Regulation of focal adhesion dynamics and disassembly by phosphorylation of FAK at tyrosine 397

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Summary

One of the major tyrosine phosphorylation activities linked to integrin signalling is that of focal adhesion kinase (FAK). High amounts of FAK are located at specialised subcellular compartments known as focal adhesions. FAK tyrosine phosphorylation at focal adhesions is increased by various stimuli including integrin engagement during migration processes, growth factors and oncogene transformation. Phosphorylation of FAK at various tyrosine residues regulates focal adhesion turnover by mechanisms that are not well understood. We made a fluorescent FAK mutant (Y397F-FAK/YCam) to analyse, in living cells, how phosphorylation of FAK regulates the turnover of focal adhesions. We found that expression of Y397F-FAK/YCam in human astrocytoma cells decreases the level of phosphorylation of FAK at endogenous Tyr-397 residues and at both endogenous and exogenous Tyr-576 residues, in the putative activation loop of the kinase. This corresponds to a decrease in phosphorylation of FAK at focal adhesions in Y397F-FAK/YCam cells, since the cellular localisation of FAK phosphoTyr-576 in cells expressing Y397F-FAK/YCam or FAK/YCam was not different. Furthermore, FRAP analysis showed that phosphorylation of FAK at Tyr-397 increases specifically the time-residency of FAK at focal adhesions but not in cytosol. This in turn induces disassembly of focal adhesions at the cell tail and promotes cell motility as shown by the decrease in microtubule-mediated turnover of Y397F-FAK/YCam-containing focal adhesions. Our data show that phosphorylation of FAK at Tyr-397 is a key determinant of how FAK controls focal adhesion turnover.

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Key words: Focal adhesion kinase, Focal adhesion dynamics, Src, FRAP

Introduction

Cellular adhesion and migration processes involve remodelling and reorganisation of specialised adhesion sites (Lauffenburger and Horwitz, 1996). In focal adhesion (FA) contacts, aggregated integrins span the plasma membrane and mediate direct interactions with components of the extracellular matrix and with cytoskeletal proteins (Burridge and Fath, 1989). In migrating fibroblasts, regarded as a slow motile cell stereotype, FAs are mainly immobile, in agreement with these adhesion sites serving as traction points, except in restricted retractile cell edges where they display centripetal movements (Smilenov et al., 1999). This localised regulation of FA movement involves asymmetric signal transduction and can be expected to direct cell polarisation and migration. One of the major kinases implicated in FA signalling is focal adhesion kinase (FAK). FAK is activated and localized at FAs upon cell adhesion to the extracellular matrix (Schaller et al., 1994; Schaller et al., 1999; Schlaepfer et al., 1999). Given the abundance of FAs and the reduced migration of fibroblasts from FAK-null mice (Ilic et al., 1995), FAK is probably involved in FA remodelling during migration. Activation of FAK requires phosphorylation of different tyrosine residues. To date, six tyrosine phosphoacceptor sites in FAK have been identified, namely tyrosines 397, 407, 576, 577, 861 and 925 (Calalb et al., 1995; Schlaepfer and Hunter, 1996a). Integrin binding to the extracellular matrix and clustering of integrins induced by antibodies or fibronectin-coated beads trigger phosphorylation of FAK at Tyr-397, which is believed to be an autophosphorylation site (Kornberg et al., 1991; Kornberg et al., 1992; Schaller et al., 1994; Calalb et al., 1995). Once phosphorylated, Tyr-397 generates a high affinity binding site for SH2-domain-containing proteins such as Src family kinases, phosphatidylinositol 3-kinase, Grb7 or phospholipase Cγ (Chen and Guan, 1994; Schaller et al., 1994; Xing et al., 1994; Zhang et al., 1999). Src binding to FAK apparently contributes to Src activation, promoting further phosphorylation of FAK at additional tyrosines. Indeed, in cellular lysates from primary chicken embryo cells expressing both Y397F-FAK and Src, no phosphorylation of Tyr-576/577 and Tyr-863 was observed, consistent with a requirement for binding of Src to FAK after phosphorylation at Tyr-397 (Maa and Leu, 1998). Subsequently, Tyr-576/577 phosphorylation

