SETA/CIN85/Ruk and its binding partner AIP1 associate with diverse cytoskeletal elements, including FAKs, and modulate cell adhesion

Mirko H. H. Schmidt, Baihua Chen, Lisa M. Randazzo and Oliver Bögler*

William and Karen Davidson Laboratory of Brain Tumor Biology, Hermelin Brain Tumor Center, Department of Neurosurgery, Henry Ford Hospital, 2799 West Grand Blvd, Detroit, MI 48202, USA

*Author for correspondence (e-mail: oliver@bogler.net)

Accepted 31 March 2003
Journal of Cell Science 116, 2845-2855 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00522

Summary

The adaptor protein SETA/CIN85/Ruk is involved in regulating diverse signal transduction pathways, including the internalization of tyrosine kinase receptors via the Cbl ubiquitin ligases, and attenuating PI3K activity by interaction with its regulatory subunit. Here we present evidence for a new aspect of SETA function, based on the initial observation that it co-localizes with actin in microfilaments and at focal adhesions, and with microtubules. Although there was no evidence for direct molecular interactions between SETA and cytoskeletal proteins, the SETA-interacting protein AIP1, which is a rat ortholog of the *Xenopus* src substrate Xp95, strongly interacted with structural proteins of the cytoskeleton, including actin and tubulins. Both SETA and AIP1 interacted with focal adhesion kinase (FAK) and proline rich tyrosine kinase 2 (PYK-2), and c-Cbl interacted with PYK-2. AIP1, which interacted more strongly than either SETA or c-Cbl, required an intact consensus tyrosine kinase phosphorylation sequence at Y319 to bind to focal adhesion kinases, which suggests that phosphorylation is an important mediator of this complex. SETA, which interacted as a dimer with focal adhesion kinases, promoted the interaction between PYK-2 and AIP1. Direct analysis of the impact of these proteins on cell adhesion, by use of an electrical cell-substrate impedance sensor (ECIS), showed that SETA promoted cell adhesion while AIP1 and c-Cbl reduced it. Furthermore, the ability of AIP1 and AIP1 mutants to decrease cell adhesion in ECIS analysis correlated with their presence in PYK-2 complexes, providing a direct link between AIP1-mediated molecular interactions and cellular behavior. Transfection of AIP1 also reduced the level of phosphorylation of endogenous PYK-2 and FAK, suggesting that this protein may directly regulate focal adhesion kinases, and thereby cell adhesion. These data are the first to implicate the adaptor protein SETA and its binding partner AIP1 as being involved with the cytoskeleton and in the regulation of cell adhesion, and suggest that they may be part of the focal adhesion kinase regulatory complex.

Key words: Glioma, Astrocytes, SETA/CIN85/Ruk, AIP1, Focal adhesion kinase, Cytoskeleton, Electrical cell substrate impedance sensor

Introduction

The gene SH3-domain encoding, expressed in tumorigenic astrocytes (*SETA*) encodes adaptor proteins characterized by the presence of src homology 3 (SH3) domains, proline rich regions with P-x-x-P motifs that bind to SH3 domains (Mayer and Eck, 1995) and a C-terminal coiled-coil. Its expression in glial cells is associated with differentiation in oligodendrocytes and the acquisition of a tumorigenic phenotype in transformed, $p53^{-/-}$ astrocytes (Bogler et al., 2000). The protein has been independently identified as a binding partner of p85· and a negative regulator of phosphatidylinositol 3-kinase [PI3K; regulator of ubiquitous kinase or *Ruk* (Gout et al., 2000)] and twice as a binding partner for c-Cbl [Cbl-interacting protein of 85 kDa or *CIN85* (Take et al., 2000; Soubeyran et al., 2002)]. SETA/CIN85/Ruk proteins exist in several isoforms (Borinstein et al., 2000; Gout et al., 2000; Buchman et al., 2002), the longest of which encodes three SH3 domains in the N-terminal half, a central proline rich region and a C-terminal coiled-coil domain involved in multimerization (Borinstein et al., 2000; Watanabe et al., 2000).

Recently SETA/CIN85/Ruk has been implicated in participating in the internalization of activated receptor tyrosine kinases together with Cbl proteins. Ligand activation of EGFR leads to the binding and phosphorylation of c-Cbl or Cbl-b proteins via their modified SH2 phosphotyrosine binding domains, and the recruitment of SETA/CIN85 by binding of its SH3 domains to the C-terminus of the Cbls (Take et al., 2000; Soubeyran et al., 2002; Szymkiewicz et al., 2002). SETA/CIN85 is monoubiquitinated at lysines in its C-terminus, while the EGFR is polyubiquitinated by the Cbls marking it for proteasomal degradation (Soubeyran et al., 2002; Haglund et al., 2002; Szymkiewicz et al., 2002). In addition, endophilins are recruited to the complex, by virtue of a constitutive interaction with SETA/CIN85 mediated by the endophilin SH3 domain and the SETA/CIN85 proline rich C-terminus

(Soubeyran et al., 2002; Szymkiewicz et al., 2002). It has been demonstrated that the internalization and ubiquitination of the EGFR can be mechanistically separated, and the interaction of the SETA/CIN85-Cbl complex with the receptor may be primarily involved in internalization into clathrin coated vesicles, while the ubiquitination state may regulate subsequent sorting into recycling or degradation pathways (Soubeyran et al., 2002; Szymkiewicz et al., 2002).

While the implication of SETA/CIN85/Ruk in receptor internalization is an important advance in our understanding of the function of this protein, it is unlikely to reflect all aspects of it. In addition to the demonstration that SETA/Ruk can inhibit PI3K activity (Gout et al., 2000), there is a growing list of interactions with other signaling molecules including regulators of the cytoskeleton and modulators of apoptosis including Crk-I, Crk-II, p130(Cas), Grb2, Sos1 and AIP1 (Chen et al., 2000; Borinstein et al., 2000; Watanabe et al., 2000). In addition, localization of SETA in the cell may provide important clues to its function. We had previously observed that SETA staining overlapped with actin staining in astrocytes (Chen et al., 2000), which prompted further investigation of the association between SETA and cytoskeletal proteins, focal adhesion kinases and whether it could modulate cellular adhesion characteristics. In addition we had shown that AIP1, a binding partner of SETA, also had a cell-staining pattern with cytoskeletal appearance, and so investigated its association with cytoskeletal proteins at the molecular level. Here we show by immunocytochemistry and confocal microscopy that SETA staining overlaps with actin and β-tubulin staining in astrocytes, but that there is no direct molecular interaction between these proteins. Similarly, intense SETA staining at focal adhesions but the presence of relatively small amounts of SETA in FAK or PYK-2 immunoprecipitates suggests that indirect binding may the most prevalent mode of interaction between these proteins. In contrast the SETA interacting protein AIP1 can be found complexed to cytoskeletal proteins and is robustly present in focal adhesion kinase immunoprecipitates, and so may be directing SETA to them. In agreement with this is the observation that SETA increases the amount of AIP1 in these complexes. Furthermore, we show that SETA promotes adhesion and AIP1 or c-Cbl antagonize adhesion as measured at steady state by an electrical cell-substrate impedance sensor [ECIS (Mitra et al., 1991)], suggesting that these proteins have an impact on cell behavior. Interestingly, the presence of AIP1 proteins in PYK-2 complexes correlates with a reduction in cell adhesion in ECIS experiments, suggesting a direct link between the interaction between these proteins and cell adhesion. Lastly, we show that AIP1 can reduce the level of endogenous focal adhesion kinase phosphorylation, providing a possible mechanism of its ability to regulate these processes.

Materials and Methods

Constructs and antibodies

Transfection experiments were performed using: full length SETA123cc and *lacZ* in pcDNA6 (Invitrogen) as previously described (Chen et al., 2000; Borinstein et al., 2000); ALG-2 and C-terminal FLAG-tagged AIP1 [(Vito et al., 1996; Vito et al., 1999) provided by Luciano D'Adamio, Albert Einstein College of Medicine, Yeshiva University, Bronx, NY] and AIP1 mutants in pcDNA3; c-Cbl carrying an N-terminal HA-tag in pCEFL [provided by Stan Lipkowitz, National Cancer Institute, National Institutes of Health, Bethesda, MD (Ettenberg et al., 1999)]. AIP1 mutants were made with the QuickChange Site Directed Mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, CA).

Antibodies used for immunohistochemistry were phalloidin-FITC and anti-β-tubulin (Sigma, St Louis, MO). For western blots and immunoprecipitations we used the following antibodies: monoclonal mouse anti-HA (F-7) and polyclonal goat anti-actin (I-19) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal anti-FLAG (M2), anti-GFAP (G-A-5), anti-MAP1 (HM-1), anti-α-tubulin (B-5-1-2), anti-β-tubulin (2-28-33), anti-γ-tubulin (GTU-88), anti-phosphotyrosine tubulin (TUB-1A2), as well as polyclonal rabbit anti-actin, anti-MAPs and anti-PKC zeta antibodies from Sigma. Monoclonal mouse anti-phosphotyrosine antibody (4G10) from Upstate Biotechnology as well as monoclonal anti-AIP1 (49), anti-FAK (77) and anti-PYK2 (11) antibodies from BD Biosciences, San Jose, CA. Polyclonal anti-SETA antibodies were as described (Chen et al., 2000).

