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The focal adhesion kinase amino-terminal domain localises to nuclei and intercellular junctions in HEK 293 and MDCK cells independently of tyrosine 397 and the carboxy-terminal domain

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Abstract

The function and intracellular localisation of the non-catalytic NH_2-terminal region of focal adhesion kinase (FAK) are unclear. We investigated the targetting of the FAK NH_2-terminal domain in HEK 293 and epithelial MDCK cells. Exogenous expression of a variety of GFP-fused and epitope-tagged NH_2 terminal domain constructs either including or lacking the major Tyr 397 autophosphorylation and Src-binding site targeted to nuclei and cell–cell junctions in HEK 293 cells and co-localised at junctions with occludin, and β1 integrin subunits at junctions. Mutation of Tyr 397 also had no effect on localisation of the NH_2-terminal domain. In contrast, constructs encoding either the kinase or focal adhesion targeting (FAT) domains but lacking the NH_2-terminal region failed to localise to intercellular junctions or nuclei. The NH_2-terminal domain was not associated with β1 integrin subunits as indicated by co-immunoprecipitation experiments, but did co-localise with cortical actin filaments. The NH_2-terminal domain also targetted to nuclei and intercellular junctions in MDCK cells, whereas full-length FAK localised only to focal adhesions in these cells. These results indicate that the FAK NH_2-terminal domain targets to epithelial intercellular junctions and nuclei and suggest novel functions for FAK NH_2-terminal domain fragments independent of Y397, kinase, and FAT domains.

Keywords: FAK; Integrin; FERM; Cell–cell adhesion

Focal adhesion kinase (FAK) is a component of focal adhesions in adherent mammalian cells [1,2] which is essential for embryonic development [3] and implicated in several cellular functions including migration, adhesion, survival, and cell cycle control [4–8]. FAK is the prototypical member of a small family of kinases, also including the proline-rich tyrosine kinase, Pyk2 [9], and the *Drosophila* gene product, Dfak56 [10], which exhibit a characteristic structure, comprising a central kinase domain (amino acids 421–680 in murine FAK) flanked by large, non-catalytic, NH_2-terminal (1–420), and COOH-terminal (681–1052) domains.

FAK tyrosine phosphorylation and activation is induced by integrin-mediated cell–matrix adhesion and has been identified as a common signalling pathway stimulated by diverse extracellular stimuli [11,12], Specific phosphorylated tyrosine residues in FAK are thought to be critical for signal relay by mediating complex formation between FAK and other signalling molecules. Y397 is the major FAK autophosphorylation site and phosphorylation at this residue creates a high-affinity binding site for the SH2 domains of $pp60^{c\text{-}src}$ and $pp59^{c\text{-}fyn}$ [13], and phosphatidylinositol-3-kinase [14]. FAK is also phosphorylated in vitro by Src at tyrosine residues 407, 576, 577, 861, and 925 [15–17]. Phosphorylation at Y576 and Y577 in the activation loop enhances FAK kinase activity [15] and Y925 is a binding site for the SH2 domain protein GRB-2 [16]. The COOH-terminal domain of FAK is expressed in some tissues as an alternative transcript encoding a 41–43 kDa protein called FRNK (for FAK-related non-kinase) [18], and this domain antagonises FAK signalling by

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competing for binding to focal contacts [4,19]. FAK localisation to focal adhesions is mediated primarily by the COOH-terminal focal adhesion targeting (FAT, residues 840–1052) domain [20].

The non-catalytic NH_2-terminal domain of FAK shares homology with the FERM domain originally identified in a family of proteins including the erythrocyte band 4.1 protein, ezrin, moesin, and radixin, which interact with the cortical erythrocyte cytoskeleton [21,22]. The FAK NH_2-terminal domain was previously reported to associate directly with the cytoplasmic domains of integrins [23], but this interaction has not been demonstrated in intact cells and the FAK motif responsible for this interaction has remained unidentified. A growing body of evidence now suggests that the FAK NH_2-terminal domain may have functions distinct from the kinase and FAT domains and autonomous of cell–matrix adhesion [24–27]. The NH_2-terminal domain also displays a distinct intracellular distribution in some cell types [28,29].

There is little information regarding the intracellular targeting of the FAK NH_2-terminal domain. In this study we investigated the intracellular localisation of GFP- and epitope-tagged constructs expressing the FAK NH_2-terminal and COOH-terminal domains. Our results demonstrate that the NH_2-terminal FAK domain uniquely localises to the nuclei and cell–cell junctions of HEK 293 cells and epithelial MDCK cells. Furthermore, localisation of this domain was independent of the Y397 autophosphorylation site. This domain also co-localised at cell–cell contacts with other junctional proteins including the β1 integrin subunits and occludin. In contrast, wild-type FAK and COOH-terminal FAK constructs failed to target to intercellular junctions or the nucleus. These findings indicate that the NH_2-terminal domain associates autonomously to cell–cell junctions and the nucleus suggesting novel cellular functions for this region of the FAK molecule in epithelial cells.

Materials and methods

FAK constructs. The full-length murine FAK cDNA cloned into pBluescript (pBS/FAK) was provided by Dr. Steven Hanks (Vanderbilt University, Nashville, TN). FAK-GFP constructs encoding NH_2-terminal residues 1–420 (ATF-420-GFP and ATF-420F-GFP) were the gift of Dr. Lucia Beviglia and Dr. W. Cance (Department of Surgery and Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill School of Medicine). Other constructs were generated from pBS/FAK as follows. Flag-tagged wild-type, full-length FAK (Flag-FAK) was generated using the 5′ primer, 5′-TAG CGG CCG CCA CCA TGG ACT ACA AGG ACG ACG ATGACA AGG CAG CTG CTT-3′, containing a *Not*I restriction site; and the 3′ primer, 5′-CAA GTA ACG AAT TCT CAA CTC-3′, which covers the internal *Eco*RI site at position 484. PCR was performed to generate a Flag-tagged 5′ product which was digested with *Not*I and *Eco*RI. pBS/FAK was then digested with *Eco*RI and *Xba*I to produce a 3′ fragment which was co-ligated with the 5′ PCR product into the *Not*I and *Xba*I sites of pcDNA 3.1 (+). FAK-GFP was generated using a similar strategy as for Flag-FAK. A modified 3′ FAK fragment was generated using the 5′ primer 5′-AAC CTT GAC CGG TCC AAT G-3′, covering the internal *Age*I site at position 2363; and the 3′ primer 5′-GGC GAT GGA TCC GTG TGG CCG TGT CTG CCC TAG-3′. An unmodified 5′ fragment was produced by digesting pBS/FAK with *Kpn*I and *Age*I. The resulting fragment was purified by agarose gel electrophoresis and co-ligated with the 5′ PCR product into the *Kpn*I and *Bam*HI sites of pEGFP-N3. Flag-ATF-386 was generated using the 5′ primer, 5′-TAG CGG CCG CCA CCA TGG ACT ACA AGG ACG ACG ATGACA AGG CAG CTG CTT-3′ and the 3′ primer, 5′-TGA CAC GGA TCC TCA CTA TGT CCG CAT GCC TTG CTT TTC ACT-3′ and cloned into the *Not*I and *Bam*HI sites of pcDNA 3.1 (−). ATF-386-GFP was produced using the 5′ primer, 5′-ACT GCT CGA GCG CCA CCA TGG CAG CTG CTT ATC TTG AC-3′ and the 3′ primer, 5′-TGA CAC GGA TCC TGT CCG CAT GCC TTG CTT TTC ACT-3′ and cloned into the *Xho*I and *Bam*HI sites of pEGFP-N3. Flag-FAT was generated using the 5′ primer, 5′-TAG CGG CCG CCA CCA TGG ACT ACA AGG ACG ACG ATG ACA AGC TCT CTC GAG GGC AGC ATC-3′ and the 3′ primer, 5′-ACG CGG ATC CTT ATC AGT GTG GCC GTG TCT GCC C-3′ and cloned into the *Not*I and *Bam*HI sites of pcDNA 3.1 (−). GFP-FAT was generated by excising the *Xho*I/*Bam*HI fragment from Flag-FAT (thus removing the Flag tag) and ligating this into the *Xho*I and *Bam*HI sites of pEGFP-C1. A construct encoding the kinase domain and lacking NH_2-terminal and FAT domains (Kin-GFP) was generated using the 5′ primer, 5′-GCA TCT CGA GCA CCA TGG CAG TCT CTG TGT CAG AGA CA-3′ and the 3′ primer, 5′-GAT GGA TCC GAG CCT CAC ATC AGG TTT CAG GAA-3′ and cloned into the *Xho*I and *Bam*HI sites of pEGFP-N3. PCR was performed using Platinum *Pfx* (Gibco BRL), according to manufacturer's instructions. All constructs were confirmed by DNA sequencing (Cambridge Bioscience).

