded the spreading of REF52 cells when plated on fibronectin. In contrast, overexpression of a truncated variant of ASAP1 that failed to bind FAK or a catalytically inactive variant lacking GAP activity resulted in less pronounced inhibition of cell spreading. Finally, transient overexpression of wild-type ASAP1 prevented efficient organization of paxillin and FAK in focal adhesions during cell

Figure 8. Transient overexpression of ASAP1 alters FAK but not vinculin localization. REF52 cells were transiently transfected with ASAP1wt, ASAP1 Δ SH3, and ASAP1R497K. Twenty-four hours after transfection, cells were treated as in Figure 6 and were stained for ASAP1 (a–c and g–i), vinculin (d–f), and FAK (j–l). The “white stars” denote the ASAP1-expressing cells.

spreading while failing to significantly alter vinculin localization and organization. These studies point out that regulation of ARF activity by ASAP1 is an important factor for the assembly and/or organization of focal adhesions by influencing mechanisms responsible for the recruitment and organization of selected focal adhesion proteins such as paxillin and FAK.

ASAP1 was originally identified as a tyrosine-phosphorylated substrate of pp60^{src} and was demonstrated to bind directly to the SH3 domain of pp60^{src} (Brown *et al.*, 1998). However, the multidomain nature of ASAP1 indicates that it may also bind other signaling molecules, thus possibly coordinating ARF activity with multiple signaling pathways. The identification of an interaction between ASAP1 and FAK links ASAP1 activity to the integrin signaling pathway and is consistent with the observation that other closely related ARF-GAPs, GIT1 and PAP α , bind to FAK and FAK-related protein Pyk2, respectively (Andreev *et al.*, 1999; Zhao *et al.*, 2000). In the case of PAP α , the interaction with Pyk2 is likely mediated by the C-terminal SH3 domain analogous to the interactions described above (Andreev *et al.*, 1999).

Previous experiments have shown that the pleckstrin homology (PH) domain and ANK repeats of ASAP1 are essential for its phospholipid-dependent ARF-GAP activity. An ASAP1 variant comprised of only the PH domain, the GAP

domain and the ANK repeats exhibits GAP activity *in vitro* (Brown *et al.*, 1998). However, in contrast to the putative role for the PH domains of ARF-GEFs (guanine nucleotide exchange factors) in intracellular targeting, the PH domain of ASAP1 appears dispensable for targeting ASAP1 to membrane ruffles induced by growth factors (Kam *et al.*, 2000). The association of ASAP1 with FAK through its C-terminal SH3 domain may provide a potential mechanism by which ASAP1 is both recruited to adhesion sites and is activated via interactions with phospholipids.

As demonstrated herein and shown previously, ASAP1 is found in focal adhesions, and overexpression of ASAP1 blocks cell spreading and platelet-derived growth factor-induced cell ruffling (Randazzo *et al.*, 2000). The ASAP1-related protein PAP α /KIAA0400 interacts with both FAK-related protein tyrosine kinase Pyk2 as well as paxillin (Andreev *et al.*, 1999; Kondo *et al.*, 2000). However, PAP α /KIAA0400 does not appear to accumulate in focal adhesions, which distinguishes this protein from ASAP1 (Kondo *et al.*, 2000). At this time, it is not clear whether PAP α /KIAA0400 function is redundant with that of ASAP1. A second family of ARF-GAPs consists of GIT1/CAT1/APP1 and GIT2/CAT2/PKL. GIT1, which shows considerable sequence homology to PKL, binds to paxillin and PIX and is also reported to bind directly to FAK (Zhao *et al.*, 2000). Interestingly, overexpression of GIT1 in fibroblasts causes the loss of paxillin from focal adhesions; however, these cells exhibit enhanced cell motility (Zhao *et al.*, 2000). The association of multiple ARF-GAPs with focal adhesion proteins indicates that ARF GTPases and associated GAPs are likely to be important regulators of protrusive events and are likely to influence integrin signaling pathways during cells attachment and migration.

The demonstration that FAK and ASAP1 coimmunoprecipitate from extracts from suspended cells indicates that these two proteins may form an adhesion-independent stable complex. We have previously noted that FAK and paxillin are found stably associated in extracts of avian embryo cells placed in suspension (Hildebrand *et al.*, 1995). These observations lead us to speculate that ARFs may participate in the organization of higher order complexes containing FAK, paxillin, Cas, and perhaps other adhesion proteins (e.g., PIX/Pac). These multi-protein complexes may exist at intracellular structures other than focal adhesions during focal adhesion turnover.

ASAP1 was shown to be tyrosine phosphorylated in cells expressing an activated form of Src (SrcF527) (Brown *et al.*, 1998). However, we have been unable to detect ASAP1 tyrosine phosphorylation of endogenous ASAP1 from lysates of continuously adherent 10T1/2 cells, cells kept in suspension, or cells replated on fibronectin (Y. Liu and J.T. Parsons, unpublished observations). Under these conditions, tyrosine phosphorylation of endogenous FAK is readily detected upon cell spreading as revealed by a FAK phospho-Tyr397-specific antibody (unpublished results). In addition, we failed to observe ASAP1 tyrosine phosphorylation when cells were treated with 50 μ M pervanadate for brief periods of time (unpublished results). These observations make less clear the role of tyrosine phosphorylation in ASAP1 regulation.

The observation that overexpression of ASAP1 retards REF52 cell spreading is consistent with the earlier studies of

Randazzo *et al.* (2000) in which overexpression of ASAP1 delayed the spreading of NIH 3T3 fibroblasts. As shown in both studies, the inhibition of ASAP1 on cell spreading is dependent upon its GAP activity, based on the comparison of a GAP-deficient mutant with wild-type ASAP1. In addition, we provide evidence that the interaction with FAK appears to be functionally important because a mutant lacking the C-terminal SH3 domain affected cell spreading less substantially than wild-type ASAP1. Because cell spreading on fibronectin requires the rapid formation of new adhesion complexes and subsequent focal adhesion remodeling, the observed inhibition of cell spreading by ASAP1 is suggestive of a role of ARF GTPase activity in focal adhesion dynamics.

In an earlier study using a serum-starved streptolysin-O-permeabilized fibroblasts system, Norman *et al.* (1998) showed that paxillin recruitment to focal adhesions was dependent upon ARF1. Leakage of endogenous ARF from permeabilized cells coincided with the loss of GTP γ S-stimulated redistribution of paxillin from perinuclear region to focal adhesions, whereas addition of ARF1 to the medium rescued paxillin redistribution. Our observation that ASAP1 overexpression perturbed paxillin localization to focal adhesions during cell spreading suggests that ASAP1 may regulate the assembly or organization of focal adhesions by modulating ARF1 activity in vivo. Using a cell-based ARF GAP assay, Furman *et al.* (2002) showed that ASAP1 functions as a GAP for ARF1 but not ARF6 in vivo, providing additional evidence with regard to the ARF specificity of ASAP1. The less potent impact on vinculin localization by ASAP1 overexpression indicates that regulatory proteins, such as paxillin or FAK, and structural proteins, such as vinculin, may be targeted to focal adhesions by different pathways. In addition of ASAP1, GIT1 also inhibits paxillin localization but not vinculin localization when overexpressed in cells (Zhao *et al.*, 2000). Norman *et al.* (1998) also showed that the redistribution of vinculin to focal adhesions and that of paxillin responded differently to cells permeabilization and GTP γ S addition, further implicating the existence of multiple recruitment pathways.

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