Cell lines and cell transfection

Primary rat cortical astrocytes were isolated as described (McCarthy and De Vellis, 1980; Chen et al., 2000) and were cultured in DMEM supplemented with antibiotics and 10% fetal calf serum (DMEM-FCS). HEK293 embryonic kidney cells were cultured under standard conditions in DMEM-FCS. Cells were transfected with plasmids by a modified calcium-phosphate procedure. The day before transfection either two or five million cells were plated in 10 cm tissue culture dishes to allow them to remain non-confluent or become confluent, respectively, two days after transfection.

Immunoprecipitation and in vitro confrontation

Cells were washed twice with ice-cold PBS and were lysed on ice for 30 minutes in a modified RIPA buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1% IGEPAL CA-630 (Sigma), 0.5% deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM EGTA, 1 mM DTT, 4 mM sodium azide, 1 mM PMSF, 5 mM benzamidine], as well as a protease inhibitor cocktail (2 μg/ml aprotinin and leupeptin, 10 μg/ml E-64 and trypsin inhibitor, 1 μg/ml pepstatin A) and a phosphatase inhibitor cocktail (2 mM sodium vanadate and sodium fluoride, 5 mM sodium molybdate and 15 mM p-nitrophenylphosphate). Following lysis, the cell suspension was sheared ten times through an 18G1½ needle, ten times through an IM1 needle, and incubated on ice for another 30 minutes. The cell solution was then cleared by centrifugation at 20,000 ***g*** at 4°C. The supernatant was used for immunoprecipitation studies. Appropriate concentrations of primary antibody were added and the solution was rotated at 4°C for at least an hour. Antibody-protein complexes were precipitated with 50 μl Protein A-agarose solution (Roche) by rotation at 4°C overnight. The agarose beads were collected by centrifugation at 12,000 ***g*** for five minutes at 4°C, and were washed seven times with precipitation buffer on ice. Finally, the sediment was boiled for five minutes at 95°C in 2× NuPAGE® LDS Sample Buffer (Invitrogen) containing 20% β-mercaptoethanol and transferred to ice immediately. The solution was cleared of agarose by centrifugation and stored at –80°C until further analysis by protein electrophoresis. Microtubule spin-down assays were performed using the Microtubule Associated Protein Spin-Down Assay Kit BK029 (Cytoskeleton Inc., Denver, CO) according to the manufacturers instructions. In vitro confrontation experiments were carried out as previously described (Chen et al., 2000; Borinstein et al., 2000).

Western blotting

Protein samples were analyzed by SDS-PAGE with NuPAGE 4-12% or 10% Bis-Tris gels (1 mm) according to the manufacturer's

guidelines. This gel system alters the relative mobility of proteins when compared with conventional Laemmli PAGE gels, and so extra care was taken to confirm the identity of all proteins identified here, by comparing non-transfected controls and using more than one antibody when possible. Proteins were blotted to PVDF and were incubated for 1 hour in blocking buffer (5% BSA and 1% Tween-20 in TBS) and overnight with appropriate dilutions of primary antibody in blocking buffer. Membranes were washed and incubated for 1 hour with alkaline-phosphatase conjugated secondary antibody solution in blocking buffer (Sigma; dilutions: anti-mouse antibody 1:3000, anti-rabbit antibody 1:5000, and anti-goat antibody 1:15,000). After additional washing steps, antibody complexes were visualized on film by Immun-Star AP substrate (Bio-Rad).

Immunocytochemistry

Primary cortical rat astrocytes were grown on glass coverslips, washed in phosphate buffered saline (PBS; pH 7.4) and fixed in 4% w/v paraformaldehyde in PBS for 10 minutes, before being rinsed in Hanks Balanced Salt Solution (HBSS; buffered with 0.04 M HEPES pH 7.4, 5% v/v calf serum) and incubated in primary antibodies in HBSS supplemented with 5% v/v goat serum for one hour in a humidified chamber. After several rinses in HBSS cells were incubated in species and isotype specific secondary fluorescently labeled antibodies (Southern Biotechnology, Birmingham, AL) or phalloidin-FITC for another hour. After the final washing steps, cells were mounted in glycerol supplemented with 2.5% w/v 1,4-diazobicyclo-2.2.2 octane (Sigma) and viewed on a Bio-Rad MRC1024 confocal microscope.

ECIS cell attachment assay

The electrical cell-substrate impedance sensor system (ECIS, Applied Biophysics, Troy, NY) (Mitra et al., 1991; Tiruppathi et al., 1992; Lo et al., 1995) was applied to analyze the ability of SETA, AIP1 and c-Cbl to alter the steady-state cell adhesive properties of HEK293 cells. Corresponding plasmids were transiently transfected 48 hours prior to each experiment. Cells were harvested after 2 days and plated at 200,000 cells per well in ECIS chambers with 400 μl DMEM-FCS to obtain a confluent layer of cells covering each electrode. Cell attachment behavior was monitored for up to 10 hours, with resistance values being collected every few seconds by the ECIS device. Complete single-layer electrode coverage was confirmed microscopically prior to data analysis. To analyze ECIS results, resistance values were adjusted by first subtracting the value of each individual electrode before addition of the cells, when only medium was present to compensate for minor differences in electrode manufacture. Values were then normalized by subtraction of the resistance value of *lacZ*-transfected control cells at each time-point. At each hour the ten readings leading up to that hour were averaged and their standard deviation calculated. Finally, the change relative to the zero time point was calculated for each data series independently, and graphed as change in resistance over time.

Results

SETA co-localizes with actin and tubulin

We previously showed that SETA appeared to co-localize with actin in primary cortical rat astrocytes under a conventional microscope (Chen et al., 2000). To extend this we performed confocal microscopy on astrocytes stained with anti-SETA antibody and phalloidin (Fig. 1A-F), which demonstrated that a portion of the endogenous SETA protein co-localized with actin microfilaments and focal adhesions in astrocytes (arrows in Fig 1A-C). However, there were also areas of SETA staining (right hand arrowhead in Fig. 1D-F) and phalloidin staining (left hand arrowhead in Fig. 1D-F) that did not appear to overlap. This allowed for the possibility that SETA could co-localize with other cytoskeletal components. Double staining with anti-GFAP antibodies, which label the major intermediate filament protein in astrocytes, failed to show convincing coincidence of signal (data not shown). However, when cells were stained with anti-β-tubulin and anti-SETA, overlap was evident (Fig. 1G-L). The overlap was particularly noticeable in the peri-nuclear area that showed the most intense β-tubulin as well as SETA staining. In addition, SETA staining was coincident with individual bundles of microtubules (Fig. 1G-L, arrows). Again, there was not complete overlap between the staining patterns for SETA and β-tubulin (Fig. 1G-L, arrowheads).

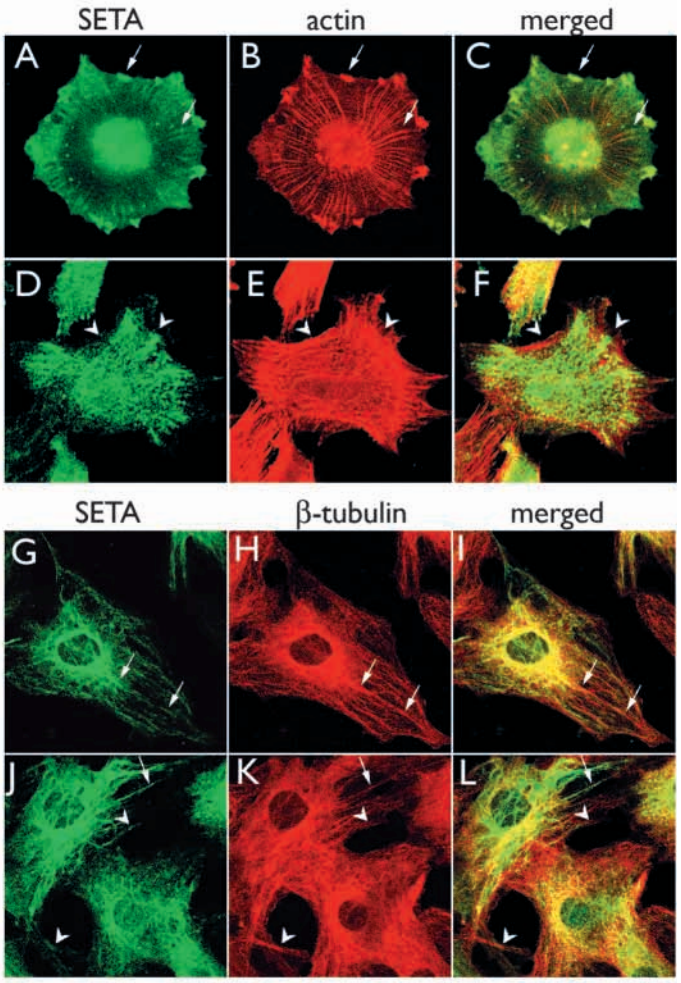

Fig. 1. SETA co-localizes with microfilaments and microtubules. Confocal microscopy of astrocytes stained with polyclonal anti-SETA antibody together with FITC-conjugated secondary antibodies (A,D,G,J), TRITC-phalloidin (B,E), or β-tubulin followed by TRITC-conjugated secondary antibodies (H,K). The analysis revealed partial co-localization of SETA and actin microfilaments, and focal adhesions (arrows in A-C). However, areas without co-localization were also detected (arrowheads in D-F). Furthermore, partial overlap of SETA and β-tubulin staining was detected in the peri-nuclear region and along individual bundles of microtubules (arrows in G-L). As shown for actin, the overlap was not complete (arrowheads in J-L).