was shown to enhance FAK activity by increasing the intermolecular trans-phosphorylation of FAK (Calalb et al., 1995; Ruest et al., 2000; Leu and Maa, 2002), whereas Tyr-863 phosphorylation increases FAK activity via intramolecular cis-phosphorylation of FAK; both events lead to greater phosphorylation of FAK at Tyr-397 (Calalb et al., 1996; Leu and Maa, 2002). These data have lead to the well-recognized model whereby integrin engagement initiates phosphorylation of FAK at Tyr-397. This creates a binding site for Src family kinases which in turn phosphorylate additional tyrosine residues of FAK, increasing FAK kinase activity and thus FAK autophosphorylation via both trans- and cis-phosphorylation. This positive loop, in turn, increases FAK-dependent signalling.

Recently, live-cell imaging of fluorescently labelled FA components was used to analyse the role of signalling molecules and protease in regulating FA disassembly (Franco et al., 2004; Webb et al., 2004). Using a talin mutant, calpain-mediated proteolysis of talin was shown to be a rate-limiting step during FA turnover (Franco et al., 2004). FAK, Src, $p130^{CAS}$, paxillin, ERK and MLCK were all found to be required for efficient FA disassembly (Webb et al., 2004). Specifically, treatment of cells with a Src inhibitor or expression of a kinase-defective mutant of Src decreased the rate constant of FA disassembly as observed using fluorescently tagged paxillin and zyxin (Webb et al., 2004). A similar effect was found after expression of a Y397F-FAK mutant, consistent with Tyr-397 phosphorylation and subsequent recruitment of Src to FAs being necessary for FA disassembly. Thus, a common signalling pathway leading to FA disassembly appears to require an obligatory phosphorylation step. In agreement, ERK/MAP kinase phosphorylation is necessary for calpain 2 activation, which leads to FA turnover (Carragher et al., 2003). Recently, we reported the existence of a rapid flux of FAK between cytosolic and FA compartments in U87 astrocytoma cells, as revealed by FRAP analysis (Giannone et al., 2004). Furthermore, compared to FAK, the dominant negative FAK-related non-kinase (FRNK), which lacks both the autophosphorylation site and the kinase domain of FAK (Schaller et al., 1993), had a shorter time-residency at FAs (Giannone et al., 2004). In view of the above actions resulting from the phosphorylation of FAK, we hypothesized that FAK-dependent signalling is correlated to the time-residency of FAK at FAs, which in turn could control FA turnover and thus cell migration.

To analyse further the role of FAK during the disassembly of FAs in living cells, we made a fluorescently tagged mutant of FAK in which Tyr-397 was replaced by phenylalanine (Y397F-FAK/YCam). We compared the specific pattern of phosphorylation of Y397F-FAK/YCam and its dynamics at FAs in U87 astrocytoma cells to those of FAK/YCam in order to address more precisely how Tyr-397 phosphorylation influences FA disassembly.

Materials and Methods

Reagents

Cell culture medium (EMEM), fetal calf serum (FCS), Hepes, L-glutamine, penicillin, streptomycin, gentamycin and trypsin-EDTA solution were from Gibco. Matrigel and the monoclonal antibody (mAb) directed against the kinase domain of FAK were from Becton-Dickinson. Polyclonal antibodies (Abs) directed against the C-terminal (Ct) domain of FAK and the FAT domain were from Upstate and Sigma, respectively. Phospho-specific FAK Abs were from Santa Cruz Biotechnology. Nocodazole was from Sigma.

Cell culture

The U87-MG human astrocytoma cell line was obtained from the American Type Culture Collection (ATCC). Cells were grown on type I collagen (0.06 mg/ml)-coated plastic dishes in EMEM supplemented with 10% heat-inactivated FCS, 0.6 mg/ml glutamine, 200 IU/ml penicillin, 200 IU/ml streptomycin and 0.1 mg/ml gentamycin. They were maintained at 37°C in a humidified incubator with 5% CO_2 in air.