Antibodies and reagents. Antibodies to β-catenin, β1 integrin subunit, and vinculin were purchased from Chemicon; ZO-1, occludin, and pan-cadherin antibodies were from Zymed; anti-FLAG antibodies were from Sigma Chemical; antibody to phosphoY397-FAK was from BioSource International; antibody to γ-catenin (plakoglobin) was from Transduction Labs; Ezrin antibody was from Upstate Biotechnology; and antibodies to E-cadherin, ZO-2, FAK NH_2-terminal and COOH-terminal antibodies, HRP-conjugated anti-mouse, anti-rabbit, and anti-goat antibodies, and protein A/G+ agarose were obtained from Santa Cruz. Rhodamine red-X- and FITC-conjugated secondary antibodies were obtained from Jackson Immunoresearch Laboratories. Fluorescent secondary antibodies were used at 7.5 μg/ml. Foetal calf serum was obtained from TCS. All restriction enzymes were obtained from Roche Molecular Biochemicals. T4 DNA ligase was purchased from New England Biolabs. All other reagents were of the highest grade available.

Cell culture and transfections. HEK 293 cells were the gift of Dr. Andrew Tinker (BHF Laboratories, Department of Medicine, University College London) and MDCK cells were provided by Professor Mike Horton (Bone and Mineral Centre, Department of Medicine, University College London). HEK 293 cells were maintained in minimal essential medium containing Earl's salts and supplemented with 10% FCS, L-glutamine, non-essential amino acids, and penicillin/streptomycin. MDCK cells were maintained in DMEM containing L-glutamine and supplemented with 10% FCS, non-essential amino acids, and penicillin/streptomycin.

For fluorescence microscopy experiments, HEK 293 cells were cultured on glass coverslips in 12-well plates at a density of 1×10^5 cells per well. Cells were seeded in normal growth medium containing no antibiotics. Transfections were performed 24 h after plating when cells had reached ~60% confluence using LipfectAMINE according to manufacturer's instructions. Briefly, 400 ng DNA and 2.5 μl LipofectAMINE were used to transfect each well in a reduced serum medium (OptiMEM, Gibco BRL). Transfection complexes were removed after

6 h and replaced with medium containing serum but no antibiotics. MDCK cells were plated on glass coverslips in 12-well plates at a density of 1.5×10^5 per well and transfected using 1 μg DNA, 2.5 μl PLUS reagent, and 4.5 μl LipofectAMINE per well, in OptiMEM reagent, 24 h after seeding when cells had reached 60% confluence. Transfection was allowed to proceed for 6 h, after which time it was stopped by the addition of medium containing 20% FCS and no antibiotics. For immunoprecipitation and Western blotting experiments, HEK 293 cells were cultured at 1×10^6 cells per well in 6-well plates and transfected with 3.2 μg DNA and 8 μl LipofectAMINE 2000 in normal growth medium containing no antibiotics, approximately 24 h after seeding when cells had reached 90–95% confluence. Cells were harvested 24 h after transfection for Western blotting and immunoprecipitation as described below.

Immunocytochemistry and confocal microscopy. Cells were fixed and stained 24–48 h post-transfection or at approximately 60% confluence for untransfected cells. For immunofluorescent staining, coverslips were washed three times in PBS; immersed in 100% anhydrous methanol at −20 °C for 5 min; and washed three times in PBS. Non-specific binding was blocked by incubation with 5% normal donkey serum (NDS) in PBS for 30 min at 37 °C. Coverslips were incubated with primary antibody diluted in 5% NDS/PBS for 1 h at 37 °C, after which coverslips were washed three times in PBS, incubated with fluorescent secondary antibody (all of donkey origin) diluted in 5% NDS in PBS for 30 min at 37 °C, and washed three times in water. Cells were slide-mounted using the Pro-Long Anti-FADE Kit (Molecular Probes). For phalloidin staining, cells seeded on glass coverslips were washed three times in PBS, immersed in 4% paraformaldehyde for 10 min at room temperature, washed three times in PBS, permeabilised in 0.2% Triton X-100 for 2 min at room temperature, washed three times in PBS and incubated with 1 μg/ml TRITC-conjugated phalloidin at 37 °C for 30 min. Coverslips were finally washed three times in water and mounted as above. Fluorescently stained cells were viewed 24 h after mounting on a Zeiss Axiophot 100M microscope. Volume deconvolution was performed using the Openlab 2.2.4 software from Improvision.

Western blotting and immunoprecipitations. Cells were harvested 24 h after the addition of transfection complexes whereas untrans-

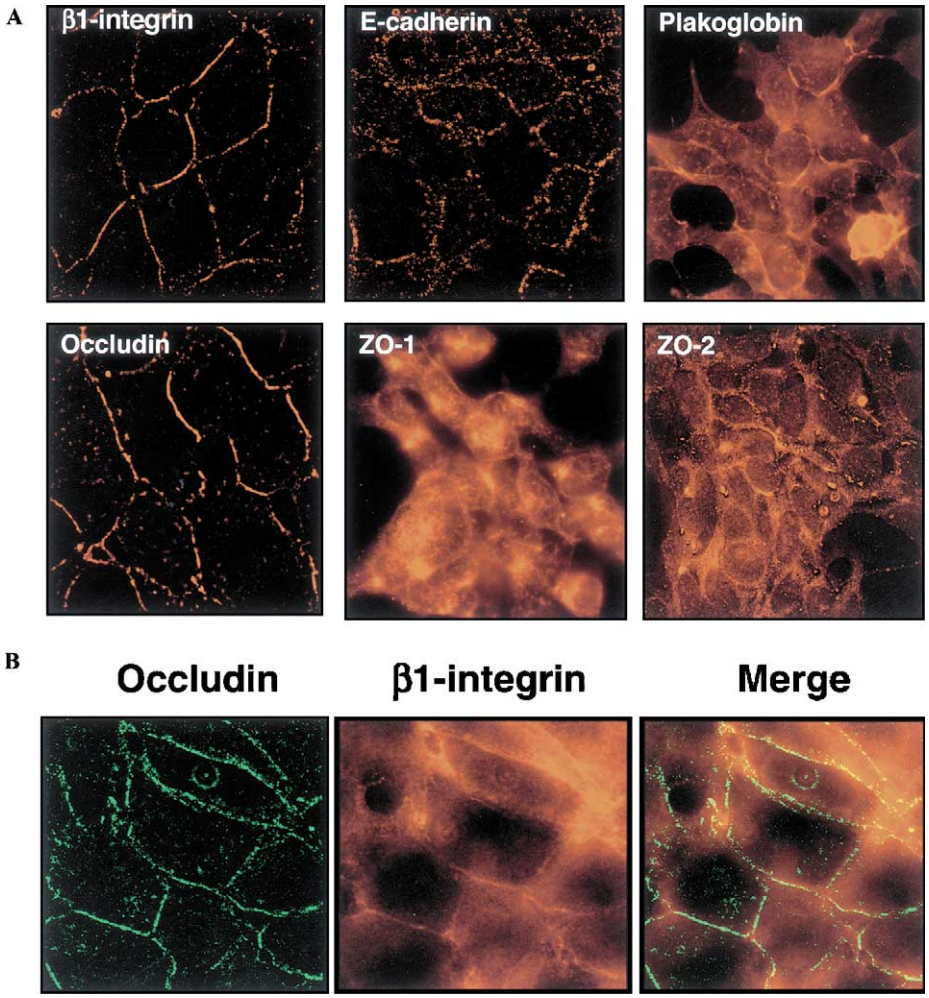

Fig. 1. Localisation of β1-integrin and cell junction components in HEK 293 cells. (A) HEK 293 cells were immunostained with antibodies for β1 integrin subunit and components of adherens junctions (E-cadherin, plakoglobin) and tight junctions (occludin, ZO-1, and ZO-2). Antibody staining was visualised using TRITC-conjugated secondary antibodies. All these components produce staining of cell–cell junctions. (B) Confluent HEK 293 cells were fixed in methanol and stained with rabbit, polyclonal anti-occludin (FITC, green), and monoclonal β1 integrin subunit (rhodamine, red) antibodies. Antibody staining was visualised with FITC-conjugated swine anti-rabbit and rhodamine red-X-conjugated donkey anti-mouse secondary antibodies.The yellow colour in the merge shows co-localisation of these components at cell–cell junctions. The results shown in (A) and (B) are representative of at least three independent experiments.