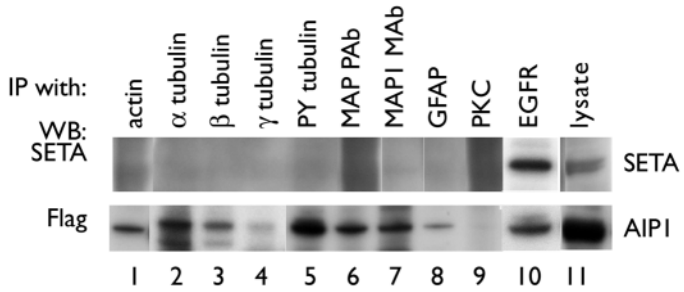

Fig. 2. AIP1 but not SETA co-immunoprecipitates with cytoskeletal proteins. SETA or Flag-tagged AIP1 were transiently transfected into HEK293 cells and various cytoskeletal proteins were immunoprecipitated with specific antibodies, as indicated. The immunoprecipitates were subsequently analyzed by PAGE and immunoblotting with polyclonal anti-SETA antibody or monoclonal anti-Flag antibody. Although SETA was present in the lysates of the cells (lane 11), and was immunoprecipitated with the positive control protein EGFR (lane 10), it was not found in any of the immunoprecipitates made with antibodies to cytoskeletal proteins (lanes 1 to 9). In contrast, AIP1 revealed a strong signal after immunoprecipitation of most cytoskeletal proteins, with actin, α-tubulin, β-tubulin and phosphotyrosine tubulin, as well as the microtubule-associated proteins (MAPs), showing particularly strong signals, and was also found in EGFR immunoprecipitates.

AIP1 but not SETA interacts directly with cytoskeletal proteins

To investigate the interaction between SETA and cytoskeleton proteins, immunoprecipitation experiments were performed in HEK293 cells, which are more efficiently transfected than primary astrocytes. Cells were transfected with full-length SETA and immunoprecipitates made with antibodies against various cytoskeletal components were analyzed by anti-SETA western blot (Fig. 2). This analysis failed to show evidence for a direct interaction between SETA and actin, any of the tubulins, microtubule associated proteins (MAPs) or GFAP (Fig. 2, lanes 1-8). In addition no interaction was demonstrable with the signal transduction associated molecule protein kinase C (PKC; Fig. 2, lane 9). SETA could be recovered by immunoprecipitating the EGFR, as has been shown previously (Soubeyran et al., 2002). Because immunoprecipitation experiments of cytoskeletal components may favor recovery of monomers, a microtubule associated protein spin-down assay was also performed. Microtubules were polymerized in vitro (Hyman et al., 1991) and confronted with bacterial SETA protein or cell extract that had been pre-cleared of microtubules but contained SETA, and the synthesized microtubules were subsequently pelleted by centrifugation. Anti-SETA western blots performed on the pellets failed to provide evidence for a direct interaction between SETA and microtubules (data not shown), suggesting that the co-localization observed in astrocytes occurs indirectly.

The SETA binding partner, apoptosis linked gene-2 interacting protein 1 [AIP1 (Chen et al., 2000)], was present in immunoprecipitates made with antibodies against various cytoskeletal components, as well as EGFR. Strong AIP1 signals were obtained in association with actin, α-tubulin, tyrosine phosphorylated tubulin and with the MAPs (Fig. 2, lanes 1,2,5,6,7), while lower but detectable signals were obtained with β-tubulin and GFAP immunoprecipitates (Fig. 2, lanes 3,8), and a weak signal was obtained with γ-tubulin (Fig. 2, lane 3). No AIP1 protein was recovered in immunoprecipitates made with PKC antibody, demonstrating the specificity of the other interactions (Fig. 2, lane 9). The ability of AIP1 to bind cytoskeletal proteins allows that it mediates their interactions with SETA.

SETA and AIP1 interact with focal adhesion kinases

The intense SETA immunoreactivity observed at focal adhesions (Fig. 1A-F) suggested that SETA may interact with proteins associated with these specialized structures. Therefore we next immunoprecipitated the endogenous focal adhesion kinases FAK and PYK-2 from SETA-transfected HEK293 cells (Fig. 3). These experiments revealed that there was a weak interaction between SETA and FAK (Fig. 3A) and PYK-2 (Fig. 3B). Cell density did not affect the strength of interaction (shown for PYK-2 in Fig. 3B) suggesting that SETA is a weak interacting partner of the focal adhesion kinases under different physiological conditions. The SETA protein associated with focal adhesion kinases was detected as a band with an apparent molecular weight of about 160 kDa in these experiments, while other SETA bands, including one at 85 kDa, that were observed in the lysates were not represented in the immunoprecipitates (Fig. 3). The 160 kDa band appeared only when gels are run under weak denaturing conditions, and likely represents a dimer of SETA mediated by the coiled-coil domain (Borinstein et al., 2000; Watanabe et al., 2000). To test this directly, we introduced either SETA full length or SETAΔcc, which lacks the C-terminal coiled-coil, into HEK293 cells, and performed western blots under strong or weak denaturing conditions (Fig. 3C). The 160 kDa SETA band was only found under weak denaturing conditions when the SETA protein encoded the coiled-coil motif (Fig. 3C, lane 3).

To determine whether a stronger interaction could again be observed between focal adhesion kinases and AIP1, we transfected AIP1 into HEK293 cells and immunoprecipitated endogeneous FAK and PYK-2 proteins (Fig. 4). High levels of AIP-1 were present in FAK (Fig. 4A, lane 1) and PYK-2 (Fig. 4Bb, lanes 1,2) immunoprecipitates. Interestingly, the amount of AIP1 recovered from precipitates of the focal adhesion kinases could be somewhat enhanced by increasing the cell density (shown for PYK-2 in Fig. 4B, lanes 1,2), while AIP1 expression levels were similar (Fig. 4B, lanes 5,6). In contrast, another binding partner of SETA, c-Cbl (Borinstein et al., 2000; Take et al., 2000; Soubeyran et al., 2002), was not found in FAK immunoprecipitates (Fig. 4A, lane 3), suggesting that not all SETA binding partners are present in this complex. However, c-Cbl was part of a complex with PYK-2, indicating an interesting difference between these two focal adhesion kinases. However, it was only present at a low intensity (Fig. 4B, lanes 3,4) as compared to AIP1. Lastly, another protein that is associated with AIP1, apoptosis linked gene 2 (ALG-2) was examined in this context, and found to be recoverable in a PYK-2 immunoprecipitate (Fig. 4C).

The interaction between focal adhesion kinases and actin is calcium dependent, and can be strengthened by high levels of intracellular calcium, which promote PYK-2 activity, autophosphorylation and complex formation (Lev et al., 1995).

To determine whether this would also mediate an increase in AIP1 association, the concentration of calcium was increased in cell lysates prior to immunoprecipitation with anti-PYK-2 antibodies (Fig. 5). In the presence of 10 mM $CaCl_2$ a noticeably higher level of AIP1 and actin was recovered from PYK-2 immunoprecipitates (Fig. 5, lane 3).