Expression plasmids and transfection

As previously described (Giannone et al., 2002), a fluorescent FA-targeted protein, FAK/YCam, was made by fusion of FAK cDNA (Whitney et al., 1993) (pCDM8-FAK plasmid kindly provided by S. B. Kanner) to the 3′ end of yellow Cameleon-2 (YCam2) (Miyawaki et al., 1997). To fuse FAK in continuity with the YCam2 reading frame, the stop codon next to enhanced yellow fluorescent protein (EYFP) was replaced by a tyrosine codon (QuickChange, Stratagene). FAK cDNA was amplified by PCR using a 5′ primer containing a *Mfe*I site and a 3′ primer containing a *Nhe*I site. The FAK PCR product was digested with *Mfe*I and *Nhe*I and cloned in the corresponding compatible sites, EcoRI and *Xba*I, located in the multiple cloning site of the newly mutated pcDNA3-YCam2 vector, adjacent to EYFP, to give FAK-Ycam. To create Y397F-FAK/YCam, the plasmid pcDNA3-FAK/YCam was amplified by PCR with Taq polymerase *Pfu* Turbo and two primers (Y397FS, Y397FAS) using a QuickChange Mutagenesis kit (Stratagene). The PCR product was digested with *Dpn*I. All constructs were verified by sequencing. The plasmids were isolated (JetStar Plasmid kit; Genomed, Lohne, Germany) before transfection with FuGENE 6. Cells were selected 24 hours later using 800 μg/ml G418 (Sigma) and maintained with 400 μg/ml G418. Cells were sorted to obtain >80% expressing cells using a FACStar cell sorter (Becton-Dickinson) before use.

Immunoblotting

FAK/YCam- and Y397F-FAK/YCam-expressing cells were plated at low density on Matrigel (178 μg/ml) for 2 days, then washed with cold PBS and incubated with lysis buffer (1% Triton X-100, 1% SDS, 100 mM NaF, 1 mM Na_3VO_4, 10 mM NaP_2O_7, in PBS, supplemented with the anti-protease cocktail Complete (Boehringer, Mannheim, Germany). Cell lysates were solubilized in Laemmli's buffer (5% glycerol, 2.5% β-mercaptoethanol, 1% SDS, 0.005% Bromophenol Blue, 50 mM Tris-HCl, pH 6.8) at 95°C and resolved by SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). After blocking overnight at room temperature with 0.1% casein in PBS/0.3% Tween 20, membranes were incubated for 1.5 hours with different Abs: anti-FAK kinase (immunogen corresponding to the kinase domain of FAK, amino acids 354-533; used at 1/1000 v/v dilution); anti-FAK Ct (immunogen corresponding to the C-terminal region of FAK, amino acids 748-1052; 1/4000 dilution); anti-FAT domain (immunogen corresponding to the C-terminal region of FAK, amino acids 1039-1052; 1/4000 dilution); anti-Y^{397}-phosphorylated FAK, anti-Y^{576}-phosphorylated FAK, (1/4000 dilution), followed by 1 hour exposure to either anti-rabbit IgG coupled to HRP (1/60,000) or anti-mouse IgG coupled to HRP (1/60,000). Specific staining was revealed using ECL kits (Amersham). Blots were analysed by densitometry, and results expressed as mean±s.e.m. Band intensities of FAK and phosphorylated FAK were determined as [OD

phosphorylated FAK/OD total FAK]×100. For each condition, blots from at least four different experiments were analysed.