fected cells were harvested at confluence. Cells were scraped into ice-cold Tris-buffered saline (TBS) and washed twice in ice-cold TBS. Cells were then lysed with gentle agitation at 4 °C for 30 min in ice-cold lysis buffer, containing 1× COMPLETE protease inhibitor cocktail (Roche Molecular Biochemicals), 1% Triton X-100, 0.5% NP-40, and 0.2 mM Na_3VO_4 in TBS. Cell lysates were centrifuged at top speed in a microcentrifuge for 15 min at 4 °C. The insoluble pellets were discarded. Protein estimation was performed using the DC Protein Assay Kit (Bio-Rad). For Western blots, 20–50 μg protein in 2× SDS–PAGE sample buffer was subjected to SDS–PAGE in 8–12% acrylamide gels. Proteins were transferred to PVDF membranes (Millipore) and blocked in 5% non-fat milk in TBS for 1 h at 37 °C. Membranes were then blotted with primary antibodies at their appropriate dilutions. Membranes were washed and incubated with HRP-conjugated secondary antibodies. After further washing, antibody binding was visualised by chemiluminescence using the ECL+ Kit (Amersham).

For immunoprecipitations, cells were harvested and lysed 24 h post-transfection, as above for Western blotting. The concentration of protein in the lysate was adjusted to 1 mg/ml and a total of 1 mg protein was used for each immunoprecipitation. The adjusted lysates were precleared by incubation with 25 μl protein A/G+ agarose for 30 min at 4 °C. Primary antibody was then added to the lysates at a final concentration of 1–5 μg/ml and incubated for 1 h at 4 °C. Twenty-five μl protein A/G+ agarose was then added to the lysate/antibody mixture and incubated for 1 h at 4 °C. The agarose/antibody complexes were collected, washed three times with lysis buffer, and extracted by heating to 95–100 °C in 2 × SDS–PAGE sample buffer. Either 25 or 50 μl eluted protein was then subjected to SDS–PAGE and Western blotting as described above.

Results

The human embryonic kidney cell line HEK 293 has previously been used to demonstrate localisation of components of epithelial cell–cell junctions [30], but intercellular junctions have not been fully characterised in these cells. HEK 293 cells possess an epithelioid 'cobblestone' morphology and form intercellular junctions which are immunostained with antibodies to several components of epithelial adherens and tight junctions including E-cadherin, plakoglobin, occludin, ZO-1, and ZO-2 (Fig. 1). E-cadherin and plakoglobin are major adherens junction proteins [31], while occludin, ZO-1, and ZO-2 are components of tight junctions [32]. The β1 integrin subunit also exhibited a striking localisation to cell–cell junctions and co-localised with occludin (Fig. 1). Western blot with the corresponding antibodies verified expression of protein bands of predicted apparent molecular weight (results not shown).

Immunostaining of HEK 293 cells with antibodies to the FAK NH_2-terminal and COOH-terminal domains produced diffuse cytoplasmic staining, but was absent from areas of intercellular adhesion and nuclei (Fig. 2A). It was a consistent finding that neither FAK antibody displayed localisation to focal adhesions or

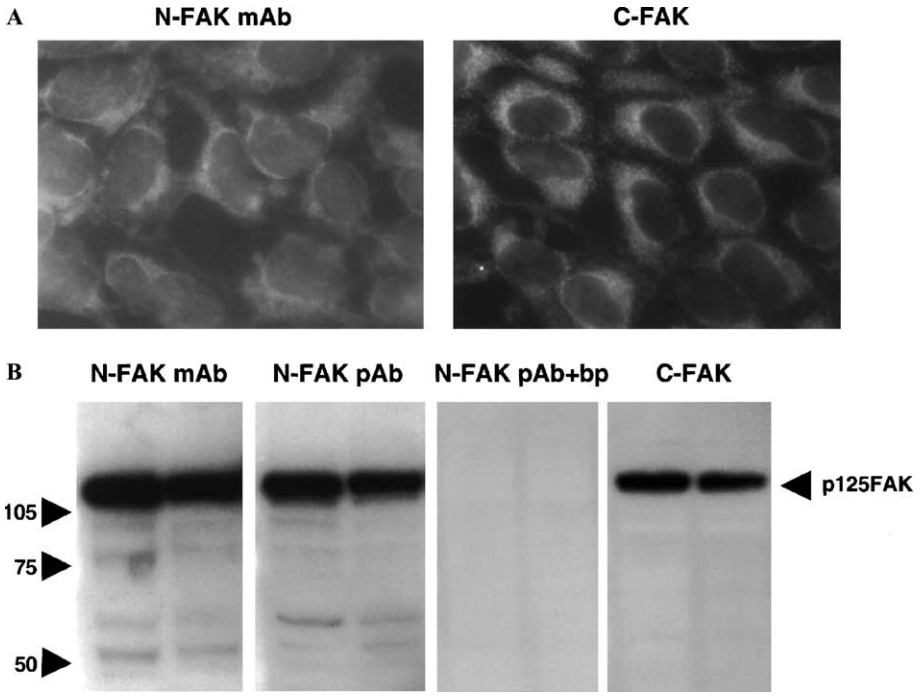

Fig. 2. Localisation and expression of endogenous FAK in HEK 293 cells. (A) HEK 293 cells were immunostained with antibodies specific for the FAK NH_2-terminus and COOH-terminus. Both FAK NH_2-terminal and COOH-terminal antibodies produce diffuse cytoplasmic immunostaining absent from areas of cell–cell contact and nuclei. (B) Western blot of whole cell HEK 293 cell extracts with monoclonal (mAb) and polyclonal (pAb) antibodies specific for the FAK NH_2-terminus (N-FAK) and polyclonal antibody to the carboxy terminus (C-FAK). Blotting with the NH_2-terminal pAb in the presence of the corresponding antigenic blocking peptide (bp) is also shown. Note that both antibodies recognise a major 125 kDa band corresponding to wild-type full-length FAK, while the NH_2-terminal antibody also detects a 55 kDa fragment, and the COOH-terminal antibody no additional bands. The positions of molecular weight markers are indicated on the right. The results shown in (A)–(C) are representative of at least three independent experiments.

structures of similar appearance in HEK 293 cells. Immunostaining for another focal adhesion-associated component, vinculin, resulted in a punctate pattern which was predominantly cytoplasmic in distribution, but did not produce staining of either cell junctions or typical focal adhesion-like structures in HEK 293 cells