SETA promotes the interaction between AIP1 and PYK-2

If SETA, AIP1 and the focal adhesion kinases participate in a multi-protein complex, it is possible that altering the level of one component may affect the interaction of the others. To test this hypothesis we first attempted to create an AIP1 mutant that

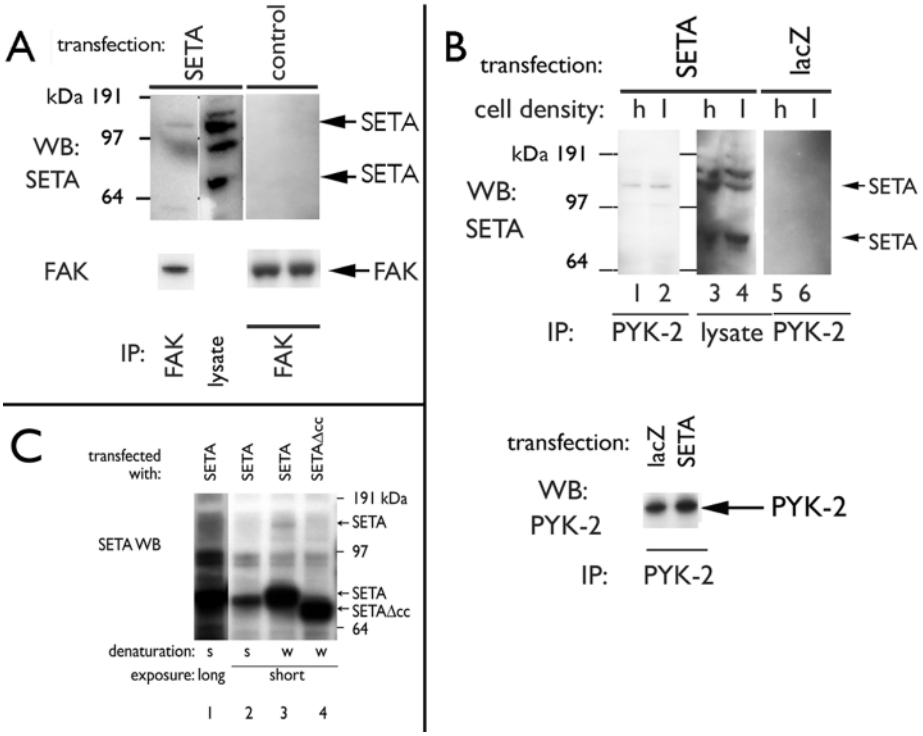

Fig. 3. SETA associates with FAK and PYK-2 as a dimer. To further analyze SETA's co-localization with focal adhesions, two major components of these structures, FAK (A) and PYK-2 (B), were immunoprecipitated from HEK293 cells that had been transiently transfected with SETA. Western blotting revealed that SETA could be recovered in both immunoprecipitates (A, lane 1; B, lanes 1,2), but was not recovered in control transfections (A, lanes 3,4; B, lanes 5,6). SETA appeared predominantly at about 160 kDa in the immunoprecipitates, while equal amounts of SETA at 85 kDa were present in lysates. Direct analysis (C) of SETA under strong (s; lanes 1,2) and weak (w; lane 3) denaturating PAGE conditions, as well as comparison with SETAΔcc (lane 4) lacking the coiled-coil domain, revealed that the 160 kDa band observed probably represents a SETA dimer mediated by homophilic interaction by this C-terminal domain, as shown previously (Borinstein et al., 2000; Watanabe et al., 2000). No SETA band at 160 kDa was found under strong denaturing conditions even when longer exposures (lane 1) were examined. This suggests that SETA preferentially associated with focal adhesion kinases as a dimer.

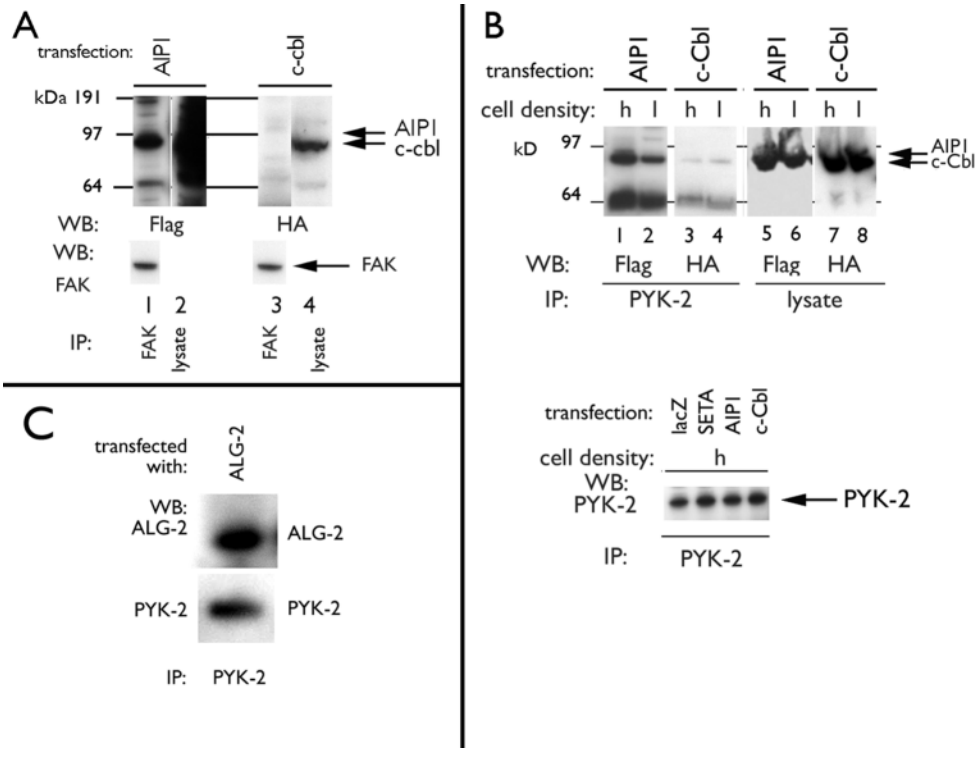

Fig. 4. The SETA binding partner AIP1 is associated with focal adhesion kinases. Analysis of the presence of the SETA-binding partners AIP1 and c-Cbl within focal adhesions revealed that AIP1 could be recovered by immunoprecipitation of FAK (A, lane 1) and PYK-2 (B, lanes 1,2) from transiently transfected HEK293 cells, whereas c-Cbl was exclusively and weakly associated with PYK-2 (B, lanes 3,4). Changing the cell density from low or non-confluent (l) to high or confluent (h) increased the amount of AIP1 associated with PYK-2, but did not influence the amount of c-Cbl. Neither protein had an influence on the concentration of PYK-2 under these conditions. Finally, the AIP1-binding partner ALG-2 was also found to co-precipitate with PYK-2 (C), indicating that an extended SETA-associated protein complex was intimately associated with focal adhesions. Please note that relative mobilities of proteins in Nu-PAGE gels is different from conventional Laemmli gels with stronger denaturation (see Materials and Methods).

was incapable of binding SETA. Two deletion constructs of the AIP1's proline rich C-terminus, where the sequences thought to interact with SETA's SH3 domains are located, were made (Fig. 6A): one lacking the C-terminal half (nonsense codon at position 784; AIP1-784Stop) and the other lacking the N-terminal half (in-frame deletion from residues 717 to 784 inclusive; AIP1-Δ717-784). In vitro binding assays between bacterial GST-SETA and in vitro transcribed and translated AIP1 showed that binding of AIP1-784Stop to SETA was indistinguishable from wild type AIP1 (Chen et al., 2000), while AIP1-Δ717-784 bound at low levels, and did not discriminate between different SETA SH3 domains (Fig. 6B). This suggests that the N-terminal half of the proline rich C-terminus of AIP1 contains the SETA binding site, and that this deletion mutant can be used to probe the role of the SETA-AIP1 interaction.

The effect of SETA on the interaction between AIP1 or AIP1 mutants and FAK or PYK-2 was then tested (Fig. 7). The transfection of SETA did not alter the amount of AIP1 detected in FAK immunoprecipitates, but did strengthen the interaction between AIP1 and PYK-2 (Fig. 7, lanes 1,2). The lower amount of SETA in the lysates of the AIP1-SETA co-transfection (Fig. 7, lane 2) as compared to the other SETA-containing co-transfections (Fig. 7, lanes 4,6,8) was a consistent observation, which we are currently investigating. It is possible that this is the reason we did not observe an effect of SETA on AIP1 in the FAK immunoprecipitations. However, this lower level did not affect the amount of SETA in the PYK-2 complexes, made from the same lysates, nor from SETA having an affect on the amount of AIP1 associated with PYK-2 (Fig. 7, lanes 1,2), implying that the amount of SETA was not necessarily limiting.

Interestingly, no interaction with either focal adhesion kinase was observed for a mutant, AIP1-Y319F, which has an alteration in the single consensus tyrosine kinase phosphorylation site, shown to be phosphorylated by src in the *Xenopus* homolog of AIP1, Xp95 (Che et al., 1999). Reduced interaction with focal adhesion kinases was also observed for the other AIP1 mutants, AIP1-Δ717-784 and AIP1-784Stop. Interestingly transfection of SETA was able to increase the amount of these two AIP1 mutants in both FAK and PYK-2 immunoprecipitates (Fig. 7, lanes 5-8). In both sets of immunoprecipitations SETA had a more pronounced effect on the amount of AIP1-784Stop than AIP1-Δ717-784 recovered (Fig. 7, lanes 6,8), in line with their relative abilities to interact with SETA. Neither wild-type AIP1 nor the deletion mutants increased the amount of SETA in focal adhesion kinase complexes.