Immunostaining and colocalisation analysis

FAK/YCam- and Y397F-FAK/YCam-expressing cells plated at low density on Matrigel for 2 days were rinsed with PBS and fixed for 15 minutes with 3% paraformaldehyde at room temperature. After 3 washes with PBS, cells were treated for 10 minutes with 0.2% Triton X-100 in PBS/0.2% BSA and incubated 30 minutes with PBS/3% BSA. Cells were washed three times with PBS/0.2% BSA and incubated for 1 hour with various phospho-specific FAK Abs in PBS/0.2% BSA (1/1000 dilution) or with an anti-human paxillin Ab (1 μg/ml). After three additional washes, cells were incubated with TRITC-conjugated secondary Ab in PBS/0.2% BSA (1/200 dilution), washed with PBS and then observed using a confocal microscope (Bio-Rad 1024, Kr-Ar laser 488 nm; Nikon Eclipse TE300, 40× oil-immersion CFI Plan-Fluor NA 1.3 objective). GFP and TRITC were excited at 488 and 568 nm, respectively, and fluorescence was collected at 522 nm (green) and 585 nm (red). For fluorescence intensities and colocalisation analyses, images were examined using LaserSharp 5.3 software. Briefly, images were first segmented and a scatter plot was created. This is a statistical representation that shows colour and intensity distributions of pixels in a pair of images (red and green). The Y-axis of the plot corresponds to green pixel intensities and the X-axis to red pixel intensities. After selecting an area in the plot having high intensity fluorescence in both channels, a new black and white image of the selected cell is generated, which represents the cellular localisation of these high-intensity pixels. To allow analysis of statistical differences between Y397F-FAK/YCam-transfected and control cells, the degree of colocalisation was also calculated using Pearson's coefficient. This describes the degree of overlap between image pairs according to the following formula: $R_r=\Sigma(S_{1i}-S_{1aver}).(S_{2i}-S_{2aver})/\sqrt{\Sigma(S_{1i}-S_{1aver})^2.(S_{2i}-S_{2aver})^2}$, where S_1 and S_2 are the pixel intensities in the first and second images, respectively, and S_{1aver} and S_{2aver} are the average intensities of first and second images.

Migration assay

Cell migration was assessed using a wound-healing model as previously described (Giannone et al., 2002). Briefly, FAK/Ycam or Y397F-FAK/Ycam cells (2×10^5 cells/ml) were grown to confluence in Matrigel-coated (178 μg/ml) Petri dishes. After 24 hours of serum starvation, a rectangular lesion was made, cells were rinsed and then incubated with culture medium supplemented with 10% heat-inactivated FCS. After 24 hours of migration, 3 fields at the lesion border were acquired using a CCD camera (Panasonic) on an inverted microscope (Olympus IMT2, 10× phase objective). In each field, the migration distance for the 10 most mobile cells was measured using Image Tool software (available by FTP from maxrad6.uthscsa.edu). The experiment was repeated four times.

Fluorescence recovery after photobleaching experiments

FRAP experiments were done on a Bio-Rad confocal microscope at 32°C with excitation at 488 nm (Giannone et al., 2004). Cells expressing FAK/YCam or Y397F-FAK/YCam were plated at low density on Matrigel (178 μg/ml) for 2 days in Petri dishes in which a 2 cm diameter hole had been cut in the base and replaced by a 0.07 mm thick coverslip. Photobleached regions consisted of a rectangle enclosing a selected FA or cytoplasmic region. Fluorescence within the rectangle was measured at low laser power before bleaching (prebleach intensity) and then photobleached with full laser power for ~6 seconds, which effectively reduced the fluorescence to background levels. Recovery was followed using low laser power at various time intervals until the intensity reached a steady plateau. Negligible bleaching occurred while imaging the recovery process at low power, as verified in control experiments. Fluorescence during recovery was normalized to the prebleach intensity. Relative recovery rates for FAK/YCam and Y397F-FAK/YCam at FAs were compared using the half-time for recovery of fluorescence towards the asymptote. Mobile and immobile fractions were calculated by comparing the intensity ratio in the bleached area just before the bleaching and after recovery.