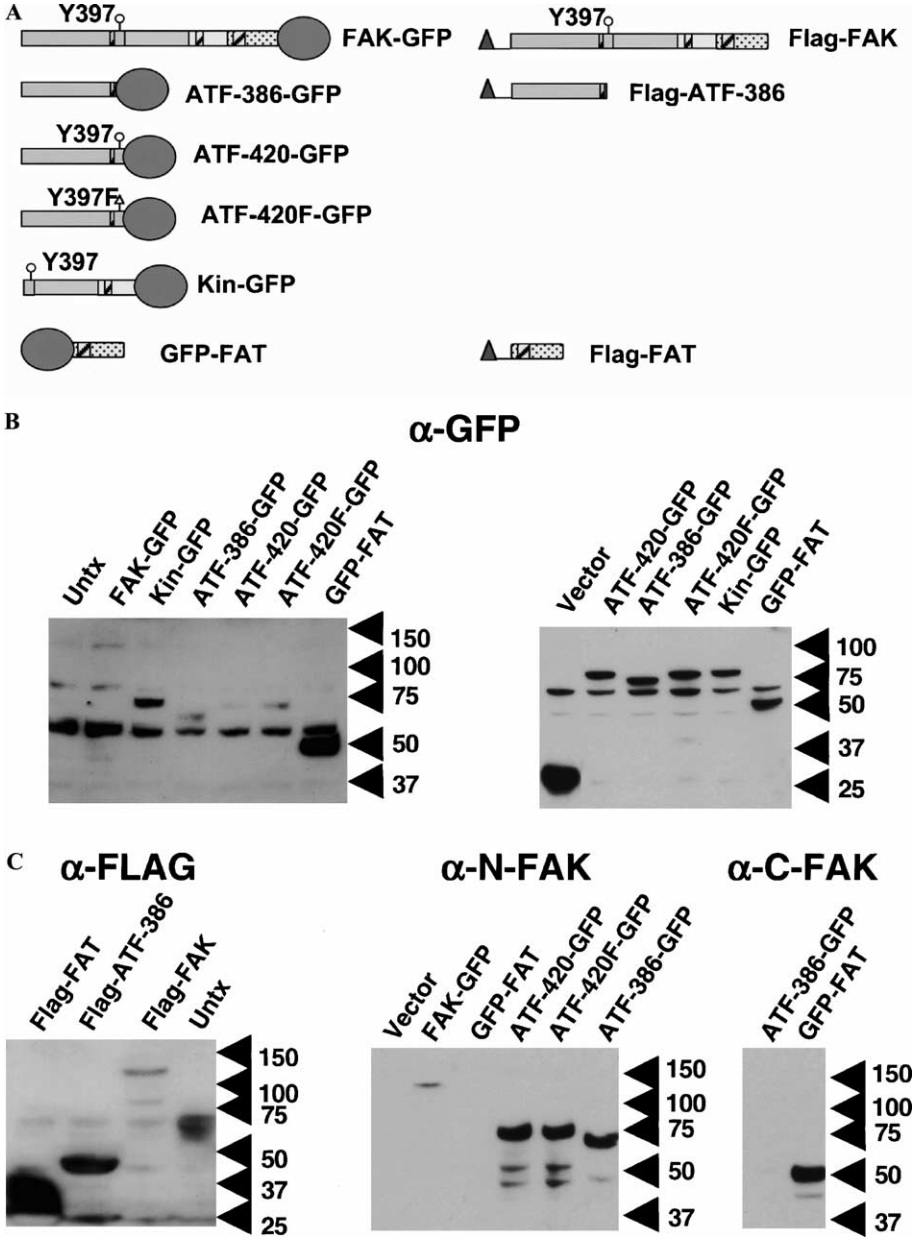

Fig. 3. Expression of GFP- and Flag-tagged FAK constructs in HEK 293 cells. (A) FAK constructs fused with GFP encoding wild-type full-length FAK (FAK-GFP), NH_2-terminal residues 1–386 (ATF-386-GFP), NH_2-terminal residues 1–420 (ATF-420-GFP), NH_2-terminal residues 1–420 with a Y397F mutation (ATF-420F-GFP), the COOH-terminal residues 840–1052 comprising the FAT domain (GFP-FAT), and residues 387–839 (kin-GFP) were generated as described in Materials and methods. Selected Flag-tagged constructs were also made encoding full-length FAK (Flag-FAK), residues 1–386 (Flag-ATF-386), and the FAT domain (Flag-FAT). (B) and (C) Cells were transfected with the GFP-FAK (B) and Flag-FAK (C) constructs as indicated and 24 h after transfection, cell lysates were prepared and blotted with either HRP-conjugated anti-GFP (α-GFP, B) or with anti-Flag (α-FLAG, C) antibodies. Untransfected cell lysates (Untx) are shown for comparison. In (B), protein bands of ~70 kDa corresponding to the kin-GFP and ATF-420-GFP constructs migrate with a similar apparent molecular weight, while the ATF-386-GFP protein migrates with a lower molecular weight. The GFP-FAT construct is expressed as a 50 kDa band while full-length FAK-GFP generates a 145 kDa band. Results of a similar experiment showing expression of the NH_2-terminal-GFP, FAT-GFP, and kin-GFP constructs compared with the GFP vector alone are given below. In (C), Flag-tagged full-length FAK, 1–386, and FAT appear as bands of approximately 125, 45, and 30 kDa, respectively. (D) Cell extracts were prepared from cells transfected with either GFP vector or the GFP FAK constructs indicated and blotted with NH_2-terminal FAK antibody (α-N-FAK). Extracts of ATF-386-GFP and FAT-GFP transfectants were also blotted with COOH-terminal FAK antibody (α-C-FAK). The positions of molecular weight markers are indicated with arrowheads.

(not shown). Western blotting showed that antibodies to the FAK NH_2-terminal and COOH-terminal domains both recognised a major 125 kDa band corresponding to full-length FAK. In addition the NH_2-terminal antibody weakly recognised several lower molecular weight bands including a 55 kDa species (Fig. 2B). The 55 kDa NH_2-terminal-immunoreactive band is similar in apparent molecular weight to an endogenous, constitutively expressed NH_2-terminal fragment in human umbilical vein endothelial [28] and glioblastoma cells [29].

To examine directly whether the NH_2-terminal domain could target specifically to intracellular junctions, a series of domain-specific FAK constructs were expressed in HEK 293 cells (Fig. 3). Because the NH_2-terminal domain also includes the major autophosphorylation and Src-binding site at Y397, we made an NH_2-terminal construct lacking Y397 corresponding to residues 1–386 of murine FAK fused with GFP (ATF-386-GFP, Fig. 3A). Expression of this construct produced an intracellular distribution characterised by distinct localisation at intercellular junctions and strong nuclear staining (Fig. 4). Since fusion of the NH_2-terminal domain with GFP could alter its normal intracellular localisation, we also examined localisation of this domain fused at its NH_2-terminus with the Flag epitope (Flag-ATF-386). As shown in Fig. 4, immunostaining of HEK 293 cells expressing Flag-ATF-386 with a Flag-specific antibody revealed a very similar intracellular distribution to that of ATF-386-GFP. The role of the Y397 phosphorylation site in intracellular targetting of the FAK NH_2-terminal domain was investigated by determining the localisation of constructs encoding NH_2-terminal residues 1–420 and 1–420 containing a Y397F mutation both fused with COOH-terminal GFP (ATF-420-GFP and ATF-420/397F-GFP, respectively). Both these constructs strongly localised to intercellular junctions and nuclei (Fig. 4) producing a similar pattern to that obtained by expression of the smaller NH_2-terminal domain proteins.

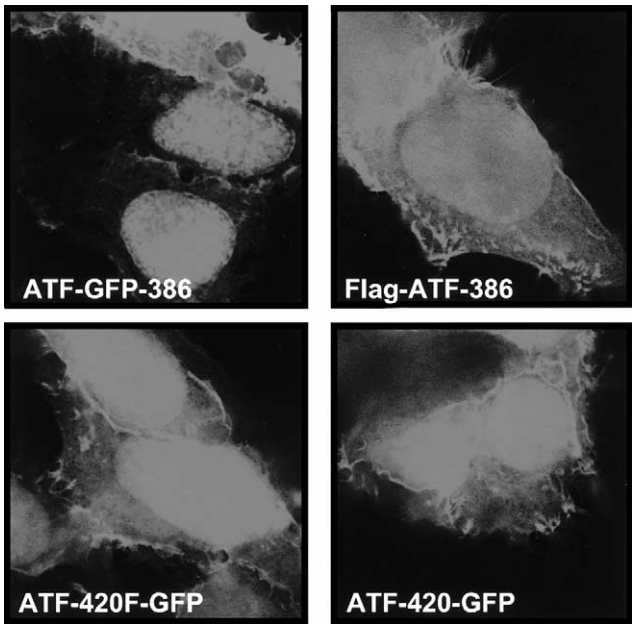

Fig. 4. Localisation of FAK NH_2-terminal domain constructs to cell–cell junctions and nuclei. HEK 293 cells were transfected with ATF-386-GFP, Flag-ATF-386, ATF-420-GFP, and ATF-420F-GFP plasmids. After 24 h, transfected cells were fixed in paraformaldehyde. Flag-ATF-386 was visualised using anti-Flag M5 and an FITC-conjugated secondary antibody. Localisation of constructs was documented as described in Materials and methods. Note the marked localisation of all NH_2-terminal constructs to cell–cell junctions and nuclei of transfected cells. The results shown are representative of at least five independent experiments.