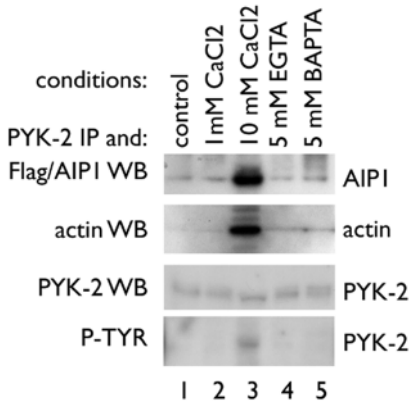

Fig. 5. Increased calcium concentration causes increased association between AIP1 and PYK-2. Increased intracellular Ca^{2+} stimulates PYK-2 phosphorylation prompting an investigation of whether increasing the level of free calcium enhanced the AIP1–PYK-2 interaction. PYK-2 immunoprecipitations from transiently AIP1-transfected HEK293 cells were performed in various immunoprecipitation buffers. The IP buffer (control; containing 1 mM EGTA) was supplemented with 1 mM (lane 2) or 10 mM (lane 3) $CaCl_2$; external Ca^{2+} was complexed by raising the EGTA concentration to 5 mM (lane 4), or protein-associated Ca^{2+} was complexed by adding 5 mM BAPTA (lane 5). No impact on the AIP1–PYK-2 or actin–PYK-2 interaction was found after moderate increases in the $CaCl_2$ concentration, nor after addition of EGTA or BAPTA. However, raising the $CaCl_2$ concentration to 10 mM resulted in a dramatic increase of AIP1 and actin in PYK-2 immunoprecipitates (lane 3). PYK-2 activity, as measured by phosphotyrosine western blot, is elevated in the presence of 10 mM $CaCl_2$. The AIP1 band appears weaker in lanes 1, 2, 4 and 5 of this figure compared with that in Fig. 4 because of the short exposure time of the film to the blot, as a result of the strong signal in lane 3.

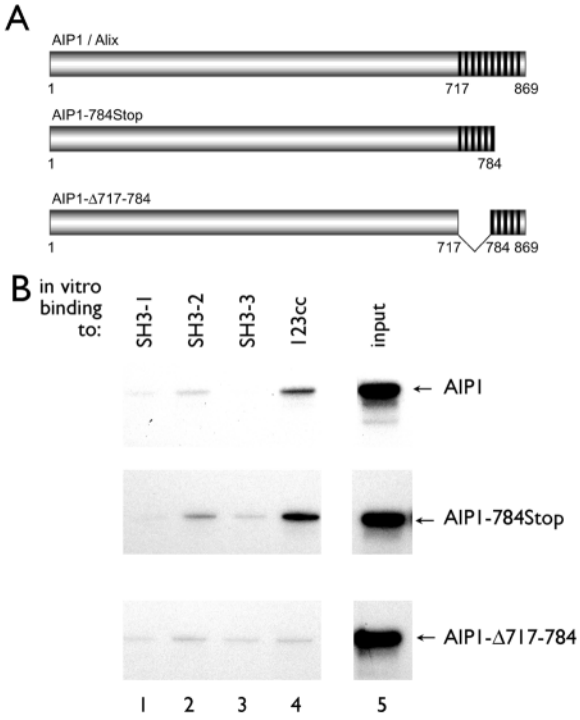

Fig. 6. The AIP1 deletion mutant AIP1-Δ717-784 shows reduced binding to SETA. (A) The C-terminal proline-rich region containing multiple P-x-x-P motifs between residues 717 and 869 (black vertical lines) in full-length AIP1, and regions deleted in two AIP1 mutants AIP1-784Stop and AIP1-Δ717-784. (B) In vitro binding assays were performed with bacterial GST-SETA fusion proteins encoding either isolated SH3 domains (SH3-1, -2 and -3) or full length SETA (123cc), and in vitro transcribed and translated, radiolabeled, full-length or mutant AIP1. Full-length AIP1 bound to the middle SH3-2 domain, and more strongly to full length SETA, as shown previously (Chen et al., 2000). AIP1-784Stop showed a similar binding profile to full-length, while AIP1-Δ717-784 showed faint, non-specific binding, indicating that the N-terminal half of the P-x-x-P domain mediated SETA binding.

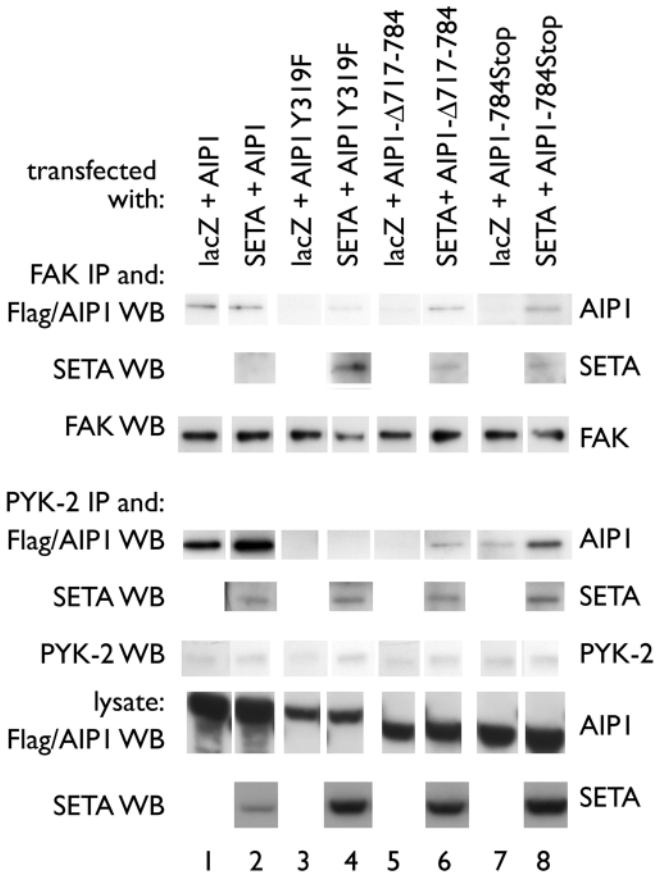

Fig. 7. SETA promotes the association between AIP1 and PYK-2. AIP1 and AIP1 mutants were transiently co-transfected into HEK293 cells with SETA or *lacZ* control plasmid. Endogenous FAK or PYK-2 was immunoprecipitated and the pellet subsequently analyzed by immunoblotting for AIP1 and SETA. When compared with wild-type AIP1, all mutants were significantly reduced in their ability to bind FAK and PYK-2, with the AIP1Y319F mutant, which is altered at a potential src phosphorylation site, showing no detectable binding to either focal adhesion kinase. The PxxP-deficient mutants were also negatively impacted in their binding capacity but remained detectable in the complex. Co-transfection of SETA enhanced wild-type AIP1 binding to PYK-2 but not to FAK. Furthermore, SETA was able to partially restore the binding of AIP1 and the two AIP1 deletion mutants to FAK/PYK-2.

SETA and AIP1 modulate cell adhesion via the attenuation of PYK-2 activity

To determine whether the association between SETA and elements of the cytoskeleton and focal adhesions had any impact on cell behavior we measured steady-state cell adhesion using an electrical cell-substrate impendence sensor [ECIS (Mitra et al., 1991; Tiruppathi et al., 1992; Lo et al., 1995)]. ECIS allows the measurement of cell-substrate adhesion both during the cell attachment phase and after cells have become attached and are in a steady state condition, rather than relying on the transient attachment of cells that have been trypsinized, are spherical and do not have well-formed focal adhesions. HEK293 cells were transfected with expression plasmids encoding SETA, AIP1, AIP1 mutants or the SETA binding partner c-Cbl, and analyzed by ECIS under confluent

