ARAP3 is transiently tyrosine phosphorylated in cells attaching to fibronectin and inhibits cell spreading in a RhoGAP-dependent manner

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Summary
ARAP3 is a GTPase activating protein (GAP) for Rho and Arf GTPases that is implicated in phosphoinositide 3-kinase (PI 3-kinase) signalling pathways controlling lamellipodia formation and actin stress fibre assembly. We have identified ARAP3 as a phosphorylated target of protein tyrosine kinases. In cells, ARAP3 was tyrosine phosphorylated when co-expressed with Src-family kinases (SFKs), upon stimulation with growth factors and during adhesion to the extracellular matrix (ECM) substrate fibronectin. Adhesion-induced phosphorylation of ARAP3 was suppressed by selective inhibitors of Src-family kinases and PI 3-kinase and by a Src dominant interfering mutant. Inducible expression of ARAP3 in HEK293 epithelial cells resulted in increased cell rounding, membrane process formation and cell clustering on ECM substrates. In contrast, ARAP3 dramatically slowed the kinetics of cell spreading on fibronectin but had no effect on cell adhesion. These effects of ARAP3 required a functional Rho GAP domain and were associated with reduced cellular levels of active RhoA and Rac1 but did not require the sterile alpha motif (SAM) or Arf GAP domains. Mutation of two phosphorylation sites, Y1399 and Y1404, enhanced some ARAP3 activities, suggesting that ARAP3 may be negatively regulated by phosphorylation on these tyrosine residues. These results implicate ARAP3 in integrin-mediated tyrosine kinase signalling pathways controlling Rho GTPases and cell spreading.

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Key words: ARAP3, GTPase activating protein, Rho, Src-family kinase, Fibronectin, Adhesion

Introduction
Attachment of cells to the extracellular matrix (ECM) is controlled by heterodimeric integrins (Hynes, 2002). As well as their role in cell adhesion, integrins functionally link the ECM to the actin cytoskeleton and are involved in cell spreading, motility, proliferation and survival (Burridge and Chrzanowska-Wodnicka, 1996; Hynes, 2002). Through their extracellular domains integrins bind specifically to ECM substrates such as fibronectin, laminins and collagens. Engagement with these proteins induces integrin clustering, promoting the assembly of intracellular protein complexes (e.g. focal complexes) that contain structural proteins (e.g. talin, α-actinin) that interact with the actin cytoskeleton, and signalling proteins important in cytoskeletal, growth and survival regulation (Burridge and Chrzanowska-Wodnicka, 1996; Hynes, 2002).

Rho-family GTPases including Rac1, Cdc42 and RhoA play central roles in controlling actin cytoskeletal dynamics (Hall, 1998). The activities of these proteins are tightly controlled during cell adhesion to the ECM (Hynes, 2002; Etienne-Manneville and Hall, 2002). For example, Rac1 and Cdc42 are activated by integrin-mediated cell adhesion and, via specific effectors, stimulate the actin-nucleating activity of the Arp2/3 complex leading to actin polymerisation and lamellipodia and filopodia formation at the leading edge of cells (van Aelst and D’Souza-Schorey, 1997; Parsons et al., 2000). In contrast, RhoA is first downregulated as cells attach to the ECM, such that the assembly of contractile actin-myosin stress fibres and associated focal adhesions is transiently suppressed (Ren et al., 1999; Arthur and Burridge, 2001). RhoA activity increases during spreading and stress fibre and focal adhesion formation is enhanced to provide contractile force for cell body and trailing edge retraction (Ren et al., 1999; Etienne-Manneville and Hall, 2002). Consistent with this dynamic regulation, several regulators of Rho GTPases, including guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) that promote GTPase cycling between active (GTP-bound) and inactive (GDP-bound) states, respectively, appear to participate in cell adhesion signalling (van Aelst and D’Souza-Schorey, 1997). Additionally, a number of GAPs controlling the activity
of a distinct family of small GTPases, the ADP-ribosylation factors (Arfs), which control membrane trafficking and cytoskeletal organization (Randazzo et al., 2000b), are also implicated in integrin signalling (Parsons et al., 2000).

Targeting to, and activation of, Src-family kinases (SFKs) and focal adhesion kinase (FAK) at sites of adhesion following integrin engagement with the ECM are critical initiating events in adhesion-signalling (Frame et al., 2002; Timpson et al., 2001; Klinghoffer et al., 1999; Schaller and Parsons, 1994; Kaplan et al., 1995). By phosphorylating and/or binding to a range of signalling proteins these kinases facilitate integrin-dependent regulation of both Rho and Arf GTPases (Parsons et al., 2000; Frame et al., 2002). For example, increased tyrosine phosphorylation of p130Cas and paxillin during cell adhesion generates binding sites for the SH2/SH3 adaptor protein Crk, and promotes the recruitment of the Crk-associated Rac GEF, DOCK180 (Parsons et al., 2000). However, Src-mediated phosphorylation and activation of p190RhoGAP is implicated in controlling RhoA activity during integrin-mediated cell adhesion (Arthur et al., 2000; Frame et al., 2002). Paxillin, via a phosphorylation-independent interaction also associates with PKL/GIT2/CAT2 (paxillin kinase linker), an Arf-specific GAP, which in turn binds the Rho and Cdc42 GEFs, PIX/COOL (p21 interacting exchange factor/cloned out of library) (Turner et al., 2001). DOCK180 and PIX/COOL probably mediate Rac1 and Cdc42 activation during cell adhesion responses. Two other GTPase regulatory proteins, GRAF and ASAP1/DEF, with GAP activity for Rho and Arf proteins, respectively, bind directly to FAK or its relative Pyk2 and are also implicated in the regulation of small GTPases during cell adhesion (Hildebrand et al., 1996; Randazzo et al., 2000a).

Human ARAP3 and the related proteins, ARAP1 and ARAP2, were described recently and together constitute a small family of bi-specific GAPS with activity towards both Rho and Arf GTPases (Miura et al., 2002; Santy and Casanova, 2002). ARAP3 has been implicated in growth factor and phosphoinositide 3-kinase (PI 3-kinase)-mediated signalling pathways controlling lamellipodia formation as well as the inhibition of actin stress fibre formation (Krugmann et al., 2002). No involvement of ARAP3 in integrin signalling has previously been reported. We have identified mouse ARAP3 in a cDNA library screen for tyrosine phosphorylated substrates of SFKs. We demonstrate that ARAP3 undergoes rapid and robust phosphorylation in cells attaching to fibronectin and show that its inducible expression in HEK293 cells antagonises cell spreading without affecting adhesion to fibronectin. A functional Rho GAP domain, but not an Arf GAP domain was required for this activity, providing evidence of a role for ARAP3 in the regulation of Rho proteins in cell spreading. Induction of ARAP3 expression in HEK293 cells also induces the outgrowth of membrane extensions and mutation of two key tyrosine phosphorylation sites enhances this activity, suggesting that ARAP3 may be negatively regulated by tyrosine phosphorylation.