FA disassembly after nocodazole wash-out

Cells expressing FAK/YCam or Y397F-FAK/YCam were plated at low density on Matrigel for 2 days and were then treated with nocodazole (2.4 μg/ml) (Kaverina et al., 1999; Bhatt et al., 2002) for 1-2 hours prior to imaging by confocal microscopy as above (488 nm excitation, 522 nm emission). A first image was obtained immediately prior to nocodazole wash-out, then cells were washed three times using EMEM supplemented with 10% FCS and 10 mM Hepes. Then, z-series stacks (0.2 μm steps) were acquired every 5 minutes for 1 hour at 32°C. Representative cells of a minimum of four independent experiments are illustrated. NIH Image software was used to assess the dynamics of FAs. FA movement and disassembly were visualized and quantified as the loss of fluorescence in a selected region of interest. Automated counting of FAs in single cells was done after noise removal by thresholding and applying a size constraint to FAs. Data are presented as mean±s.e.m. of the percentage of disassembled, newly formed or constant (immobile) FAs compared to the total number of FAs.

Results

Expression of FAK/YCam and Y397F-FAK/YCam in human astrocytoma cells

In order to visualize the effect of FAK Tyr-397 phosphorylation on the dynamics of FAK at FAs, we made a FAK mutant in which Tyr-397 was mutated to Phe (Y397F-FAK), and fused to YCam, a fluorescent construction. Expression of FAK/YCam and Y397F-FAK/YCam in human U87 astrocytoma cells was detected in lysates immunoblotted with FAK anti-kinase, FAK anti-Ct and FAK anti-FAT antibodies (Fig. 1A). Using these three different antibodies, endogenous FAK was detected at 125 kDa in control and transfected cells, whereas FAK/YCam and Y397F-FAK/YCam were detected at approximately 200 kDa in transfected cells (Fig. 1B). No significant differences were found by densitometric analysis between the exogenous expression levels of FAK/YCam and Y397F-FAK/YCam compared to the relative level of endogenous FAK. Indeed, using the antibody raised against the kinase domain of FAK, the level of exogenous FAK over endogenous FAK was 2.63±0.47 (n=9) in FAK/YCam-transfected cells and 2.29±0.34 (n=9) in Y397F-FAK/YCam-transfected cells. Similar results were obtained using the antibodies raised against the C-terminal and the FAT domain of FAK (not shown).

Analysis of the pattern of phosphorylation of FAK in Y397F-FAK/YCam-transfected cells

It is well-accepted that autophosphorylation of FAK at Tyr-397 upon integrin-ligand binding creates a binding site for Src family kinases via their SH2 domains. This allows phosphorylation of FAK at additional Tyr residues to increase FAK kinase activity. We therefore compared phosphorylation