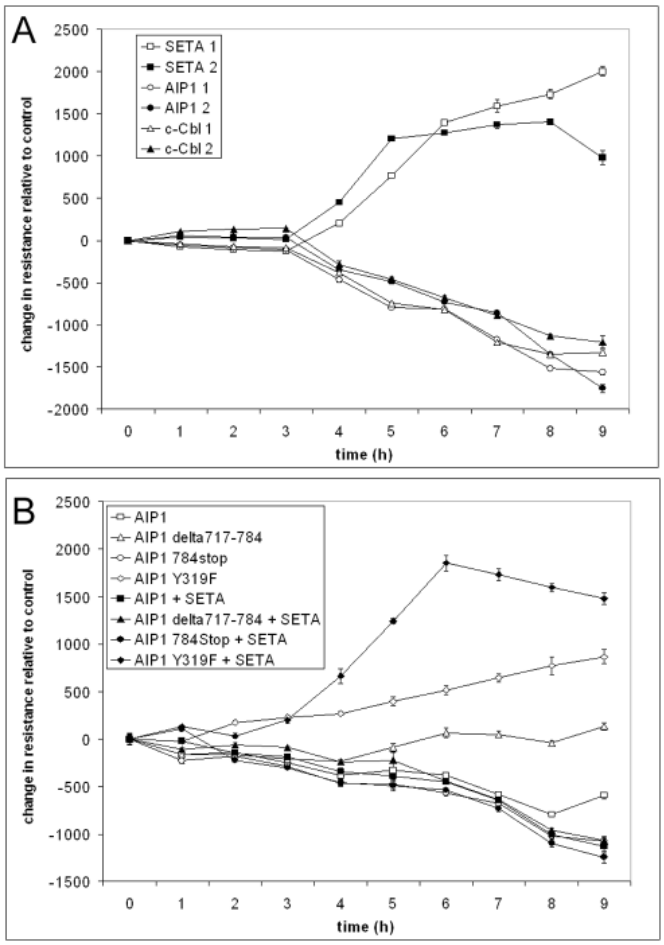

Fig. 8. Reduction in cell adhesion by AIP1 proteins correlates with their presence in PYK-2 immunoprecipitates. HEK293 cells were transiently transfected with SETA, AIP1, AIP1-Δ717-784, AIP1-784Stop, AIP1Y319F c-Cbl and *lacZ* as a control, as indicated. Two days after transfection cultures were harvested and underwent an ECIS cell attachment assay under confluent conditions on the electrode, over a period of 9 hours (see Materials and Methods for details). The values at each time point for the *lacZ*-transfected cultures from each experiment were averaged, taken as background and subtracted from the other data sets. Ten values around each hour were then taken, averaged and the standard deviation calculated. The values for each curve were set to zero at the zero time point and changes in resistance over time plotted. After an initial settling period of 3 hours the graphs reflect changes in resistance as cells attach and reach a steady state. (A) The resistance of SETA and AIP1 or c-Cbl-transfected cells moved in opposite directions over time. While SETA increased the resistance of the cell layer up to +1500 ohm, AIP1 and c-Cbl decreased it to about –1500 ohm. Accordingly, SETA mediates a pro-adhesive effect on HEK293 cells, while AIP1 and c-Cbl negatively affect cell adhesion. Cell density at the time of transfection had no major influence on these effects. (B) AIP1-Δ717-784 and AIP1Y319F induced no major change in cell adhesion, while AIP1 and AIP1-784Stop induced a reduction similar to that observed in AIP1 (A). While SETA co-transfection did not alter the pattern of AIP1 and AIP1-784Stop cell adhesion, it caused an increase in adhesion when co-transfected with AIP1Y319F, which suggests that it's effect was dominant over this mutant, which had no effect on its own. Interestingly, co-transfection of SETA and AIP1-Δ717-784 caused a reduction in adhesion. Error bars are smaller than plot symbols at some time points.

conditions over a period of nine hours. Changes in resistance after subtraction of the measurements obtained from *lacZ*-transfected control cells, and after setting the zero time point to a zero value were calculated over time (Fig. 8). Transfection of full length SETA resulted in an increase in resistance, after an initial three hour period as cells settled on the electrode, and a reaching of a steady state of close to 1500 ohm over control (Fig. 8A), indicating a pro-adhesive effect of SETA. In contrast, transfection of c-Cbl or AIP1 had the opposite effect and diminished resistance and cell adhesion. Again changes were manifested after an initial period of settlement, and resulted in these cells reaching a steady state close to –1500 ohm relative to the *lacZ*-transfected control (Fig. 8A). This suggests that AIP1 and c-Cbl have anti-adhesive effects. To compare the impact of AIP1 mutants to AIP1, AIP1-Δ717-784, AIP1-784Stop and AIP1Y319F were also analyzed. AIP1Y319F had no prominent effect on adhesion, and a slight increase over time was observed (Fig. 8B). Similarly, AIP1-Δ717-784 showed essentially no difference when compared to control cells (Fig. 8B). In contrast, AIP1-784Stop mediated a similar decrease in adhesion to wild-type AIP1. These data correlated very well with what was observed in Fig. 7 in terms of AIP1 protein in PYK-2 immunoprecipitates: a reduction in adhesion was only observed in the case of AIP1 and AIP1-784Stop, which were present in PYK-2 complexes, but not in the case of AIP1Y319F or AIP1-Δ717-784 which were not recruited to this kinase. These immunoprecipitation studies had also demonstrated the ability of SETA to recruit AIP1-Δ717-784 but not AIP1Y319F proteins to focal adhesion kinase complexes, and so SETA's ability to modulate cell adhesion decreases by AIP1 proteins was tested by ECIS. This analysis further supported the correspondence between a reduction of cell adhesion and the presence of AIP1 protein in PYK-2 complexes. In the case of co-transfection of SETA and AIP1Y319F, which did not result in the recruitment of the AIP1 mutant to PYK-2 (Fig. 7, lanes 2,4) an increase in adhesion similar to that observed when SETA was transfected alone was observed (Fig. 8A,B), suggesting that SETA's effect was the dominant phenomenon. Interestingly, when SETA was co-transfected with AIP1-Δ717-784 a reduction in cell adhesion relative to control was observed (Fig. 8B), paralleling the ability of SETA to increase the amount of AIP1-Δ717-784 in PYK-2 complexes (Fig. 7, lanes 5,6). Co-transfection of SETA and AIP1 or AIP1-784Stop did not further decrease adhesion, suggesting that it did not promote an effect beyond that already induced by these AIP1 proteins alone.

Finally, to determine whether the presence of AIP1 proteins in focal adhesion kinases had any consequences on the biochemical nature of these proteins, cells were transfected with AIP1 proteins, and the endogenous PYK-2 or FAK protein was immunoprecipitated and analyzed for phosphotyrosine levels (Fig. 9). This study demonstrated that co-transfection of AIP1 resulted in a significant attenuation of both PYK-2 and FAK tyrosine phosphorylation levels, suggesting that this protein may regulate the activity of these protein kinases. AIP1-784Stop also reduced PYK-2 phosphotyrosine levels, but to a smaller extent, and appeared to have little if any effect on FAK phosphorylation levels. This mutant is also present in PYK-2 immunoprecipitates (Fig. 7) and capable of reducing adhesion in HEK293 cells (Fig. 8). As expected from the cell adhesion and the PYK-2 immunoprecipitation data, neither

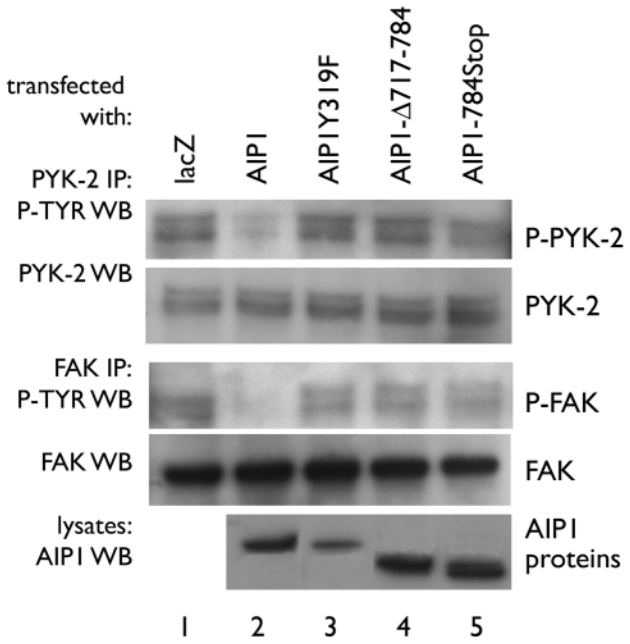

Fig. 9. AIP1 reduces PYK-2 and FAK phosphotyrosine levels. HEK293 cells were transfected with lacZ or AIP1 proteins as indicated, and the endogenous PYK-2 or FAK proteins were immunoprecipitated. Western blots of the immunoprecipitates demonstrate similar levels of focal adhesion kinase proteins, but different levels of phosphotyrosine when AIP1 or, to a lesser extent, AIP1-784Stop are co-transfected.

AIP1Y319F nor AIP1-Δ717-784 affected focal adhesion kinase phosphorylation levels (Fig. 9). This experiment suggests that negative modulation of PYK-2 phosphorylation levels is a consequence of AIP1 protein binding and a possible mediator of AIP1 action on cell adhesion.

Discussion

Confocal analysis (Fig. 1) showed that SETA non-exclusively co-localized with actin microfilaments, focal adhesions and microtubules, suggesting that interaction between SETA and different elements of the cytoskeleton is not obligate and so dynamic and possibly regulated. This is underpinned by its presence at focal adhesions, which are short-lived, appear at contact points between the cell and extra-cellular substrates and are active signaling complexes. At the molecular level, SETA could not be recovered in immunoprecipitations of various cytoskeletal proteins (Fig. 2), suggesting an indirect and perhaps labile association with SETA. One possible candidate to mediate these interactions is the SETA-binding protein AIP1 (Chen et al., 2000), which showed strong interaction with actin and tubulin, the two components SETA was preferentially associated with at the immunohistochemical level. This suggests that AIP1 mediates the interaction between SETA and the cytoskeleton, but does not exclude the possibility that other proteins are involved.