Two cDNAs encoding ARAP3 (residues 1278-1538 and 1380-1538) were identified. Nine other clones, collectively encoding amino acids 6-1538, were isolated by hybridization of mouse embryo (Novagen), lung and spleen cDNA libraries (Stratagene) with 32P-labelled ARAP3 cDNA fragments. Expressed Sequence Tags (ESTs) (GenBank acc. no. BB647198 and BB663769), identified using BLAST searches of the NCBI database, extended the ARAP3 open reading frame by 24 nucleotides (TGAT, CCC, AATG, GCT, GCC, CCT, CAG) to include a translational start site (underlined) preceded by an in-frame stop codon (italicised). The full length cDNA predicted a protein of 1538 amino acids (aa) (GenBank acc. no. AF469621). One cDNA contained an alternative 5'-end and is predicted to result from atypical mRNA splicing of a non-coding exon to a ‘cryptic’ splice acceptor site between nucleotides (nt) 22 and 23 in the AF469621 sequence. This cDNA (GenBank acc. no. AF469622) has an alternative start site (position 244 in the AF469621 sequence) and encodes a protein of 1460 aa, lacking the SAM domain (see Fig. 1). ESTs utilizing this cryptic splice site were identified (e.g. GenBank acc. nos BY014006, BY245530, BB629587). Coding sequences for a SAM domain and proline-rich sequence in human ARAP1 were identified in human genomic sequences in the Celera database by searching for putative coding sequences similar to those in ARAP2 (Miura et al., 2002). ESTs (GenBank acc. no. BU430651 and BQ921498) and a computer predicted coding sequence based on annotated genomic sequences (GenBank acc. no. XM_355966) confirmed this region of ARAP1 is transcribed. Mouse ESTs (GenBank acc. nos B903770, Bf661009) corresponding to the entire N-terminal coding region of ARAP1 (residues 1-278) were identified by homology searches.

DNA constructs
Mammalian expression vectors encoding N-terminal FLAG-tagged and untagged forms of ARAP3 were generated by subcloning EcoRI-ClaI (aa 6-1076) and Clal-HindIII fragments (aa 1077-1538) from overlapping ARAP3 cDNA clones into pEXlox (Novagen). PCR was then used to generate DNA fragments encoding full length ARAP3 (aa 1-1538) or ARAP3 lacking its initiating methionine (aa 2-1538) flanked by Nhel or MluI sites, respectively. Fragments were subcloned into pEF-BOS or pEF-BOS FLAG (a gift from D. Huang), respectively. Vectors encoding C-terminal FLAG-tagged ARAP3 (and mutants) were generated by subcloning an EcoRI-ClaI fragment (aa 1-1076) together with a Clal-Spel fragment (aa 1077-1537, lacking stop codon) into pEF-BOS C-term FLAG (a gift from A. Verhagen). ARAP3 C-terminal FLAG variants containing point mutations in the Arf GAP domain (R527K), Rho GAP domain (R938L), tyrosine phosphorylation sites (Y1399/1404F) or combinations thereof were generated with specific oligonucleotides by overlap PCR mutagenesis as described previously (Lock et al., 1999) or using a QuikChange® II XL mutagenesis kit (Stratagene). ARAP3 expression constructs for generating inducible Fip-In™ 293 cell lines (Invitrogen) were created by digesting pEF-BOS constructs with EcoRI and Nol and subcloning the inserts into pFRT/TO (Invitrogen), pOG44 (Invitrogen) encoding Flp recombinase and pEGFP-N1 (Clontech) were purchased. An expression vector encoding activated RhoA (RhoA V14) was a generous gift from C. Hovens. A bacterial expression construct encoding glutathione S-transferase (GST) fused to ARAP3 residues 1278-1538 was generated by insertion of an EcoRI-HindIII fragment spanning this region into pGEX 4T1 (Pharmacia). PCR reactions were performed using a 4:1 ratio of Pfu (Stratagene) and Taq (Promega) DNA polymerases and all PCR products verified by sequencing.

Materials and Methods
Identification of mouse ARAP3 and a novel human ARAP1 variant
A day-16 mouse embryo cDNA expression library (Novagen) was screened for SFK substrates as described previously (Lock et al., 1999).
ARAP3 inhibits cell spreading but not adhesion

Incubator containing 10% CO₂. LNCaP cells were grown in RPMI (Invitrogen) containing 10% FCS, penicillin and streptomycin in an incubator containing 5% CO₂. To generate stable isogenic tet-inducible Flp-In™ 293 cell lines, 2×10⁶ cells were transfected with 4.5 µg pOG44 and 0.5 µg pRT/TO ARAP3 (or mutant) using the calcium phosphate procedure (Lock et al., 1999). At 24 hours post-transfection cells were split 1:10 and at 48 hours, hygromycin was added to 100 µg/ml. Hygromycin-resistant isogenic colonies were pooled (>20 colonies per pool). ARAP3 expression was induced in the Flp-In™ 293 cells by treatment with 2 µg/ml doxycycline (Sigma) for 16 hours. 293T cells were transfected by the calcium phosphate procedure (Lock et al., 1999). NIH 3T3 cell lines stably expressing ARAP3 were generated by co-transfection with pEF-BOS ARAP3 plus pGK-Puro (10:1) and selection in 5 µg/ml puromycin (Sigma). For growth factor stimulations, NIH 3T3 cell lines expressing ARAP3 or nontransfected LNCaP cells were serum-starved overnight then stimulated with 20 ng/ml human platelet-derived growth factor-BB (PDGF-BB; Invitrogen) or 100 ng/ml human epidermal growth factor (EGF; Invitrogen), respectively, for various intervals. Plasticware was coated with matrix proteins by incubation overnight with 10 µg/ml human fibronectin or laminin (both Invitrogen) in PBS at 4°C. Plates were washed with PBS, then incubated with serum-free GM for 1 hour at 37°C.

GTPase activating protein assays

Recombinant Arf proteins were preloaded with [γ-32P]GTP by incubation at 30°C for 30 minutes in 25 mM Hepes, pH 7.4, 100 mM NaCl, 2.5 mM MgCl₂, 1 mM EDTA, 1 mM ATP, 25 mM KCl, 1.25 units/ml pyruvate kinase and 3 mM phosphoenolpyruvate. The preloaded Arf proteins were diluted in a reaction buffer containing 25 mM Hepes, pH 7.4, 100 mM NaCl, 2 mM MgCl₂, 1 mM GTP, 1 mM diithiothreitol. ARAP3-FLAG or the FLAG-tagged Arf GAP regions of AGAP1 (Nie et al., 2002) or ACAP1 (Jackson et al., 2000a) were expressed in HEK 293T cells and immunoprecipitated using anti-FLAG beads. Equivalent aliquots of the purified proteins as assessed by western blotting (data not shown) were added to GTP-loaded Arfs in the presence of 360 µM phosphatidic acid (PA), 45 µM phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P²] or 1 µM trisphosphate [PtdIns(3,4,5)P³] (all from Sigma). Phospholipids were presented as mixed micelles in Triton X-100. Reactions were terminated after 2 minutes by adding ice-cold buffer containing 10 mM Tris, pH 8.0, 100 mM NaCl, 10 mM MgCl₂, 1 mM diithiothreitol. Protein-bound nucleotide was trapped on nitrocellulose filters, released into 2 M formic acid, fractionated by chromatography on polyethyleneimine cellulose plates and quantified using a PhosphorImager (Molecular Dynamics). Rho GAP activity assays were performed as described previously (Miura et al., 2002) with the exception that purified His-tagged (rather than GST-tagged) Rho-family proteins were used and assays were done in the presence or absence of 1.5 µl preloaded FLAG-ARAP3 (PH3-PH5). The integrity of the ARAP3-FLAG and FLAG ARAP3 (PH3-PH5) proteins used in the Arf GAP and Rho GAP assays was confirmed by western blotting with anti-FLAG M5 antibody (Sigma) (data not shown).