Immunostaining of cells expressing ATF-386-GFP with an antibody specific for the FAK COOH-terminal domain showed no co-localisation at intercellular junctions or the nucleus (Fig. 5), indicating that NH_2-terminal domain targetting was independent of the FAT domain. Furthermore, in contrast to the FAK NH_2-terminal domain constructs, expression of either full-length wild-type FAK (FAK-GFP and Flag-FAK), the FAT domain (residues 840–1052; Flag-FAT and GFP-FAT), or a construct encoding residues 387–839 containing the kinase domain and lacking both the NH_2-terminal and FAT domains (Kin-GFP) (Fig. 1), produced markedly different patterns of intracellular localisation characterised by strong perinuclear, juxtanuclear, and cytoplasmic staining, and a marked absence of staining at areas of cell–cell contact (Fig. 5).

To further investigate localisation of the FAK NH_2-terminal domain to cell–cell junctions, co-immunostaining was performed for FAK constructs and junctional components in HEK 293 cells transfected with ATF-386-GFP. The GFP-tagged FAK NH_2-terminal domain co-localised strongly with both occludin and β1 integrin subunit at cell–cell junctions (Fig. 6). The relationship between FAK NH_2-terminal domain localisation and organisation of actin filaments was also examined. Rhodamine-conjugated phalloidin produced a cortical mesh-like belt of staining in regions of cell–cell contact which exhibited a strong degree of co-localisation with the GFP-NH_2-terminal domain (Fig. 6).

Since the β1 integrin subunit and FAK NH_2-terminal domain co-localised at intercellular junctions, it was investigated whether they were associated. Immunoprecipitation of FAK constructs or the β1 subunit from HEK 293 cells transfected with empty vector, Flag-ATF-386, Flag-FAT, or Flag-FAK, followed by immunoblotting with β1 subunit or FAK antibodies revealed no specific co-immunoprecipitation of these components (results not shown).

To determine whether junctional localisation of the FAK NH_2-terminal domain was typical of other cell

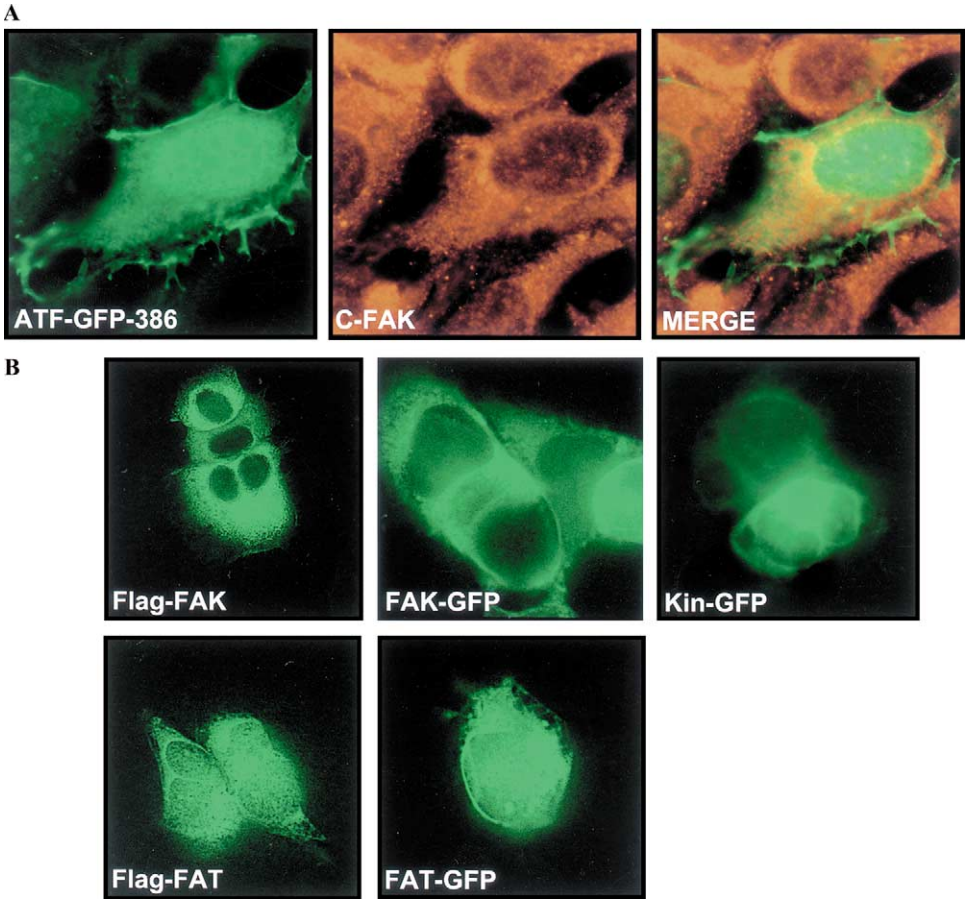

Fig. 5. Differential localisation of FAK NH_2-terminal and COOH-terminal domains. (A) HEK 293 cells were transfected with ATF-386-GFP and 24 h later immunostained with a COOH-terminal specific antibody (C-FAK). ATF-386-GFP localisation is shown in the left panel (green) and COOH-terminal antibody staining was visualised with a rhodamine-conjugated secondary (red, middle panel). The merge between AF-386-GFP and C-FAK (right panel) shows no or little co-localisation between COOH-terminal antibody staining and ATF-386-GFP at cell–cell junctions or in the nucleus. (B) HEK 293 cells were transfected with either Flag-FAK, FAK-GFP, Kin-GFP, Flag-FAT, or FAT-GFP. Flag-tagged constructs were visualised 24 h later using anti-Flag M5 and an FITC-conjugated secondary antibody. Localisation is primarily perinuclear and cytoplasmic. Staining of cell–cell contacts and nuclei is largely absent. The results in (A) and (B) are representative of at least three independent experiments.

types, intracellular targetting of NH_2-terminal domain constructs was investigated in epithelial MDCK cells. In contrast to HEK293 cells, immunostaining of MDCK cells with antibodies to the FAK NH_2-terminal and COOH-terminal domains showed strong focal adhesion localisation of FAK (Fig. 7A). Western blotting with these antibodies detected a major 125 kDa band corresponding to full-length FAK and in addition the NH_2-terminal antibody weakly recognised several lower molecular weight bands including a 55 kDa species (Fig. 7B).

Immunostaining of MDCK cells with antibodies to β1-integrin subunit, plakoglobin, occludin, and ZO-1 showed strong localisation of these components to cell–cell junctions (Fig. 7C). Very similar to results obtained in HEK293 cells, the ATF-386-GFP construct also localised strongly to intercellular junctions and nuclei in MDCK cells. In contrast, expression of Flag-tagged wild-type full-length FAK produced no localisation at intercellular junctions in MDCK cells, but did result in strong anti-Flag staining of focal adhesions (Fig. 7C).

