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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other signaling molecules, including focal adhesion kinase (FAK) (Schaller et al., 1992), pp60^src family kinases (Rohrschneider, 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 (Lipert 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 (GAP) homology that bind to focal adhesion. The structure of FAK has also provided insight to 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 (GAP) homology that bind to focal adhesion. The structure of FAK has also provided insight to 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 (GAP) homology that bind to focal adhesion.

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'-GCCGATCCCTCCAGGGGCCAGCT-3') and FAT2 (5'-GCCAATTCGTGCGGGCCTGACTG-3'). The resultant PCR product was cloned into the BamHI-EcoRI sites of pGBT10, which was derived from pGBT9 (CLONTECH, Palo Alto, CA) by insertion of a multiple cloning site (5'-BamHI-AatII-EcoRI-Sall-PstI-3').

The glutathione S-transferase (GST)-ASAP1 SH3 and CasL SH3 constructs were generated by subcloning the BamHI/EcoRI 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-Asp31 mutant, ASAP1 cDNA was amplified by PCR using primers A5Full (5'-ATAAGCTTGGATGAGATCTCGATCCCACTTTCGCG-3') and A3SH3 (5'-CCTGGATCTGACCGCTGTTTTTCTC-3'). The resultant PCR product was digested with HindIII and EcoRI and was sub-
plasmid and clones identified from two-hybrid screen. The resultant transformants were grown on Leu plates supplemented with 5 mM 3-aminotriazole to test HIS3 reporter activity as described in “Materials and Methods.”

**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). Anti-Flag mAb M5 and anti-vinculin mAb were purchased from Sigma. Anti-FAK mAb, anti-FAK mAb, and anti-phosphotyrosine mAb were purchased from BD Transduction Laboratories (Lexington, KY). Anti-FAK phosphotyrosine mAb RC20 was purchased from BioSource Inc. (Camarillo, CA).

**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 mAb3, and anti-phosphotyrosine mAb were purchased from BD Transduction Laboratories (Lexington, KY). Anti-FAK phosphotyrosine mAb RC20 was purchased from BioSource Inc. (Camarillo, CA).

**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 μg) were incubated with 500 μl of 1 mg/ml cell lysate 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 μg/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).
CA). Anti-Cas was a generous gift from Amy Bouton (University of Virginia).

For immunoprecipitation endogenous FAK, 5 μg of anti-FAK mAB 2A7 was incubated with 500 μl of phosphate-buffered saline containing 50 μl 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 μg of clarified cell lysates in lysis buffer (20 mM HEPES, pH 7.8, 50 mM KCl, 1 mM EDTA, 1% NP-40, 10 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.05U/ml apro- tinin, 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 horse- radish peroxidase-conjugated protein A, followed by enhanced chemiluminescence (Amersham Pharmacia).

Cell Culture, Transfection, and Immuno-fluorescence Microscopy

REF52 and CH1H10T1/2 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 10 μg/ml penicillin, and 0.25 μg/ml streptomycin (Invitrogen). For transient transfection experiments, cells were grown to 80% confluence/H9262 and were incubated with 500 ng/ml of DNA for 4 h. The transfected and untransfected cells were distinguished by the level of ASAP1 stain- ing. To immunoprecipitate endogenous FAK, 5 μg of anti-FAK mAb 2A7 was incubated with 500 μg 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 domains of ASAP1 forms a stable complex with FAK in vitro.

The C terminus of FAK contains two proline-rich sequences, 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 domain was the second proline-rich motif. To test this, Pro→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).

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, PAPA/IKAAL4 (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 (Joberly 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 pax- illin 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 PAPα 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 μg 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 domains of ASAP1 forms a stable complex with FAK in vitro.

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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
carried out. REF52 or 10T1/2 cells were grown to 90% confluency, and endogenous FAK was immunoprecipitated from the whole cell lysates 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 µg) 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 µg/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, 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).
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). ASAP1ΔSH3 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→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 5C and D). Four hours after initial plating, ∼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 ΔSH3 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 ΔSH3 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 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 localization of...
Cells were then transfected with ASAP1wt, ASAP1∆SH3, and ASAP1R497K. Twenty-four hours after transfection, cells were trypsinized and replated on FN-coated coverslip (2.5 μg/cm²) 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.

**Figure 6.** Transient overexpression of ASAP1 alters the focal adhesion localization of paxillin. REF52 cells were transiently transfected with ASAP1wt, ASAP1∆SH3, or GAP-deficient ASAP1R497K. Twenty-four hours after transfection, cells were trypsinized and replated on FN-coated coverslip (2.5 μg/cm²) 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.

Phosphorylation of paxillin and other focal adhesion components, REF52 cells were transiently transfected with ASAP1wt, ASAP1∆SH3, 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 ASAP1∆SH3 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%) ASAP1∆SH3 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 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 ASAP1∆SH3 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

ASAP1 Association with FAK
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/KIAA0400 interacts with both FAK-related protein tyrosine kinase Pyk2 as well as paxillin (Andreev et al., 1999; Kondo et al., 2000). However, PAPa/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 PAPa/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 communoprecipitate 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 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 spreading while failing to significantly alter vinculin localization. REF52 cells were transiently transfected with ASAP1wt, ASAP1ΔSH3, and ASAP1R497K. Twenty-four hours after transfection, cells were treated as in Figure 6 and were stained with a phospho-Tyr397-specific antibody (unpublished results). In Figure 6, cells were stained for ASAP1 (a–c and g–i), vinculin (d–f), and FAK (j–l). The “white stars” denote the ASAP1-expressing cells.

Figure 8. Transient overexpression of ASAP1 alters FAK but not vinculin localization. REF52 cells were transiently transfected with ASAP1wt, ASAP1ΔSH3, 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.
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 adhesions complexed 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 GTPγS-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 permeabilized and GTPγS addition, further implicating the existence of multiple recruitment pathways.

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