Cell spreading, adhesion and migration assays

Doxycycline-treated Flp-In™ 293 cell lines were harvested by trypsinization (3 minutes), washed once with growth medium (GM), washed three times with serum-free GM and retained in suspension at 37°C for 1 hour. Cells were then seeded at a density of 0.5-2×10⁶ cells/well on plasticware coated with fibronectin (10 µg/ml) for various times. The fraction of spreading cells (defined as cells with lamellipodia-like extension(s)) was determined by digital imaging of live cells under phase contrast microscopy with an Olympus inverted microscope (IX-50). At least 200 cells were counted for each cell type in duplicate samples in two independent experiments. An unpaired t-test was used to compare the mean rate of spreading of the Flp-In 293 control cells at different time points with those of the other cell lines. Cell spreading was also evaluated by plating cells for 0, 20, 60 and 180 minutes on coverslips treated with 10 µg/ml fibronectin. Cells were fixed in 3% paraformaldehyde (10 minutes) and stained with 1% crystal violet (30 minutes). Coverslips were mounted on microscope slides, sealed with nail polish and cells imaged by differential interference contrast (DIC) microscopy with a Nikon TE2000-E microscope fitted with a 60× oil immersion objective and an Olympus DP-70 camera. For cell adhesion determinations, 5×10⁵ doxycycline-treated cells in a volume of 100 µl prepared as described above, were seeded in quadruplicate on flat bottom 96-well plates (Nunc) that had been coated with different concentrations of fibronectin (2.5, 5, 10 and 20 µg/ml). After 35 minutes, non adherent cells were removed by washing the cells gently four times with 100 µl of pre-warmed GM using a multichannel pipette (Costar). Cells were incubated in GM at 37°C in 10% CO₂ for 1 hour after which MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) was added to 0.8 µg/ml. Cells were incubated for 2 hours at 37°C then lysed with 1 volume of lysis buffer (10% Triton X-100, 0.86% HCl) for 12 hours at 37°C. Relative numbers of attached cells were determined by measuring the absorbance at 595 nm of individual wells using an automated plate reader (PathTech Diagnostics) and Labsystems software. All cell lines displayed similar adhesion maxima when plated on 20 µg/ml fibronectin. Adhesion to lower doses of fibronectin was expressed as a percentage of that occurring on 20 µg/ml fibronectin. For cell migration assays, equivalent numbers of serum-starved and doxycycline-treated or non-treated Flp-In™ 293 cell lines (5×10³ cells) were plated in triplicate on the upper side of 8 µm pore Transwell filters (Corning Inc.) coated with fibronectin (10 µg/ml) and suspended in serum-free GM. Cells were incubated for 8 hours at 37°C, those on the upper side of filters were removed and those migrating to the underside were fixed with 4% paraformaldehyde for 6 minutes at 4°C, then stained with 1% crystal violet in 10% ethanol. Cells were washed three times in PBS, dye was extracted with 0.05 M sodium phosphate (pH 4.5) in 50% ethanol and the absorbance at 590 nm was determined. The experiment was repeated three times.

Antibodies, immunoprecipitation, immunoblot analysis and in vitro kinase assays

Polyclonal ARAP3 antibodies were raised by immunizing NZ white rabbits with purified GST-ARAP3 fusion protein (residues 1278-1538) in Freund’s complete adjuvant (Commonwealth Serum Laboratory). The specificity of this antibody was confirmed in experiments showing that pre-incubation of the ARAP3 antibodies with GST-ARAP3 immunogen (1 µg/ml) prevented detection of ARAP3 immunoprecipitated from mouse lung tissue extract (not shown). Monoclonal 4G10 phosphotyrosine (pY), Rac1 and Cdc42 antibodies (Upstate Biotechnology) and polyclonal Lyn antibodies (Santa Cruz Biotechnology) were purchased. The anti-Src monoclonal antibody has been described previously (Lock et al., 1998). Monoclonal anti-FLAG M2-agarose beads and anti-FLAG M5 antibodies were purchased from Sigma, and protein A-Sepharose from Pharmacia. Preparation of tissue and cell extracts, immunoprecipitation and immunoblot analyses have been described previously (Lock et al., 1999). For in vitro kinase assays, ARAP3 was expressed in 293T cells, immunoprecipitated with a FLAG antibody, washed in lysis buffer three times and transferred into kinase buffer (20 mM Hepes, pH 7.5, 10 mM MnCl₂, 1 mM DTT, 250 µM ATP) together with purified human Src catalytic domain (a gift from A. Weijland) or immunopurified pS653 (expressed using a baculoviral vector in S9 insect cells) (Lock et al., 1999). Samples were incubated at 30°C for 20 minutes, boiled in Laemmli buffer then analysed by SDS-PAGE and immunoblotting with a phosphotyrosine antibody.

Src Inhibitor and dominant interfering mutant experiments

For Src inhibitor experiments, replicate 6 cm dishes of 293T cells were transfected with plasmids encoding ARAP3-FLAG (2 µg) and
serum-starved overnight. For experiments examining the effects of the Src dominant negative mutant, 293T cells were transfected with expression vectors encoding ARAP3 (2 µg) and 0-10 µg of a dominant interfering Src mutant (Src K295M/Y527F) (a gift from G. Superti-Furga). Cells were trypsinised and held in suspension for 1 hour in serum-free medium at 37°C. For inhibitor studies cells were further incubated for 30 minutes in the presence of 0.2% DMSO containing PP2, SU6656 or PP3 (0-10 µM) (Calbiochem). Cells were then plated onto fibronectin-coated plasticware (10^6 cells) for 20 minutes, lysed and samples analysed by SDS-PAGE and immunoblotting.

RhoA, Rac1 and Cdc42 activity assays

Cellular levels of GTP-bound RhoA were determined using a RhoA activation assay kit (Cytoskeleton). Levels of GTP-bound Rac1 and Cdc42 were determined using the reagents specified below. Inducible Flp-In™ 293 cell lines (10^7 cells) were grown on 15 cm dishes containing GM with or without 2 µg/ml doxycycline for 16 hours. Cells were lysed in 1.5 ml cell lysis buffer (50 mM Tris pH 7.5, 0.5 M NaCl, 10 mM MgCl2, 1% Triton X-100, 0.1 µg/ml leupeptin, 0.1 µg/ml aprotinin, 5 µg/ml tosyl arginine methyl ester) and centrifuged at 13,000 g for 5 minutes at 4°C. Aliquots of clarified lysate (30 µl) were retained to evaluate total levels of RhoA, Rac1 and Cdc42. Cell lysate was mixed by rotation for 1 hour at 4°C with 45 µl of GST fusion proteins containing the mouse Rhotekin RBD (residues 7-89) (to precipitate RhoA.GTP) or residues 69-108 of human PAK1 (Maruta et al, 2002) (to capture GTP-bound Rac1 and Cdc42). Affinity complexes were washed twice with wash buffer (25 mM Tris, pH 7.5, 30 mM MgCl2, 40 mM NaCl, 150 mM EDTA), denatured in sample buffer then analysed by SDS-PAGE and immunoblotting with specific antibodies.