The localization of SETA to focal adhesions prompted us to further explore interactions with the focal adhesion kinases FAK (Cary and Guan, 1999; Parsons et al., 2000) and PYK-2/Cakβ/RAFTK/CADTK/FAK2 (Lev et al., 1995).

Immunoprecipitation studies of endogenous focal adhesion kinases revealed the presence of small amounts of a higher molecular weight isoform of transfected SETA, which most likely represented a dimer (Fig. 3). While it has previously been demonstrated that the coiled-coil domain of SETA can mediate multimerization (Borinstein et al., 2000; Watanabe et al., 2000; Verdier et al., 2002) this is the first instance to our knowledge, of a SETA-binding partner showing a preference for SETA dimers.

Although the association between SETA and FAK or PYK-2 could occur directly via SETA's SH3 domains and the proline-rich regions of both kinases (Lev et al., 1995; Cary and Guan, 1999), the amount of SETA observed in FAK or PYK-2 immunoprecipitates was relatively low and contrasted with the robust signal obtained by cell staining. This suggests that SETA may also be interacting with focal adhesions via other proteins, and prompted an investigation of AIP1 and c-Cbl (Fig. 4). It has previously been shown that c-Cbl associates with PYK-2 via src (Sanjay et al., 2001), and localizes to actin lamellae in fibroblasts (Scaife and Langdon, 2000). Immunoprecipitation studies showed a stronger association between AIP1 and FAK or PYK-2 than was found for SETA, mirroring the results obtained for cytoskeletal proteins, and again allowing for a role for AIP1 in mediating SETA localization. As reported by others (Sanjay et al., 2001) c-Cbl was associated with PYK-2, however it bound more weakly than AIP1, and did not associate with FAK. This suggests the interaction between SETA and the focal adhesion kinases does not resemble SETA's recruitment to the activated EGFR complex, which occurs via Cbl proteins (Soubeyran et al., 2002), but is likely to involve other molecules including AIP1.

The strong presence of AIP1 in focal adhesion kinase complexes prompted an investigation of its binding partner ALG-2 (Vito et al., 1999), which was found to be associated with PYK-2 as well. Interestingly, ALG-2 binding to AIP1 is Ca^{2+} regulated (Vito et al., 1999) as is PYK-2 activation (Lev et al., 1995). ALG-2 shares some sequence homology with the calpain protease family, which disassembles focal adhesions by cleaving the activated FAK complex, and reduces cell adhesion (Frame et al., 2002), allowing for the possibility that it has a regulatory role, and that association with AIP1, and perhaps indirectly SETA, modulates its activity. Furthermore, analysis of the calcium-dependency of the AIP1-PYK-2 interaction (Fig. 5) showed that a large increase in calcium concentration resulted in an enhanced attraction of AIP1 and actin to PYK-2. This may reflect the ability of increased intracellular Ca^{2+} to stimulate the formation of focal adhesions in intact cells (Lev et al., 1995; Blaukat et al., 1999).

To test the role of the interaction between SETA and AIP1 in the presence of AIP1 in focal adhesion kinase complexes we created mutants in AIP1. All AIP1 mutants were restricted in their capacity to bind FAK or PYK-2. The AIP1Y319F mutation, which alters the single consensus tyrosine phosphorylation site, eliminated interaction between AIP1 and PYK-2 completely, allowing for a critical role for tyrosine kinase signaling in the formation of this complex. It has been established that the interaction between activated PYK-2 and src results in the src-mediated phosphorylation of other associated proteins (Blaukat et al., 1999; Parsons et al., 2000), allowing for the possibility that the stability of the AIP1-PYK-2 interaction is dependent on the presence of src and its ability to phosphorylate AIP1 at Y319F.

Analysis of AIP1 mutants also showed that deletion of the SETA binding site in AIP1-Δ717-784 strongly diminished the amount of AIP1 in focal adhesion kinase immunoprecipitations, making it undetectable in the case of PYK-2. Furthermore, elimination of the other half of the proline rich C-terminus in AIP1-784Stop also significantly diminished binding to FAK or PYK-2. Taken together this suggests that the interaction between AIP1 and focal adhesion kinases is not SETA dependent. However, SETA was capable of increasing the amount of AIP1 associated with PYK-2 and partially restoring the interaction of the C-terminal deletion mutants of AIP1 with FAK or PYK-2. This included AIP1-Δ717-784, which lacks the SETA binding site, suggesting that it may be mediated indirectly, and possibly represents the stabilization of the focal adhesion protein complex via other associated proteins. It is worth noting in this context that, in addition to the interactions discussed above, there are other binding partners of the focal adhesion kinases that are also known to interact with SETA. These include the adaptor protein Grb2 (Lev et al., 1995; Cary et al., 1998; Cary and Guan, 1999; Borinstein et al., 2000) and the p85 regulatory subunit of PI3K (Cary and Guan, 1999; Gout et al., 2000; Parsons et al., 2000). In addition, receptor tyrosine kinases, including the EGFR may play a role, as they interact with the very N-terminus of FAK and PYK-2 (Parsons et al., 2000) and are internalized by a complex that contains SETA/CIN85 (Soubeyran et al., 2002).

To examine the impact of the molecular events described above on cells we performed ECIS cell adhesion assays with HEK293 cells transfected with AIP1, AIP1 mutants, SETA or c-Cbl. First, we compared the impact of AIP1, SETA and c-Cbl, and found that all three molecules had profound effects on adhesion, with SETA mediating increased cell adhesion, while AIP1 and c-Cbl caused a similar decrease (Fig. 8A). Analysis of AIP1 mutants, and the modulation of their impact by SETA, provided further evidence that the presence of AIP1 in PYK-2 complexes is an important negative modulator of adhesion. For example, AIP1Y319F, which was not found in focal adhesion kinase complexes, did not reduce cell adhesion, and was incapable of antagonizing SETA's stimulation of cell adhesion in co-transfection experiments. In contrast, AIP1 or AIP1-784Stop, which bound PYK-2 in the presence or absence of SETA, reduced adhesion regardless of whether SETA was co-transfected. Interestingly, AIP1-Δ717-784, which alone was not detectable in PYK-2 immunoprecipitates did not modulate cell adhesion. However, when SETA was co-transfected, and increased the amount of AIP1-Δ717-784 in the complex (Fig. 7), this resulted in a reduction in adhesion as measured by ECIS (Fig. 8B). These data demonstrate a direct connection between the composition of the PYK-2 complex and cell behavior, and suggest that the presence of AIP1 has a negative impact on cell adhesion.

The molecular basis for AIP1's negative impact on cell adhesion is not yet fully understood but has been reported in a different assay for the human homologue Hp95 (Wu et al., 2002). Our experiments show that AIP1 is capable of reducing the level of tyrosine phosphorylation of both FAK and PYK-2 (Fig. 9). Focal adhesion kinase phosphorylation on tyrosine has been linked to increased cell adhesion in another cellular

context (McDonald et al., 2000). Similarly, it has been shown that c-Cbl negatively regulates cell adhesion in a complex with src and PYK-2, and does so by binding the auto-phosphorylation site of c-src, which would otherwise be activated by integrin signaling via focal adhesion kinases (Blaukat et al., 1999; Sanjay et al., 2001). This illustrates a possible mechanism for c-Cbl action in our experiments, and raises the possibility that AIP1 could reduce focal adhesion kinase phosphorylation on tyrosine by interacting with src. AIP1 is likely to be a src kinase substrate by analogy to its *Xenopus* homologue Xp95 (Che et al., 1999). Indeed, the apparent requirement for a tyrosine phosphorylation site (Y319) in AIP1 for interaction with focal adhesion kinases suggests that AIP1 may be an adaptor protein between src and focal adhesion kinases. An alternative mechanism of action may relate to AIP1's potential of recruiting of ALG-2, a relative of calpain proteases that are known to negatively affect focal adhesions (Frame et al., 2002). Similarly SETA's mechanism of action on cell adhesion is not yet clear, although it appears to work in part independently to promote adhesion when no AIP1 is present. When AIP1 proteins are present, this effect is masked by its promotion of AIP1 protein presence in PYK-2 immunoprecipitates. The molecular structure of SETA, as well as the wide range of other interactions described for it, allow that SETA may have additional functions in integrating focal adhesions with other signal transduction pathways (Chen et al., 2000; Borinstein et al., 2000; Take et al., 2000; Watanabe et al., 2000; Dikic, 2002).

In summary, SETA and AIP1 have been identified as proteins that are associated with diverse cytoskeletal elements, including focal adhesion kinases and that have the capacity of modulating cell attachment, which in the case of AIP1 may relate to its modulation of focal adhesion kinase phosphorylation levels and activity. The SETA relative CD2AP/CMS has been reported to co-localize with actin in membrane ruffles (Kirsch et al., 1999), suggesting that this family of adaptor molecules is involved in mediating cytoskeletal function in a variety of settings. Because these proteins have also been implicated in other important cellular functions, including the regulation of apoptosis [AIP1 (Vito et al., 1999; Chen et al., 2000)] and the internalization of receptor tyrosine kinases [SETA/CIN85 (Take et al., 2000; Soubeyran et al., 2002)], they may represent novel points of integration between cell shape, adhesion and motility, and cell division and survival.