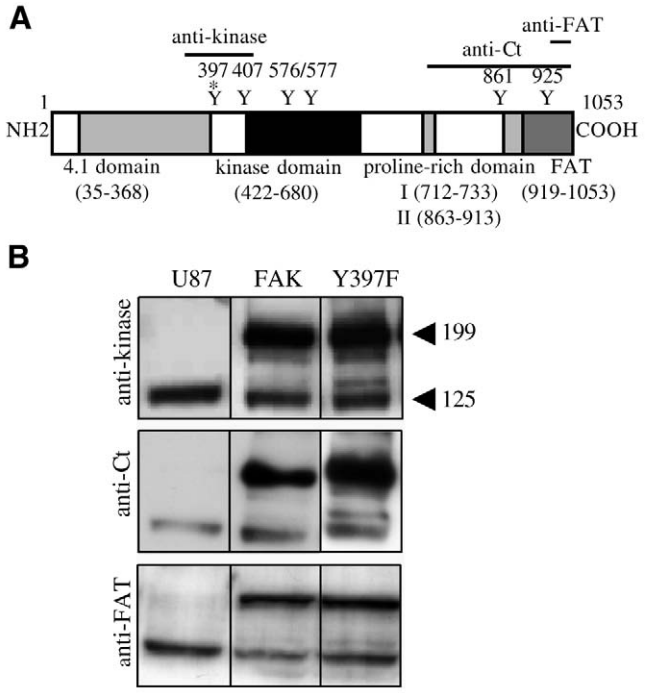

Fig. 1. Expression of FAK/YCam and Y397F-FAK/YCam in U87 astrocytoma cells. (A) Schematic representation of FAK with the phospho-acceptor residues and the epitopes recognized by the different antibodies used in this study. (B) Control U87, FAK/YCam (FAK) and Y397F-FAK/YCam (Y397) cells were lysed and immunoblotted for FAK with antibodies that recognize the kinase domain (top panel), the C-terminal domain (Ct; middle panel) or the FAT domain (lower panel). The lower bands correspond to endogenous FAK (125 kDa), while the upper bands correspond to the fusion proteins FAK/YCam or Y397F-FAK/YCam (199 kDa).

of FAK using phospho-specific antibodies in control U87 cells and after transfection either with FAK/YCam or Y397F-FAK/YCam.

In subconfluent cells migrating on Matrigel, endogenous FAK was phosphorylated at Tyr-397 in both control and transfected cells (125 kDa bands, Fig. 2A). As expected, exogenous FAK (from expression of FAK/YCam and Y397F-FAK/Yam; 199 kDa bands) was phosphorylated only in cells transfected with FAK/YCam. However, analysis of the percentage of endogenous FAK-Tyr-397 phosphorylation compared to total FAK shows that in Y397F-FAK/YCam-transfected cells, autophosphorylation of endogenous FAK (125 kDa bands) is decreased by 40% compared to control U87 cells or to cells transfected with FAK/YCam (Fig. 2B), consistent with a decrease in intermolecular trans-phosphorylation of FAK. Immunocytochemical analysis (Fig. 2C) revealed that Tyr-397 is not necessary for FAK localisation at FAs, as Y397F cells exhibit YCam staining (green) at FAs without specific phosphotyrosine staining (red). Indeed, the absence of exogenous FAK-Tyr-397 phosphorylation observed in western blots of Y397F cells (Fig. 2A) is accounted for by the absence of P-Tyr-397 staining at FAs in Y397F-transfected cells (Fig. 2C). The cellular localisation of YCam and P-Tyr-397 signals can be revealed by extracting high intensity pixels (white circles in Fig. 2D) from two-colour scatter plots (representing the intensity distribution of pixels in a pair of images: green for YCam, red for P-Tyr-397; Fig. 2D), to form a new image. The corresponding images (white images, upper right corners; Fig. 2D) indicate that these high intensity pixels are located at FAs in FAK/YCam cells but not in Y397F-FAK/YCam cells. Thus, phosphorylation of FAK-Tyr-397 is considerably decreased at FAs in Y397F-transfected cells. This difference was confirmed by quantification using Pearson's coefficient analysis, which calculates, as a measure of colocalisation, the degree of overlap between paired images (Fig. 2E). As expected, the coefficient is relatively low for Y397F-FAK/YCam cells (R=0.52; the two colours are not highly present in the same structures) and significantly smaller compared with FAK/YCam cells (R=0.81).