Results

Identification of mouse ARAP3

To identify possible tyrosine phosphorylated substrates of Src-family kinases (SFKs), we screened a murine embryonic cDNA expression library for products that could be phosphorylated by Lyn (Lock et al., 1998; Lock et al., 1999). Two of the cDNA products identified were C-terminal fragments of the mouse homologue of a recently described human protein, ARAP3 (Arf GAP Rho GAP-Ankyrin repeat, PH protein 3) (Krugmann et al., 2002). Both products contained just two tyrosines (Y1399 and Y1404) located within a sequence context that closely resembles the optimal phosphorylation sites for vSrc and Lyn (Zhou and Cantley, 1995; Ruzzene et al., 1997). cDNAs spanning the entire ARAP3 coding region were subsequently isolated and predicted a protein of 1538 amino acids (GenBank acc. no. AF469621) sharing 88% identity overall with human ARAP3. Like its human counterpart (Krugmann et al., 2002) mouse ARAP3 contains a sterile alpha motif (SAM) domain, five pleckstrin homology (PH) domains, an Arf GAP domain, a Rho GAP domain and a Ras association (RA) domain (Fig. 1A). The putative phosphorylation sites, Y1399 and Y1404 (Fig. 1A), are conserved in human ARAP3 but are not present in the related ARAP1 and ARAP2 proteins (see Fig. S1 in supplementary material) (Miura et al., 2002). An ARAP3 cDNA with a unique 5’-end (GenBank acc. no. AF469622) with a ‘cryptic’ splice acceptor site was also isolated (Fig. 1B). The ATG start site that initiates full length ARAP3 is absent from this cDNA and translation is therefore predicted to initiate at an alternative Kozak consensus start site (corresponding to methionine 79) to generate a 1460 residue ARAP3 isoform lacking a SAM domain (designated ARAP3ΔSAM, see Materials and Methods). When transfected in 293T cells these cDNAs were found to encode proteins of 190 and 180 kDa, respectively (Fig. 1B), species which co-migrate precisely with the two endogenous ARAP3 proteins found in murine tissues (not shown) and cells (Fig. 1B). Several mammalian cell lines were also found to express two ARAP3 isoforms (see Fig. S2 in supplementary material; and not shown). These results suggest that the two major forms of ARAP3 detected in mammalian cells are ARAP3 and ARAP3ΔSAM.

ARAP3 belongs to a family of bi-specific GAPs that includes ARAP1 and ARAP2 (Krugmann et al., 2002; Miura et al., 2002). However, while ARAP2 and ARAP3 have an N-terminal SAM domain, no analogous region has been identified in ARAP1 (Miura et al., 2002). We wondered if an alternative form of ARAP1 with a SAM domain might also exist. Genomic database searches revealed that the human and mouse ARAP1 genes do indeed contain putative coding sequences for a SAM domain as well as an extended proline-rich linker region. ESTs further confirmed that this region is transcribed and predict an ARAP1 variant where the first nine residues of...
ARAP1 (Miura et al., 2002) are replaced with 249 unique amino acids. Fig. S1 in supplementary material shows a sequence alignment of the human ARAP proteins (Miura et al., 2002; Krugmann et al., 2002) with their mouse counterparts (this work).

Mouse ARAP3 is a bi-functional GAP in vitro
Human ARAP3 displays phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3]-dependent GAP activity for Arf6, as well as phosphoinositide-independent GAP activity for RhoA, Rac1 and Cdc42 in vitro (Krugmann et al., 2002). Mouse ARAP3 also exhibited PtdIns(3,4,5)P3-dependent Arf GAP activity, which moreover was independent of other phospholipids including phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P2/PIP2) or phosphatidic acid (PA; Fig. 2A). Interestingly, however, mouse ARAP3 showed a preference for Arf5 rather than Arf6 (Fig. 2B). The low Arf6 GAP activity detected for ARAP3 was in marked contrast to that of another Arf GAP protein, AGAP1, preferentially stimulated on Arf6, as well as phosphoinositide-independent GAP activity (Krugmann et al., 2002). Mouse ARAP3 also exhibited PtdIns(3,4,5)P3-dependent GAP activity in vitro (Fig. 2B). Like human ARAP3, mouse ARAP3 was an efficient GAP for RhoA, but in contrast to human ARAP3, it lacked Rac1 and Cdc42 GAP activity in vitro (Fig. 2C). The relatively minor sequence differences between the mouse and human Arf GAP and Rho GAP domains (which share 90 and 97% identity, respectively; Fig. 1A) seem unlikely to account for the apparent difference in specificity. However, several differences in the assays used to measure GAP activity may have a bearing on this. In particular, human ARAP3 Rho GAP activity was assessed by measuring its ability to stimulate the release of inorganic phosphate from GST-tagged Rho proteins (caused by GTP hydrolysis) at a single time point. By contrast mouse ARAP3 Rho GAP activity was assessed more directly by determining the levels of non hydrolysed GTP remaining associated with His-tagged Rho proteins at several time points. Despite apparent differences in GAP specificity, it is clear that mouse ARAP3 has GAP activity for Arf and Rho proteins in vitro.

Src-kinases phosphorylate ARAP3 in cells and in vitro
We identified ARAP3 cDNA products based on their ability to serve as substrates for Src-family kinases when immobilized on a solid nitrocellulose support. Given the artificial nature of this system, we tested whether full length ARAP3 could also serve as a target for SFKs when expressed in mammalian cells. FLAG-tagged or non-tagged forms of ARAP3 were co-expressed with Lyn, Src or a control vector in 293T cells. Immunoprecipitates of the ARAP3 proteins were then analysed by SDS-PAGE followed by immunoblotting with a phosphotyrosine (pY) antibody. Fig. 3A shows that epitope-tagged and untagged ARAP3 proteins were readily tyrosine phosphorylated under these conditions. Immunoprecipitates were then analysed by SDS-PAGE followed by immunoblotting with a phosphotyrosine (pY) antibody. Fig. 3A shows that epitope-tagged and untagged ARAP3 proteins were readily tyrosine phosphorylated under these conditions.
phosphorylated by Lyn and Src. Additionally, both SFKs co-immunoprecipitated with ARAP3, showing that these SFKs can stably interact with ARAP3 in cells (Fig. 3A). In vitro pull-down assays further revealed that the SH2 and SH3 domains of Lyn could co-operatively bind ARAP3 in a tyrosine phosphorylation-dependent manner (not shown). Substitution of Y1399 and Y1404 with phenylalanine residues (Y1399/1404F) dramatically reduced the levels of ARAP3 phosphorylation mediated by Src, suggesting that these residues are the major phosphorylation sites recognized by Src in cells (Fig. 3B). By comparison, Lyn-mediated phosphorylation of the Y1399/1404F mutant was only slightly reduced, implying that ARAP3 contains additional tyrosines that are phosphorylated efficiently by Lyn but not by Src (Fig. 3B). We also tested whether ARAP3 could be directly phosphorylated on tyrosine residues by Src kinases using in vitro kinase assays. As shown in Fig. 3C, tyrosine phosphorylation of ARAP3 was negligible in the absence of Src kinases but increased in a dose-dependent manner when ARAP3 was incubated with purified c-Src (kinase domain) or baculovirally expressed Lyn kinase.