Discussion

In this paper we demonstrate that constructs encoding the FAK NH_2-terminal domain localise to nuclei and cell–cell junctions in HEK 293 cells and co-localise with other components of intercellular junctions including the β1 integrin subunit and occludin. Targeting of the NH_2-terminal domain to cell–cell junctions was also independent of Y397, the major autophosphorylation and Src-binding site, since constructs lacking this residue or in which Y397 was substituted produced a very similar pattern of intracellular localisation. The FAK NH_2-terminal domain exhibited a similarly well-defined pattern of localisation to cell–cell junctions and nuclei in

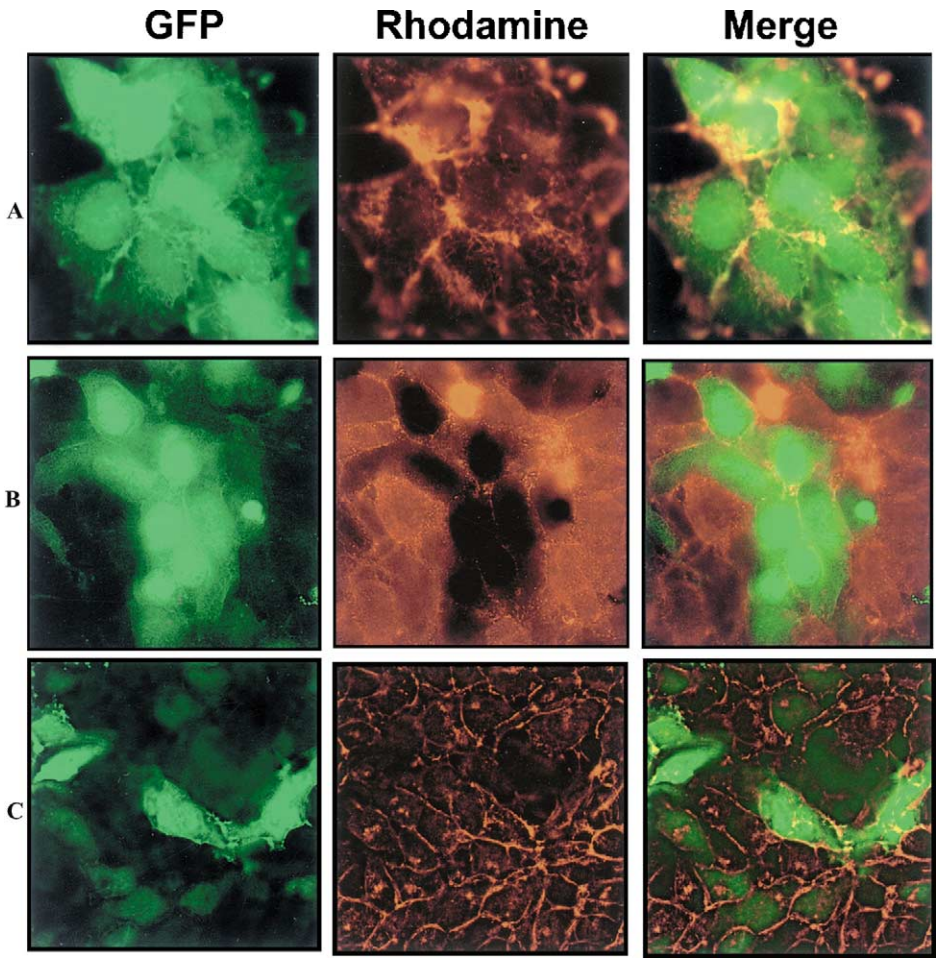

Fig. 6. The NH_2-terminal domain of FAK co-localises with actin, β1 integrin, and occludin in HEK 293 cells. HEK 293 cells were transfected with ATF-386-GFP. Twenty-four hours after transfection, cells were fixed and stained with either rhodamine-conjugated phalloidin (A) or antibodies against β1 integrin subunit (B) or occludin (C). Antibody staining was visualised using rhodamine-conjugated secondary antibodies. The merge shows co-localisation (yellow) of ATF-386-GFP with actin filaments (A), β1 integrin (B), and occludin (C) at cell–cell junctions. The results shown are representative of at least three independent experiments.

epithelial MDCK cells, whereas wild-type FAK localised to focal adhesions and produced no junctional or nuclear staining. Thus, although neither wild-type FAK nor FAT constructs localised to typical focal adhesions in HEK 293 cells, a disruption of focal adhesion architecture in these cells is unlikely to account for the targeting of the NH_2-terminal domain to cell–cell junctions.

We previously provided evidence for nuclear localisation of an endogenous 45 kDa NH_2-terminal band in non-transfected human umbilical vein endothelial cells [28], and a similar endogenous NH_2-terminal FAK fragment has been identified in glioblastoma cells [29]. The level of expression of both NH_2-terminal and COOH-terminal FAK fragments in HEK293 and MDCK cells was very low, and no endogenous nuclear or junctional localisation of endogenous FAK-immunoreactive species could be detected in these cells. Though it remains unclear whether there is a function for constitutively expressed NH_2-terminal FAK species, these findings support the conclusion that FAK NH_2-terminal domain fragments are specifically targeted to the nucleus and in some cell types to intercellular junctions independently of other FAK domains. Since FAK NH_2-terminal fragments are generated during apoptosis [28,29], nuclear or junctional targetting of these species may play a role during cell death.

An association of integrins with cell–cell junctions has not been unambiguously established. Our results indicate that β1 integrin subunits localise strongly to cell–cell junctions in HEK 293 and MDCK cells, a finding that is of interest in the context of recent studies suggesting a regulatory role for integrins in the function of intercellular junctions [33,34]. However, we found no evidence for an association of the FAK NH_2-terminal domain with the β1 integrin subunit in HEK 293 cells, despite the co-localisation of these proteins at cell–cell contacts. Though FAK has been reported to associate with peptides corresponding to the cytoplasmic domains

of β1 and β3 integrin subunits in vitro, confirmation of these findings in intact cells is so far lacking and neither the regions of FAK that interact with integrins nor the role of FAK-integrin associations has been defined. The fact that no association of FAK with β1 integrin subunits was evident in cells strongly expressing the NH_2-terminal domain suggests that a direct interaction between these components is unlikely to mediate FAK localisation to cell–cell junctions.

The NH_2-terminal FERM domain is a strong candidate to mediate the association of FAK with cell–cell junctions given that this domain targets other FERM family members to the plasma membrane via associations with the cytoplasmic regions of transmembrane proteins [22]. Furthermore, the inability of both wild-type FAK and the COOH-terminal domain to localise to intercellular junctions indicates that the ability of the FAK FERM domain to target to junctions when it is part of the full-length molecule is suppressed by other regions of FAK. This is a property shared with other FERM family proteins which display distinct intracellular distributions and functions when expressed as separate NH_2- and COOH-terminal domains or as full-length molecules, and is consistent with a model of FERM protein regulation in which binding sites in the FERM domains are masked by an intramolecular interaction [35–38]. Identification of the proteins associated with the FAK FERM domain and the mechanism(s) regulating availability of its binding sites warrants further work.

The marked nuclear localisation of the NH_2-terminal domain was a striking feature of our findings. Moreover, expression of the NH_2-terminal domain in endothelial cells also results in striking nuclear localisation, as well as localisation to endothelial adherens junctions (J. Kabir and I. Zachary, unpublished findings). Dfak56, a *Drosophila* homologue of FAK, was also reported to localise in part to the nucleus [10]. Though the role of nuclear localisation of the FAK NH_2-terminal domain is unclear, recent findings indicate that other components originally