We next analysed the effect of the Y397F mutant on the phosphorylation status of FAK-Tyr-576, which lies in the kinase domain of FAK (Fig. 1A) and thus has been viewed as a good reporter for FAK kinase activity (Calalb et al., 1995; Maa and Leu, 1998). Since Tyr-576 is a putative Src-specific phosphorylation site, according to the model of FAK-Src interaction (Maa and Leu, 1998), its level of phosphorylation should decrease upon Y397F-FAK/YCam transfection, Indeed, in Y397F cells, phosphorylation of both endogenous (125 kDa) and exogenous (199 kDa) FAK-Tyr-576 (Fig. 3A) was decreased by 25% and 50%, respectively (upper and lower panels, respectively, Fig. 3B) compared with control U87 and FAK/YCam cells. The decrease in phosphorylation of endogenous FAK-Tyr-576 in Y397F cells is consistent with a decreased trans-phosphorylation of FAK in these cells. Since phosphorylation at Tyr-576 mediates an increase in FAK activity as observed by higher levels of FAK autophosphorylation (Calalb et al., 1995; Leu and Maa, 2002), a reduction in endogenous phospho-Tyr-397-FAK levels could alter FAK-Src binding-mediated Tyr-576 phosphorylation, thus leading to further reduction in FAK activity via a positive loop. The reduction in Tyr-576 phosphorylation was observed at FAs in Y397F cells as seen by confocal imaging (Fig. 3C, lower panel; compare transfected with non transfected cells). This is confirmed in scatter-plots where intensities of P-Tyr-576 signals (red) are lower than YCam signals (green) in Y397F-FAK/YCam cells but not in FAK/YCam cells (Fig. 3D). However, high-intensity pixels in both cell types were located at FAs (white images, upper right corners, Fig. 3D). Moreover, colocalisation of P-Tyr-576-FAK and FAK/YCam or Y397F/FAK-YCam was not different, as quantified by Pearson's coefficient analysis (Fig. 3E). Taken together, these results indicate that the preferential localisation of P-Tyr-576-FAK is at FAs (Fig. 3C) and therefore, the decrease in Tyr-576 phosphorylation in Y397F-FAK/YCam cells (Fig. 3D) is specifically localised to FAs.

Local redistribution of FAK from FA to cytosol is impaired in transfected Y397F-FAK cells

The overall effects observed on tyrosine phosphorylation in Y397F cells are in accord with a global reduction of FAK activity which should alter the turnover of FAs. In motile U87 cells, we observed predominantly static FAs, together with restricted zones where FAs disassembled (Giannone et al., 2002; Giannone et al., 2004). Therefore, to analyse how expression of Y397F-FAK might affect the turnover of FAs, we compared the molecular dynamics of FAK/YCam and