Growth factors stimulate ARAP3 tyrosine phosphorylation

Stimulation of PAE endothelial cells with platelet-derived growth factor (PDGF), or PC12 cells with epidermal growth factor (EGF) was previously shown to promote a PI 3-kinase-dependent increase in membrane ruffle-localized ARAP3 (Krugmann et al., 2002). We suspected that these growth factors, by activating their cognate receptor tyrosine kinases (RTKs), might also regulate ARAP3 tyrosine phosphorylation. Indeed, PDGF stimulated the phosphorylation of ectopically expressed ARAP3 in NIH 3T3 cells, while EGF promoted the tyrosine phosphorylation of endogenous ARAP3 (both isoforms) in human tumour cell lines including LNCaP prostate cancer cells (see Fig. S2 in supplementary material) and A431 epidermoid carcinoma cells (not shown). These data suggest that ARAP3 as well as being a substrate of SFKs, is a novel target in growth factor-stimulated signal transduction pathways.

ARAP3 is transiently tyrosine phosphorylated in cells adhering to fibronectin

Because ARAP3 can serve as a Lyn and Src substrate in vitro and in cells, and SFKs together with focal adhesion kinase (FAK) are known to be activated as a consequence of integrin binding to extracellular matrix (ECM) substrates (e.g. fibronectin) (Schaller and Parsons, 1994; Kaplan et al., 1995), we investigated whether the phosphorylation state of ARAP3 was modulated by cell adhesion to the ECM. For these studies the tetracycline-inducible Flp-InTM T-RExTM system was used to express ARAP3 in HEK293 cells. The 293 cell line has been used previously as a model system to study fibronectin-stimulated signalling mediated by endogenous integrin receptors and protein tyrosine kinases including Src and FAK (Schlaepfer Fig. 3. Src-kinases phosphorylate ARAP3 at sites including Y1399 and/or Y1404 in cells and can phosphorylate ARAP3 in vitro. (A) ARAP3 (left panels) or FLAG-ARAP3 (right panels) were expressed with Lyn, Src or alone (none) in 293T cells. Cell lysates were immunoprecipitated (IP) with an ARAP3 antibody and analysed by SDS-PAGE and western blotting (WB) with phosphotyrosine (pY), ARAP3, Src or Lyn antibodies. (B) FLAG-ARAP3 (ARAP3 WT) and FLAG-ARAP3 with tyrosines 1399 and 1404 substituted for phenylalanines (Y1399/1404F) were expressed (+) with Lyn (L) or Src (S) in 293T cells. Cell lysates were immunoprecipitated (IP) with anti-pY and anti-ARAP3 antibodies. (C) FLAG-ARAP3 expressed in 293T cells and purified with a FLAG antibody was incubated with the indicated concentrations of purified Src kinase domain (upper panels) or with increasing amounts of immunopurified baculovirally expressed Lyn (lower panels) in in vitro kinase buffer containing ATP. Samples were analysed by SDS-PAGE and immunoblotting with the indicated antibodies.

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and Hunter, 1997). In addition to cells expressing wild-type ARAP3, we also generated cells expressing ARAP3ΔSAM (ΔSAM) or ARAP3 variants with inactivating mutations in the Arf GAP and/or Rho GAP domains (R527K, R938L and R527K/R938L) or with mutated tyrosine phosphorylation sites (Y1399/1404F) (Fig. 4A). Because the FlpIn™ 293 cell line contains a single Flp recombinase target (FRT) site for insertion of the different ARAP3 cDNAs, all cell lines generated were isogenic, having the same chromosomal integration site. Treatment of the cell lines with doxycycline (Dox), a tetracycline analogue, for 16 hours induced the expression of all six ARAP3 proteins to identical levels (Fig. 4B). Contrasting with this, the levels of endogenous p120RasGAP (RasGAP) and p85α, the regulatory subunit of PI 3-kinase, remained constant (Fig. 4B). Immunofluorescent staining of cell lines with an anti-ARAP3 antibody revealed that >99% of the treated cells expressed the given ARAP3 protein (not shown). ARAP3 proteins localized in the cytoplasm as expected (data not shown) (Krugmann et al., 2002) but could also be readily detected in F-actin-rich membrane ruffles and lamellipodia at the cell periphery in some cells (not shown).

To examine ARAP3 tyrosine phosphorylation during

Fig. 4. ARAP3 is tyrosine phosphorylated during attachment of HEK293 cells to the ECM substrate fibronectin. (A) Diagram of ARAP3 proteins introduced into tet-inducible Flp-In™ T-REx™ HEK293 cell lines. Crosses designate domains with mutated ‘catalytic arginine’ residues required for GAP activity. (B) Stable Flp-In™ T-REx™ HEK293 cell lines (pools of >20 isogenic clones) harbouring stably integrated cDNAs for the indicated ARAP3 proteins were treated (+) or not treated (–) with 2 µg/ml doxycycline (DOX) for 16 hours. Cell lysates (20 µg protein) were analysed by SDS-PAGE and western blotting (WB) with antibodies against ARAP3, p120RasGAP and p85α (the PI 3-kinase regulatory subunit). (C) HEK293 Flp-In™ cell lines treated for 16 hours with doxycycline to induce expression of the indicated ARAP3 proteins were trypsinised, washed and resuspended in serum-free medium for 1 hour. Suspension cells (0 minutes) or cells plated onto fibronectin (FN)-coated plates for the indicated times were lysed and immunoprecipitated with anti-FLAG then subjected to SDS-PAGE and western analysis with anti-phosphotyrosine (pY) then anti-FLAG antibodies (right panels). (D) The indicated HEK293 Flp-In™ cell lines were treated as in C. Suspension cells were pre-incubated with 15 µM LY294002 (+) or DMSO (–) for 20 minutes and the cells plated onto fibronectin for 30 minutes. FLAG-ARAP3 proteins were analysed as described in C. (E) Serum-starved 293T cells expressing ARAP3 were harvested and held in suspension for 1 hour then incubated for 30 minutes with the indicated concentrations of the Src inhibitors PP2 and SU6656 and the negative control compound PP3. Cells were plated onto fibronectin-coated plastic (10 µg/ml) for 30 minutes and lysed. Samples were analysed by western blotting with phosphotyrosine and ARAP3 antibodies. (F) 293T cells were transfected with plasmids encoding ARAP3 (2 µg) and the indicated amounts of a dominant interfering Src mutant (Src KD). Cells were plated on fibronectin for 30 minutes and samples of the cell lysate analysed as described in E.
ARAP3 affects HEK293 cell morphology via its Rho GAP domain

We observed that expression of ARAP3 had a profound effect on HEK293 cell morphology either when the cells were cultured on tissue culture plastic (not shown) or on surfaces coated with ECM substrates. Fig. 5 shows that cells grown on fibronectin in the presence of doxycycline for 16 hours (+Dox) to induce ARAP3 expression, had a highly refractive, rounded appearance and a marked propensity to form foci-like clusters. By contrast, cells not expressing ARAP3 (−Dox) formed a monolayer and included only a low proportion of refractive and rounded cells. Cells expressing the naturally occurring ARAP3ASAM isoform, the Arf GAP mutant, R527K, or the mutant lacking the putative phosphorylation sites that were identified, Y1399/1404F, appeared similar to those expressing ARAP3. By contrast, mutation of the Rho GAP domain, either in wild-type ARAP3 (R938L, Fig. 5 and K977A, not shown) or in an Arf GAP-defective ARAP3 (R527K/R938L) abolished the morphological effects of these proteins.