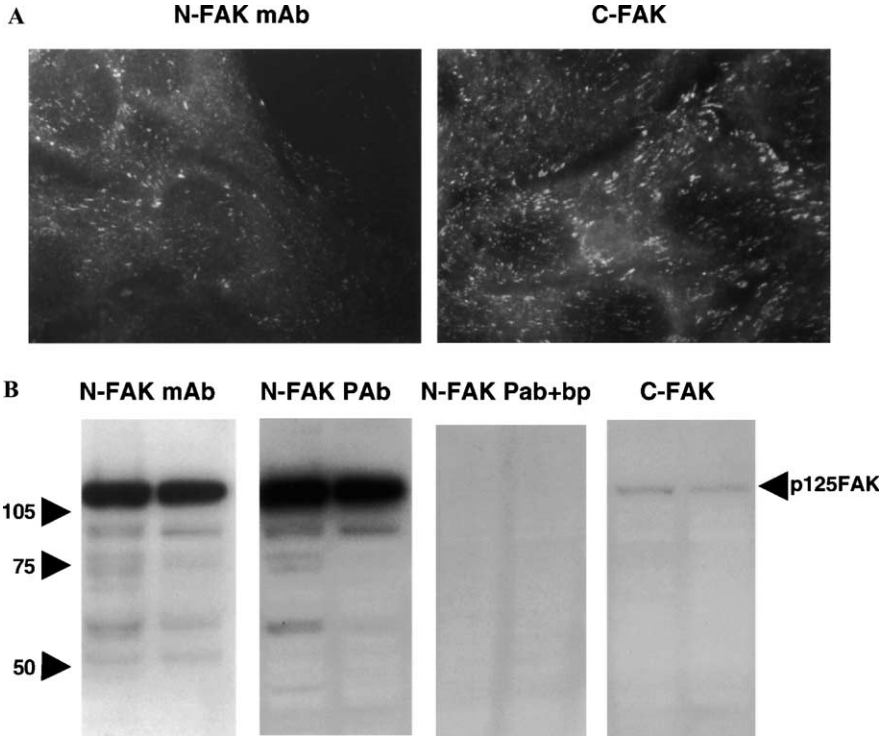

Fig. 7. The FAK NH_2-terminal domain localises to areas of cell–cell contact and nuclei in MDCK cells. (A) MDCK cells were immunostained with antibodies specific for the FAK NH_2-terminus and COOH-terminus. Both FAK NH_2-terminal and COOH-terminal antibodies produce strong focal adhesion immunostaining absent from areas of cell–cell contact and nuclei. (B) Western blot of whole cell MDCK extracts with monoclonal (mAb) and polyclonal (pAb) antibodies specific for the FAK NH_2-terminus (N-FAK) and polyclonal antibody to the carboxy terminus (C-FAK). Blotting with the NH_2-terminal pAb in the presence of the corresponding antigenic blocking peptide (bp) is also shown. Both antibodies recognise a major 125 kDa band corresponding to wild-type full-length FAK, while the NH_2-terminal antibody also detects a 55 kDa fragment, and the COOH-terminal antibody no additional bands. (C) Confluent MDCK cells were fixed and stained with antibodies to β1 integrin, plakoglobin, ZO-1, and occludin. Antibody staining was visualised using rhodamine-conjugated secondary antibodies and demonstrates localisation of these components to cell–cell junctions. Flag-FAK and ATF-386-GFP were expressed in parallel in confluent MDCK cells and localisation of Flag-FAK was determined using anti-Flag M5 antibody and an FITC-conjugated secondary. Flag-FAK localised strongly to basolateral focal adhesions, but was absent from cell–cell junctions and nuclei, whereas ATF-386-GFP localised strongly to cell–cell contacts and nuclei. The results shown are representative of at least two independent experiments.

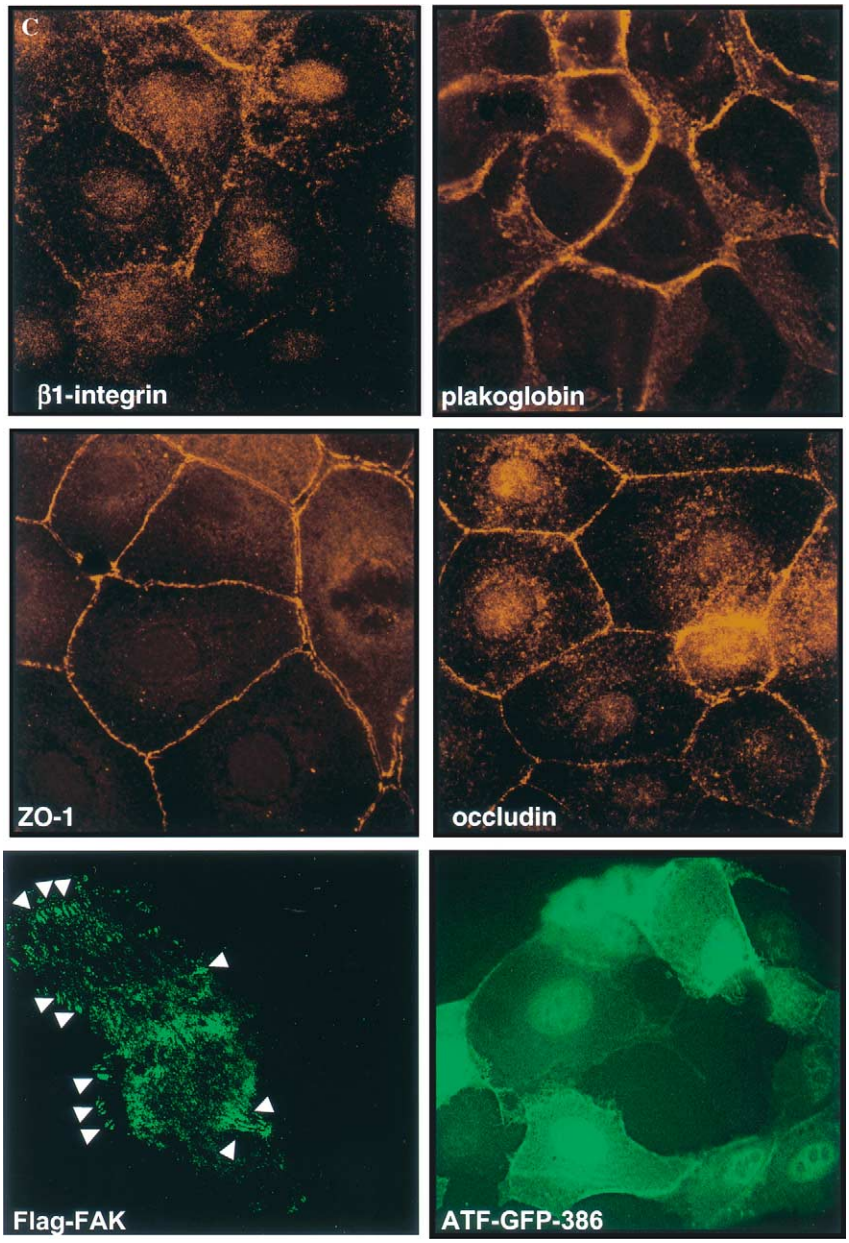

Fig. 7 (*continued*)

identified as focal adhesion-associated proteins, including Zyxin [39] and the p130Cas-related protein HEF1 [40], also localise to the nucleus. FAK does not contain a classical nuclear localisation signal, though a highly conserved consensus nuclear export signal, IALKLG CLEI [41] is present between residues 166 and 176. The distribution of the FAK NH_2-terminal domain between the nuclei and cell–cell junctions of HEK 293 and MDCK cells points to a dual role for this domain in regulating cell functions.

The demonstration that the FAK NH_2-terminal domain localises to epithelial cell–cell junctions and the nucleus raises the possibility that the generation of NH_2-terminal FAK fragments by post-translational processing may provide a novel mechanism for modulating cell behaviour. Alternatively, different FAK domains may be postulated to play distinct cell type-specific roles. Elucidation of the mechanisms controlling production of NH_2-terminal domain fragments and their cellular functions is likely to generate novel insights into the biological role(s) of FAK.

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References

[1] M.D. Schaller, C.A. Borgman, B.S. Cobb, R.R. Vines, A.B. Reynolds, J.T. Parsons, pp125^{FAK}, a structurally distinctive protein-tyrosine kinase associated with focal adhesions, Proc. Natl. Acad. Sci. USA 89 (1992) 5192–5196.

[2] S.K. Hanks, M.B. Calalb, M.C. Harper, S.K. Patel, Focal adhesion protein tyrosine kinase phosphorylated in response to cell attachment to fibronectin, Proc. Natl. Acad. Sci. USA 89 (1992) 8487–8491.

[3] D. Ilic, Y. Furuta, S. Kanazawa, N. Takeda, K. Sobue, N. Nakatsuji, S. Nomukira, J. Fujimoto, M. Okada, T. Yamamoto, S. Aizawa, Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice, Nature 377 (1995) 539–544.

[4] A. Richardson, J.T. Parsons, A mechanism for Regulation of the adhesion-associated protein tyrosine kinase pp125^{FAK}, Nature 380 (1996) 538–540.

[5] L.A. Cary, J.F. Chang, J.-L. Guan, Stimulation of cell migration by over-expression of focal adhesion kinase and its association with Src and Fyn, J. Cell Sci. 109 (1996) 1787–1794.

[6] S.M. Frisch, K. Vuori, E. Ruoslahti, P.Y. Chan-Hui, Control of adhesion dependent cell survival by focal adhesion kinase, J. Biol. Chem. 134 (1996) 793–799.

[7] J.E. Hungerford, M.T. Compton, M.L. Matter, B.G. Hoffstrom, C.A. Otey, Inhibition of pp125FAK in cultured fibroblasts results in apoptosis, J. Biol. Chem. 135 (1996) 1383–1390.