This work was supported by CA-R01-84109 from the National Cancer Institute, 98-62 BC-GLO.05 from the James S. McDonnell Foundation, as well as by generosity of the Hermelin Brain Tumor Center donors with particular thanks to William and Karen Davidson. We thank Stan Lipkowitz (National Cancer Institute, National Institutes of Health, Bethesda, MD) for the c-Cbl expression plasmid and Luciano D'Adamio (Albert Einstein College of Medicine, Yeshiva University, Bronx, NY) for AIP1 and ALG-2 constructs.

References

Blaukat, A., Ivankovic-Dikic, I., Gronroos, E., Dolfi, F., Tokiwa, G., Vuori, K. and Dikic, I. (1999). Adaptor proteins Grb2 and Crk couple Pyk2 with activation of specific mitogen-activated protein kinase cascades. *J. Biol. Chem.* **274**, 14893-14901.

Bogler, O., Furnari, F. B., Kindler-Roehrborn, A., Sykes, V. W., Yung, R., Huang, H.-J. S. and Cavenee, W. K. (2000). SETA: a novel SH3 domain-containing adapter molecule associated with malignancy in astrocytes. *Neuro. Oncol.* **2**, 6-15.

Borinstein, S. C., Hyatt, M. A., Sykes, V. W., Straub, R. E., Lipkowitz, S., Boulter, J. and Bogler, O. (2000). SETA is a multifunctional adapter protein with three SH3 domains that binds Grb2, Cbl and the novel SB1 proteins. *Cellular Signalling* **12**, 769-779.

Buchman, V., Luke, C., Borthwick, E., Gout, I. and Ninkina, N. (2002). Organization of the mouse Ruk locus and expression of isoforms in mouse tissues. *Gene* **295**, 13-17.

Cary, L. A. and Guan, J. L. (1999). Focal adhesion kinase in integrin-mediated signaling. *Front. Biosci.* **4**, D102-D113.

Cary, L. A., Han, D. C., Polte, T. R., Hanks, S. K. and Guan, J. L. (1998). Identification of p130Cas as a mediator of focal adhesion kinase-promoted cell migration. *J. Cell Biol.* **140**, 211-221.

Che, S., El-Hodiri, H. M., Wu, C. F., Nelman-Gonzalez, M., Weil, M. M., Etkin, L. D., Clark, R. B. and Kuang, J. (1999). Identification and cloning of xp95, a putative signal transduction protein in Xenopus oocytes. *J. Biol. Chem.* **274**, 5522-5531.

Chen, B., Borinstein, S. C., Gillis, J., Sykes, V. W. and Bogler, O. (2000). The glioma associated protein SETA interacts with AIP1/Alix and ALG-2 and modulates apoptosis in astrocytes. *J. Biol. Chem.* **275**, 19275-19281.

Dikic, I. (2002). CIN85/CMS family of adaptor molecules. *FEBS Lett.* **529**, 110-115.

Ettenberg, S. A., Keane, M. M., Nau, M. M., Frankel, M., Wang, L. M., Pierce, J. H. and Lipkowitz, S. (1999). cbl-b inhibits epidermal growth factor receptor signaling. *Oncogene* **18**, 1855-1866.

Frame, M. C., Fincham, V. J., Carragher, N. O. and Wyke, J. A. (2002). v-Src's hold over actin and cell adhesions. *Nat. Rev. Mol Cell Biol.* **3**, 233-245.

Gout, I., Middleton, G., Adu, J., Ninkina, N. N., Drobot, L. B., Filonenko, V., Matsuka, G., Davies, A. M., Waterfield, M. and Buchman, V. L. (2000). Negative regulation of PI 3-kinase by Ruk, a novel adaptor protein. *EMBO J.* **19**, 4015-4025.

Haglund, K., Shimokawa, N., Szymkiewicz, I. and Dikic, I. (2002). Cbl-directed monoubiquitination of CIN85 is involved in regulation of ligand-induced degradation of EGF receptors. *Proc. Natl. Acad. Sci. USA* **99**, 12191-12196.

Hyman, A., Drechsel, D., Kellogg, D., Salser, S., Sawin, K., Steffen, P., Wordeman, L. and Mitchison, T. (1991). Preparation of modified tubulins. *Methods Enzymol.* **196**, 478-485.

Kirsch, K. H., Georgescu, M. M., Ishimaru, S. and Hanafusa, H. (1999). CMS: an adapter molecule involved in cytoskeletal rearrangements. *Proc. Natl. Acad. Sci. USA* **96**, 6211-6216.

Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B. and Schlessinger, J. (1995). Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions. *Nature* **376**, 737-745.

Lo, C. M., Keese, C. R. and Giaever, I. (1995). Impedance analysis of MDCK cells measured by electric cell-substrate impedance sensing. *Biophys. J.* **69**, 2800-2807.

Mayer, B. J. and Eck, M. J. (1995). Minding your p's and q's. *Curr. Biol.* **5**, 364-367.

McCarthy, K. D. and de Vellis, J. (1980). Preparation of separate astrocyte and oligodendrocyte cultures from rat cerebral tissue. *J. Cell Biol.* **85**, 890-902.

McDonald, J. T., Teague, R. M., Benedict, S. H. and Chan, M. A. (2000). Induction of PYK-2 phosphorylation during LFA-1/ICAM-1-dependent homotypic adhesion of fresh human B-cells. *Immunol. Invest* **29**, 71-80.

Mitra, P., Keese, C. R. and Giaever, I. (1991). Electric measurements can be used to monitor the attachment and spreading of cells in tissue culture. *Biotechniques* **11**, 504-510.

Parsons, J. T., Martin, K. H., Slack, J. K., Taylor, J. M. and Weed, S. A. (2000). Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. *Oncogene* **19**, 5606-5613.

Sanjay, A., Houghton, A., Neff, L., DiDomenico, E., Bardelay, C., Antoine, E., Levy, J., Gailit, J., Bowtell, D., Horne, W. C. and Baron, R. (2001). Cbl associates with Pyk2 and Src to regulate Src kinase activity, alpha(v)beta(3) integrin-mediated signaling, cell adhesion and osteoclast motility. *J. Cell Biol.* **152**, 181-195.

Scaife, R. M. and Langdon, W. Y. (2000). c-Cbl localizes to actin lamellae and regulates lamellipodia formation and cell morphology. *J. Cell Sci.* **113**, 215-226.

Soubeyran, P., Kowanetz, K., Szymkiewicz, I., Langdon, W. Y. and Dikic, I. (2002). Cbl-CIN85-endophilin complex mediates ligand-induced downregulation of EGF receptors. *Nature* **416**, 183-187.

Szymkiewicz, I., Kowanetz, K., Soubeyran, P., Dinarina, A., Lipkowitz, S. and Dikic, I. (2002). CIN85 participates in Cbl-b-mediated downregulation of receptor tyrosine kinases. *J. Biol. Chem.* **277**, 39666-39672.

Take, H., Watanabe, S., Takeda, K., Yu, Z. X., Iwata, N. and Kajigaya, S. (2000). Cloning and characterization of a novel adaptor protein, CIN85, that interacts with c-Cbl. *Biochem. Biophys. Res. Commun.* **268**, 321-328.

Tiruppathi, C., Malik, A. B., del Vecchio, P. J., Keese, C. R. and Giaever, I. (1992). Electrical method for detection of endothelial cell shape change in real time: assessment of endothelial barrier function. *Proc. Natl. Acad. Sci. USA* **89**, 7919-7923.

Verdier, F., Valovka, T., Zhyvoloup, A., Drobot, L. B., Buchman, V., Waterfield, M. and Gout, I. (2002). Ruk is ubiquitinated but not degraded by the proteasome. *Eur. J. Biochem.* **269**, 3402-3408.

Vito, P., Lacana, E. and D'Adamio, L. (1996). Interfering with apoptosis: Ca2+-binding protein ALG-2 and Alzheimer's disease gene ALG-3. *Science* **271**, 521-525.

Vito, P., Pellegrini, L., Guiet, C. and D'Adamio, L. (1999). Cloning of AIP1, a novel protein that associates with the apoptosis-linked gene ALG-2 in a Ca2+-dependent reaction. *J. Biol. Chem.* **274**, 1533-1540.

Watanabe, S., Take, H., Takeda, K., Yu, Z. X., Iwata, N. and Kajigaya, S. (2000). Characterization of the CIN85 adaptor protein and identification of components involved in CIN85 complexes. *Biochem. Biophys. Res. Commun.* **278**, 167-174.

Wu, Y., Pan, S., Luo, W., Lin, S. H. and Kuang, J. (2002). Hp95 promotes anoikis and inhibits tumorigenicity of HeLa cells. *Oncogene* **21**, 6801-6808.