Expression of ARAP3 in the Flp-In™ 293 cells also promoted the outgrowth of elongated and irregular membrane projections when compared to untreated cells or control Flp-In™ 293 cells treated with doxycycline (not shown, also see Fig. 7B). To quantify this effect, stable cell lines inducibly expressing ARAP3 proteins, were grown on the ECM substrates, fibronectin or laminin, for 16 hours. The proportion of cells with one or more membrane processes of >1 cell diameter were then scored. Fig. 6A shows that ARAP3, promoted dramatic increases in the formation of elongated processes, irrespective of whether cells were grown on laminin or fibronectin. The Arf GAP mutant R527K was even slightly more efficient at promoting the formation of elongated processes than wild-type ARAP3 but, strikingly, its capacity to induce membrane extensions was completely abolished by a further point mutation in the Rho GAP domain (R527K/R938L) (Fig. 6A). These results imply that a functional Rho GAP but not an Arf GAP domain is necessary for ARAP3-mediated membrane process formation. The Y1399/1404F mutant was also more effective than wild-type ARAP3 in promoting the outgrowth of membrane processes, suggesting that intact tyrosine residues may, upon phosphorylation, negatively regulate the activity of ARAP3.

Both mouse and human ARAP3 exhibited GAP activity towards RhoA in vitro (Fig. 2C) (Krugmann et al., 2002). Therefore we tested whether the increased formation of membrane projections in cells expressing ARAP3 might be related to an ability to antagonize RhoA activation in cells. Doxycycline-treated stable cell lines expressing ARAP3, ARAP3ASAM, R527K, R938L or Y1399/1404F were co-transfected with plasmids encoding enhanced green fluorescent protein (GFP) (to reveal transfected cells) plus a constitutively active form of RhoA (RhoA V14), which is insensitive to Rho GAP-mediated downregulation. As a control, cells were transfected with the GFP marker plasmid and an empty protein (GFP) (to reveal transfected cells) plus a constitutively active form of RhoA (RhoA V14), which is insensitive to Rho GAP-mediated downregulation. As a control, cells were transfected with the GFP marker plasmid and an empty
ARAP3 perturbs cell spreading in a Rho GAP-dependent manner

Our findings showing that ARAP3 is rapidly phosphorylated on tyrosine residues during cell adhesion and that constitutive expression of ARAP3 dramatically alters cell morphology suggested that ARAP3 may regulate cell spreading or adhesion. To evaluate the effects of ARAP3 on cell spreading, cell lines were treated overnight with doxycycline, suspended in serum-free medium for 1 hour then seeded onto fibronectin-coated coverslips or cell culture dishes. Cell spreading was monitored at fixed time intervals by phase contrast microscopy of live cells (data presented graphically in Fig. 7A) or by differential interference contrast (DIC) microscopy of fixed cells stained with crystal violet (Fig. 7B). As expected, cells of all six genotypes showed no evidence of spreading at

Fig. 5. Inducible expression of ARAP3 in HEK293 cells affects cell morphology in a Rho GAP-dependent manner. The stable Flp-In™ T-REX™ cell lines indicated were plated onto fibronectin-coated (10 µg/ml) plasticware in the presence (+ Dox) or absence of 2 µg/ml doxycycline (–Dox) for 16 hours. Digital phase contrast microscopic images representative of more than five experiments are shown.

Fig. 6. ARAP3-mediated membrane process formation is Rho GAP dependent, blocked by activated RhoA expression, and enhanced by mutation of the tyrosine phosphorylation sites Y1399 and Y1404. (A) The indicated Flp-In™ T-REX™ 293 cell lines were grown overnight on laminin- or fibronectin-coated dishes in the presence (+) or absence (–) of 2 µg/ml doxycycline (Dox). Multiple random fields of cells were imaged as in Fig. 5 and the proportion of cells with process(es) >1 cell diameter was scored. Between 200 and 350 cells were scored per sample. Data are means ± s.e.m. from duplicate determinations in two independent experiments. (B) Cell lines treated with doxycycline overnight to induce ARAP3 proteins were transfected with plasmids encoding green fluorescent protein (GFP) plus a 10-fold excess of RhoA V14 or empty vector (Vector). Live transfected cells expressing GFP were visualised 24 hours post-transfection by confocal fluorescence microscopy using a water immersion 60× objective.
t=0 minutes (Fig. 7A). However, by 20 minutes, striking differences in the rates of cell spreading by the different cell lines were apparent. Whereas approximately 20±6% of the control Flp-In™ 293 cells exhibited clearly discernible lamellipodia-like protrusions after 20 minutes, significantly fewer ARAP3-expressing cells (6.7±4%) and even fewer Y1399/1404F and R527K cells, (3.9±3% and 3.5±2%, respectively) were spreading on the fibronectin at this time. However, the impaired spreading of the ARAP3- and R527K-expressing cells was reversed by the R938L mutation abolishing Rho GAP function. Remarkably, cell spreading was significantly faster in R938L and R527K/R938L-expressing cells, with 52±8% and 61±7% of cells, respectively, forming lamellipodia within 20 minutes (Fig. 7A). Maximum levels of cell spreading in the Flp-In™ control cells and the R938L and R527K/R938L cell lines was apparent at 60 minutes (with 78±15%, 89±7% and 88±9%, respectively, of the cells spreading) and remained relatively constant for 3 hours. By comparison, cell spreading by the cell lines expressing ARAP3, R527K and Y1399/1404F was significantly slower during the first 60 minutes of the time course, with maximal cell spreading only evident after 3 hours (85±7%, 85±11% and 87±3%, respectively). The morphological changes of the Flp-In™ 293 cell lines spreading on fibronectin are shown in Fig. 7B. Cells expressing ARAP3 or the R527K and Y1399/1404F mutants had a much lower capacity to form broad lamellipodia-like protrusions and to spread on fibronectin than the control Flp-In™ 293, R938L and R527K/R938L cells. Differences in cell spreading were clearly evident by t=20 minutes and further accentuated at ≥60 minutes with ARAP3, R527K and Y1399/1404F cells retaining a more rounded phenotype and displaying highly developed and branched membrane processes. By contrast, control cells and those expressing ARAP3 forms lacking Rho GAP activity (R938L and R527K/R938L) were considerably flatter and more regular in shape and had few if any membrane outgrowths.

ARAP3 does not interfere with cell adhesion but inhibits cell migration

Given the impaired capacity of cells expressing ARAP3 to spread on fibronectin and the loosely adherent appearance of these cells, we were interested in determining whether this was associated with altered adhesion of the cells to fibronectin. We examined this by quantitatively and qualitatively evaluating cell spreading and adhesion using a fibronectin-coated Boyden chamber assay and differential interference contrast (DIC) light microscopy. These data are shown in Figs. 7A and B.
ARAP3 inhibits cell spreading but not adhesion

In preliminary experiments we found no clear effect of any ARAP3 protein on adhesion of the Flp-In™ 293 cell lines to plastic coated with 10 µg/ml fibronectin (not shown). However, it was possible that small differences in adhesiveness could be masked at this concentration of fibronectin. We therefore examined the ability of cells to adhere to a range of concentrations of fibronectin (2.5, 5, 10 or 20 µg/ml). Cells were allowed to adhere for 35 minutes, a time selected because it reveals clear differences in the cell spreading properties of the different cell lines (Fig. 7A). Cells were washed to remove non-adherent cells and the relative numbers of cells adhering to the fibronectin was quantified by using the MTT metabolic activity assay. Values were then normalized by expressing them as a percentage of the levels of adhesion observed on 20 µg/ml fibronectin (arbitrarily set at 100%). Fig. 8A shows a uniform decline in the adhesive capacity of all cell lines with the decreasing concentrations of fibronectin. No clear differences in the adhesive capacity were apparent even at the lowest concentration of fibronectin tested. Notably, at concentrations below 2.5 µg/ml, fibronectin-dependent and -independent (background) adhesion were indistinguishable (not shown). We conclude from these data that ARAP3, while perturbing cell spreading, does not significantly inhibit cell adhesion to fibronectin.