[8] J.-H. Zhao, H. Reiske, J.-L. Guan, Regulation of the cell cycle by focal adhesion kinase, J. Cell Biol. 143 (1998) 1997–2008.

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

[10] J. Fujimoto, K. Sawamoto, M. Okabe, Y. Takagi, T. Tezuka, S. Yoshikawa, H. Ryo, H. Okano, T. Yamamoto, Cloning and characterization of Dfak56, a homolog of focal adhesion kinase, in *Drosophila melanogaster*, J. Biol. Chem. 274 (1999) 29196–29201.

[11] I. Zachary, E. Rozengurt, Focal adhesion kinase (p125^{FAK}): a point of convergence in the action of neuropeptide, integrins and oncogenes, Cell 71 (1992) 891–894.

[12] S.K. Hanks, T.R. Polte, Signalling through focal adhesion kinase, Bioessays 19 (1997) 137–145.

[13] M.D. Schaller, J.D. Hildebrand, J.D. Shannon, J.W. Fox, R.R. Vines, J.T. Parsons, Autophosphorylation of the focal adhesion kinase, pp125^{FAK}, directs SH2-dependent binding of pp60Src, Mol. Cell. Biol. 14 (1994) 1680–1688.

[14] H.-C. Chen, P.A. Appeddu, H. Isoda, J.-L. Guan, Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase, J. Biol. Chem. 271 (1996) 26329–26334.

[15] M.B. Calalb, T.R. Polte, S.K. Hanks, Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases, Mol. Cell. Biol. 15 (1995) 954–963.

[16] D.D. Schlaepfer, S.K. Hanks, T. Hunter, P. van der Geer, Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase, Nature 372 (1994) 786–791.

[17] M.B. Calalb, X. Zhang, T.R. Polte, S.K. Hanks, Focal adhesion kinase tyrosine-861 is a major site of phosphorylation by Src, Biochem. Biophys. Res. Commun. 228 (1996) 662–668.

[18] M.D. Schaller, C.A. Borgman, J.T. Parsons, Autonomous expression of a noncatalytic domain of the focal adhesion-associated protein tyrosine kinase pp125^{FAK}, Mol. Cell. Biol. 13 (1993) 785–791.

[19] J.M. Taylor, C.P. Mack, K. Nolan, C.P. Regan, G.K. Owens, J.T. Parson, Selective expression of an endogenous inhibitor of FAK regulates proliferation and migration of vascular smooth muscle cells, Mol. Cell. Biol. 21 (2001) 1565–1572.

[20] J.D. Hildebrand, M.D. Schaller, J.T. Parsons, Identification of sequences required for the efficient localisation of the focal adhesion kinase, pp125$^{(FAK)}$, to cellular focal adhesions, J. Cell Biol. 123 (1993) 993–1005.

[21] A.H. Chishti, A.C. Kim, S.M. Marfatia, M. Lutchman, M. Hanspal, H. Jindal, S.C. Liu, P.S. Low, G.A. Rouleau, N. Mohandas, J.A. Chasis, J.G. Conboy, P. Gascard, Y. Takakuwa, S.C. Huang, E.J. Benz Jr., A. Bretscher, R.G. Fehon, J.F. Gusella, V. Ramesh, F. Solomon, V.T. Marchesi, S. Tsukita, K.B. Hoover, et al., The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane, Trends Biochem. Sci. 238 (1998) 281–282.

[22] J.-A. Girault, G. Labesse, J.-P. Mornon, I. Callebaut, The N-termini of FAK and JAKs contain divergent band 4.1 domains, Trends. Biochem. Sci. 24 (1999) 54–57.

[23] M.D. Schaller, C.A. Otey, J.D. Hildebrand, J.T. Parsons, Focal adhesion kinase and paxillin bind to peptides mimicking β integrin cytoplasmic domains, J. Cell Biol. 130 (1995) 1181–1187.

[24] D. Vial, H. Okazaki, R.P. Siraganian, The NH_2-terminal region of focal adhesion kinase reconstitutes high affinity IgE receptor-induced secretion in mast cells, J. Biol. Chem. 275 (2000) 28269–28275.

[25] D.J. Sieg, C.R. Hauck, D. Ilic, C.K. Klingbeil, E. Schlaepfer, C.H. Damsky, D.D. Schlaepfer, FAK integrates growth-factor and integrin signals to promote cell migration, Nat. Cell. Biol. 2 (2000) 249–256.

[26] R. Chen, O. Kim, M. Li, X. Xiong, J.L. Guan, H.J. Kung, H. Chen, Y. Shimizu, Y. Qiu, Regulation of the PH-domain-containing tyrosine kinase Etk by focal adhesion kinase through the FERM domain, Nat. Cell. Biol. 3 (2001) 439–444.

[27] P. Poullet, A. Gautreau, G. Kadare, J.-A. Girault, D. Louvard, M. Arpin, Ezrin interacts with focal adhesion kinase and induces its activation independently of cell–matrix adhesion, J. Biol. Chem. 276 (2001) 37686–37691.

[28] M. Lobo, I. Zachary, Nuclear localization and apoptotic regulation of an amino-terminal domain focal adhesion kinase (FAK) fragment in endothelial cells, Biochem. Biophys. Res. Commun. 276 (2000) 1068–1074.

[29] G. Jones, J. Machado Jr., A. Merlo, Loss of focal adhesion kinase (FAK) inhibits epidermal growth factor receptor-dependent migration and induces aggregation of NH_2-terminal FAK in the nuclei of apoptotic glioblastoma cells, Cancer Res. 61 (2001) 4978–4981.

[30] K. Turksen, T.C. Troy, Claudin-6: a novel tight junction molecule is developmentally regulated in mouse embryonic epithelium, Dev. Dyn. 222 (2001) 292–300.

[31] A.S. Yap, W.M. Brieher, B.M. Gumbiner, Molecular and functional analysis of cadherin-based adherens junctions, Annu. Rev. Cell. Dev. Biol. 13 (1997) 119–146.

[32] B.R. Stevenson, B.H. Keon, The tight junction: morphology to molecules, Annu. Rev. Cell. Dev. Biol. 14 (1998) 89–109.

[33] G.K. Ojakian, D.R. Ratcliffe, R. Schwimmer, Integrin regulation of cell–cell adhesion during epithelial tubule formation, J. Cell Sci. 114 (2001) 941–952.

[34] S. Levenberg, B.Z. Katz, K.M. Yamada, B. Geiger, Long-range and selective autoregulation of cell–cell or cell–matrix adhesions by cadherin or integrin ligands, J. Cell Sci. 111 (1998) 347–357.

[35] M. Algrain, O. Turunen, A. Vaheri, D. Louvard, M. Arpin, Ezrin contains cytoskeleton and membrane binding domains accounting

for its proposed role as a membrane-cytoskeletal linker, J. Cell Biol. 120 (1993) 129–139.
[36] M.D. Henry, C. Gonzalez Agosti, F. Solomon, Molecular dissection of radixin: distinct and interdependent functions of the amino- and carboxy-terminal domains, J. Cell Biol. 129 (1995) 1007–1022.
[37] M. Martin, C. Andreoli, A. Sahuquet, P. Montcourrier, M. Algrain, P. Mangeat, Ezrin NH_2-terminal domain inhibits the cell extension activity of the COOH-terminal domain, J. Cell Biol. 128 (1995) 1081–1093.
[38] R. Gary, A. Bretscher, Ezrin self-association involves binding of an N-terminal domain to a normally masked C-terminal domain that includes the F-actin binding site, Mol. Biol. Cell. 6 (1995) 1061–1075.
[39] D.A. Nix, M.C. Beckerle, Nuclear-cyoplasmic shuttling of the focal contact protein, Zyxin: a potential mechanism for communication between sites of cell adhesion and the nucleus, J. Cell Biol. 138 (1997) 1139–1147.
[40] S.F. Law, Y.-Z. Zhang, A.J.P. Klein-Szanto, E.A. Golemis, Cell cycle-regulated processing of HEF1 to multiple protein forms differentially targetted to multiple subcellular compartments, Mol. Cell. Biol. 18 (1998) 3540–3551.
[41] E.A. Nigg, Nucleocytoplasmic transport: signals, mechanisms and regulation, Nature 386 (1997) 779–787.