Cell spreading is functionally related to the early stages of cell migration (Small et al., 2002) so we examined the effects of ARAP3 induction on cell motility. A Transwell filter migration assay was used. The ARAP3 and R938L cell lines were treated (or not treated) with doxycycline and serum-starved, then allowed to migrate through Transwell filters coated on both sides with fibronectin (10 µg/ml). After 8 hours, migrating cells that had reached the lower side of the filters were stained with crystal violet and the dye extracted and quantified spectrophotometrically. Fig. 8B shows that expression of ARAP3 weakly but significantly suppressed migration, whereas expression of the Rho GAP defective mutant, R938L, had no effect on cell motility. These results imply that ARAP3 inhibits cell migration in a Rho GAP-dependent fashion.
ARAP3 causes a Rho GAP-dependent decrease in active RhoA and Rac1 in vivo

We examined the consequences of ARAP3 overexpression on the cellular levels of active (GTP-bound) RhoA, Rac1 and Cdc42. The ARAP3 and R938L inducible Flp-In™ cell lines were treated or not treated with doxycycline overnight. Cell extracts were then incubated with GST-fusion proteins containing either the rhotekin Rho binding domain (RBD), which binds specifically to the active GTP-bound form of RhoA, or with the Rac/Cdc42 binding domain of PAK1, which binds the GTP-bound forms of Rac1 and Cdc42. Levels of active RhoA, Rac1 and Cdc42 were then compared with the total levels of the GTPases by western analysis with specific antibodies. Fig. 9A shows that doxycycline-induced expression of ARAP3 was associated with a marked reduction in active RhoA and to a lesser degree Rac1, but not Cdc42. A functional Rho GAP domain was required for this activity as expression of the R938L mutant did not alter the levels of GTP-bound RhoA, Rac1 or Cdc42.

Discussion

A multitude of cytoplasmic proteins with the ability to positively or negatively regulate the activities of small GTPases of the Rho and Arf families have been identified. Approximately 60 Rho GEFs and over 70 Rho GAPs are expressed in mammalian cells (Etienne-Manneville and Hall, 2002) while genes for a further nine mammalian Arf GEFs (Jackson and Casanova, 2000; Jackson et al., 2000b) and at least 26 Arf GAPs have been reported (Bernards, 2003). Cellular regulation of GTPases is undoubtedly complex with the number of GTPases (six mammalian Arfs and 16 Rho GTPases) far outnumbered by their putative regulators (Randazzo et al., 2000b; Etienne-Manneville and Hall, 2002). Several GEFs and GAPs are implicated in adhesion-dependent signalling following integrin engagement with the ECM. Among these regulatory proteins are the Rac1- and Rac1/Cdc42-specific GEFs, Dock180 and PIX/COOL, which are recruited to focal complexes via indirect associations with p130Cas and paxillin (Parsons et al., 2000; Turner et al., 2001). Arf GAPs such as PKL/GIT2/CAT2, ASAP1/DEF1 and PAPz/PAG3 (Turner et al., 2001) and Rho GAPs including p190RhoGAP and GRAF are also known to associate with integrin-linked focal complexes and structures such as invadopodia (Nakahara et al., 1998; Hildebrand et al., 1996). We present evidence that ARAP3, a bi-specific GAP for Arf and Rho proteins may also contribute to the regulation of adhesion-stimulated signalling pathways.

Human ARAP3 was identified recently using a unique affinity-based method to purify proteins capable of binding specifically to phosphoinositide second messengers, such as PtdIns(3,4,5)P3, generated by PI 3-kinase (Krugmann et al., 2002). Functional studies confirmed that human ARAP3 Arf GAP catalytic activity and its ability to translocate to, and perturb, lamellipodia formation in growth factor-stimulated cells required PtdIns(3,4,5)P3, binding (Krugmann et al., 2002). We cloned the cDNA for mouse ARAP3 in a screen for SFK substrates and show that alternative splicing generates two distinct ARAP3 isoforms containing, or lacking, an N-terminal SAM domain. Intriguingly, SAM domain-containing and -lacking ARAP1 variants also appear to exist. Until now, tyrosine phosphorylation of ARAP proteins has not been documented. Our results indicate that ARAP3 undergoes tyrosine phosphorylation in cells under a variety of conditions; when co-expressed with SFKs, in response to growth factor stimulation with PDGF and EGF, and in cells adhering to the ECM substrate fibronectin. Src-kinases are likely to play a role in phosphorylating ARAP3, based on evidence that ARAP3 can be phosphorylated by Src kinases in vitro and that adhesion-dependent phosphorylation of ARAP3 is selectively inhibited by a dominant interfering Src mutant and the Src-family inhibitors, PP2 and SU6656. These findings suggest a general involvement of ARAP3 in growth factor and integrin receptor signalling pathways. Interestingly, adhesion-dependent tyrosine phosphorylation of ARAP3 was also partially inhibited by LY294002, implying that ARAP3 binding to products of PI 3-kinase may facilitate recruitment of ARAP3 to sites of PTK activation. The Arf GAP proteins ASAP1/DEF1 and PAPz/PAG belong to a large family of PH domain-containing proteins that also includes ARAP3 (Jackson et al., 2000b). It is therefore interesting that these proteins also interact with and undergo phosphorylation by cytoplasmic tyrosine kinases including Src, FAK and the FAK-related kinase, Pyk2 (Andreev et al., 1999; Brown et al., 1998; King et al., 1999). Adhesion-dependent or growth factor-stimulated changes in the phosphorylation state of these proteins have not been reported, whereas tyrosine phosphorylation of the PH domain-lacking Arf GAP protein, CAT1/GIT1, in response to integrin engagement has been observed (Bagrodia et al., 1999). Very recently it was shown that Pyk2-mediated phosphorylation of ASAP1/DEF1 altered its phosphoinositide-binding specificity and inhibited its Arf GAP catalytic activity, providing evidence that tyrosine phosphorylation is important in ASAP1 regulation (Krujlac-Letunic et al., 2003).

ARAP3 tyrosines Y1399 and Y1404 were identified in this investigation as in vitro phosphorylation sites in our initial screen for SFK substrates. We also find that these tyrosines serve as in vivo phosphorylation sites in full length ARAP3; their substitution with phenylalanines (Y1399/1404F) clearly inhibited ARAP3 phosphorylation by SFKs (particularly by Src, but also to a lesser extent Lyn). Significantly, phosphorylation of the Y1399/1404F mutant, mediated by endogenous PTKs was also reduced compared to wild-type ARAP3 in 293 cells spreading on fibronectin (and in NIH 3T3 cells stimulated with PDGF; data not shown). However, ARAP3 clearly contains additional phosphorylation sites as mutation of Y1399 and Y1404 reduced, but did not abolish, Lyn-mediated and adhesion-dependent phosphorylation of ARAP3. There are 23 tyrosine residues in ARAP3 and it remains to be identified which of these is also phosphorylated in vivo. Intriguingly, mutating the Y1399 and Y1404 residues appeared to enhance the ability of ARAP3 to induce the formation of elongated membrane projections on ECM substrates, suggesting that phosphorylation of these sites could be a mechanism for downregulating ARAP3 activity. This seems plausible given that maximal levels of ARAP3 tyrosine phosphorylation, in response to adhesion, coincide with time points when cell spreading is most affected; for efficient spreading, cells would require ARAP3 activity to be suppressed during this phase. The relatively small difference in activity between ARAP3 and the Y1399/1404F mutant in promoting the formation of elongated membrane processes, and the similar abilities of these proteins to inhibit cell spreading could be due to overexpression such that only a fraction of the total ARAP3...
protein is tyrosine phosphorylated (and regulated) at a given time. The overall contribution of the unidentified tyrosine phosphorylation sites to ARAP3 regulation also remains to be determined.

ARAP3 strongly suppressed cell spreading and clearly required its Rho GAP function for this activity as mutation of the ‘catalytic arginine’ (R938), a residue conserved in all Rho GAP proteins and essential for activity (Scheffzek et al., 1998), completely abolished its ability to inhibit spreading. In fact the R938L mutant appeared to enhance cell spreading, presumably by interfering in a dominant negative manner with endogenous ARAP proteins. By contrast, a functional Arf GAP domain was dispensable for ARAP3 biological activity. This finding is perhaps unexpected since ARAP1, which can perturb spreading of NIH 3T3 cells, required both its Rho GAP and Arf GAP domains for efficient activity (Miura et al., 2002). This apparent functional difference between ARAP1 and ARAP3 is interesting and could reflect distinct (non-redundant) specificities of ARAP1 and ARAP3 for their GTPase targets in vivo. It is also possible that NIH 3T3 cells, used to study ARAP1 (Miura et al., 2002), express a different subset of Arf and Rho proteins to the HEK293 cells used in our analysis of ARAP3. For example, HEK293 cells might lack an Arf protein inhibited by ARAP1 that is important for efficient spreading of NIH 3T3 cells.

Formation of lamellipodia is a common response of cells stimulated with soluble growth factors or spreading on ECM substrates like fibronectin (Small et al., 2002). Our data showing that ARAP3 inhibits lamellipodia formation and cell spreading on fibronectin therefore extend the earlier finding that PDGF-induced lamellipodia in PAE cells was antagonised by ARAP3 (Krugmann et al., 2002). Mouse ARAP3 (this study) and human ARAP3 (Krugmann et al., 2002) required a functional Rho GAP domain for these effects. Activation of Rac1 is known to induce lamellipodia formation in growth factor-stimulated cells and those adhering to fibronectin (Hall, 1998; Price et al., 1998). While mouse ARAP3 lacked Rac1 GAP activity in vitro, it nevertheless caused a modest decrease in the levels of active Rac1 in Flp-In™ 293 cells. Potentially, this effect on Rac1 may contribute to ARAP3’s ability to inhibit cell spreading. How ARAP3 alters Rac1 activation in cells, given its lack of Rac1 GAP activity in vitro, is open to speculation. This may be an indirect effect of ARAP3, perhaps involving inhibition of an endogenous Rac1 GEF or stimulation of a Rac1 GAP. Alternatively, post-translational modification(s) and/or interactions of ARAP3 with endogenous proteins could alter ARAP3 activity, enabling it to function as a Rac1 GAP in vivo. In this context, it was reported recently that MgcRacGAP, which exhibits GAP activity for Rac and Cdc42 in vitro, can be functionally converted into a RhoA GAP in vivo by site-specific serine phosphorylation (Minoshima et al., 2003).

ARAP3 stimulated a marked decrease in active RhoA levels in cells. Consistent with this, ARAP3 showed GAP activity towards RhoA in vitro (our results) (Krugmann et al., 2002), and when overexpressed, inhibited actin stress fibre formation in PAE (Krugmann et al., 2002) and NIH3T3 cells (not shown). These data imply that ARAP3 regulates RhoA levels via its own Rho GAP function. The dynamic regulation of RhoA activity is essential for normal spreading of some cell types on fibronectin (Clark et al., 1998; Ren et al., 1999; Arthur and Burridge, 2001). For example, constitutive inhibition of RhoA in Rat1 fibroblasts by overexpression of p190RhoGAP enhances cell spreading, whereas a dominant interfering p190RhoGAP mutant inhibits this process (Arthur and Burridge, 2001). Our results with ARAP3, showing inhibition of HEK293 cell spreading, are in apparent contrast to the findings with p190RhoGAP and suggest (cell type differences aside) that inhibition of RhoA may not contribute to ARAP3’s inhibitory effect on cell spreading. Nevertheless, given that RhoA activation is reduced in response to ARAP3 we cannot exclude some role for this effect of ARAP3 in inhibiting spreading of HEK293 cells.

In addition to its effects on cell spreading ARAP3 also enhanced the outgrowth of elaborate membrane projections by HEK293 cells. This effect of ARAP3 may be mediated via inhibition of endogenous RhoA since expression of an activated RhoA mutant (RhoA V14), which is insensitive to Rho GAP-mediated inactivation, suppressed the formation of membrane outgrowths induced by ARAP3 proteins with normal Rho GAP activity. Interestingly, whereas ARAP3 exhibited an opposing action to p190RhoGAP in the context of cell spreading, ARAP3’s ability to enhance the formation of membrane projections is highly analogous to that of p190RhoGAP in stimulating neurite outgrowth in neuroblastoma cells by inhibiting endogenous RhoA (Brouns et al., 2001; Hernandez et al., 2004).

ARAP3 also promoted increased cell rounding as was reported for ARAP1 (Miura et al., 2002), raising the possibility that ARAP3 might impact on cell adhesion. However, we could detect no significant effects of any ARAP3 proteins on the adhesive properties of cells even when seeded on low concentrations of fibronectin that might be expected to reveal subtle differences in adhesion. This suggests that ARAP3 does not alter the net levels or binding avidity of cell surface integrins mediating cell attachment to fibronectin. This finding is consistent with evidence that inhibition of RhoA, Rac1 or Cdc42, while perturbing cell spreading, has no affect on cell adhesion (Clark et al., 1998). Perhaps surprisingly, given the dramatic effects of ARAP3 on cell morphology and spreading, ARAP3 only modestly inhibited cell migration. This may reflect ARAP3’s ability to inhibit both RhoA and Rac1. Whereas inhibiting RhoA or its downstream effector, p160ROCK, can enhance fibroblast migration (Nobes and Hall, 1999; Arthur and Burridge, 2001), inhibiting Rac1 markedly suppresses fibroblast migration (Nobes and Hall, 1999). Suppression of Rac1 and RhoA activity by ARAP3 may cancel out, to some extent, the cellular responses that would result from inhibition of RhoA or Rac1 alone. Future studies will investigate whether ARAP3 affects the activity of Rho proteins globally or in a more spatially restricted manner within cells. These studies will be aided by new techniques to visualize active Rho proteins in situ (Berdeaux et al., 2004).

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