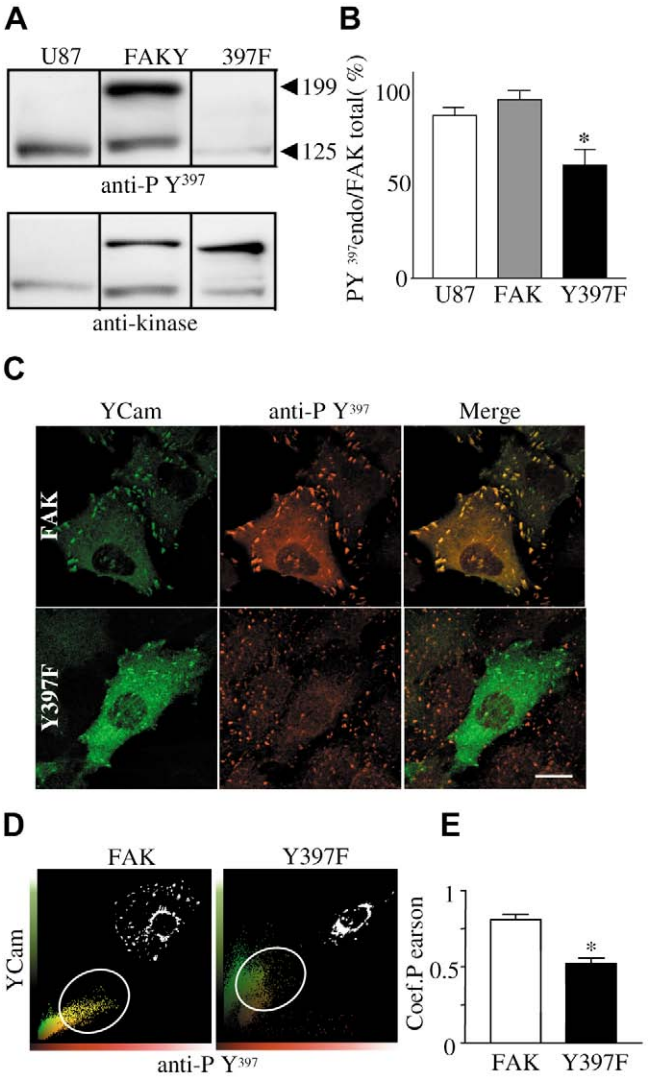

Fig. 2. Expression of Y397F-FAK/YCam decreases endogenous FAK-Tyr-397 phosphorylation at FAs. (A) Control U87, FAK/YCam (FAK) and Y397F-FAK/YCam (Y397) cells were lysed and site-specific FAK phosphorylation was analysed on western blots using anti-FAK[P-Tyr-397] antibody. The amounts of proteins were monitored by stripping and reblotting the membranes with anti-FAK antibody. In Y397F cells, note the absence of phosphorylation of exogenous FAK-Tyr-397 (199 kDa) and the reduced phosphorylation of endogenous FAK-Tyr-397 (125 kDa). (B) Quantification of the percentage of endogenous FAK-P-Tyr-397/total FAK in control and transfected cells. Data are mean±s.e.m. (n=5); *P<0.05, paired t-test. (C) FAK/YCam- and Y397F-FAK/YCam-transfected cells plated for 2 days on Matrigel were fixed, permeabilized and FAK-Tyr-397 phosphorylation was analysed by immunocytochemistry. Y397F-FAK/YCam transfected cells (lower panel) display very low levels of FAK-Tyr-397 phosphorylation at FAs compared to adjacent non-transfected cells and to FAK/YCam-transfected cells (upper panel). Scale bar: 10 μm. (D) Analysis of the localisation pattern of YCam (green) and P-Tyr-397 (red) pixels in FAK/YCam- and Y397F-FAK/YCam-transfected cells. The scatter plots (lower left corners) show colour and intensity distributions of pixels from a pair of images (YCam and P-Tyr-397). By selecting a ROI (outlined in white), a group of pixels with high intensities was extracted to form a new image of the cellular localisation of these pixels (white images in upper right corners). (E) Quantification using Pearson's coefficient (which describes the extent of overlap between image pairs) reveals a significant reduction (*P<0.05, paired t-test) of correlation in YCam and P-Tyr-397 images for Y397F-FAK/YCam-transfected cells compared to FAK/YCam–transfected cells.

Y397F-FAK/YCam in two compartments (cytosol and at FAs) using fluorescence recovery after photobleaching (FRAP) experiments. It is now well accepted that during their lifetime, FAs are highly dynamic structures that can vary in morphology and molecular composition (Zaidel-Bar et al., 2003). This molecular heterogeneity at integrin-mediated adhesion sites has lead several researchers to attempt to classify FAs into different groups, including 'classical FAs', fibrillar adhesions (FBs) under the cell centre and focal complexes (FXs) (Zaidel-Bar et al., 2004) in lamellipodia at leading edges. Therefore, to avoid heterogeneity in the molecular composition of focal structures that might alter their dynamic response, we chose to selectively study only peripheral FAs, thereby excluding FXs and FBs. The YFP moiety of YCam2 was bleached with short high-power excitation using the 488 nm line of a Kr/Ar laser in small FA-containing or cytosolic regions of interest (ROIs). Subsequent time-lapse imaging of bleached regions allowed visualisation of the movement of FAK/YCam or Y397F-FAK/YCam during recovery after bleaching (Fig. 4A). The recovery kinetics corresponds to the replacement of bleached FAK/YCam or Y397F-FAK/YCam by surrounding unbleached FAK/YCam or Y397F-FAK/YCam. Recovery after photobleaching of FAK/YCam at immobile FAs was fast [comparable with our previous observations (Giannone et al., 2004)], with a recovery half-time of 16.3±2.7 seconds (n=9 FAs; Fig. 4B). However, a much faster recovery (half-time of 5.2±0.5 seconds, n=7) was observed when FRAP experiments were carried out on small peripheral cytosolic ROIs, indicating that additional factors are involved in regulating FAK exchange between FAs and the cytosol. This regulation at FAs is apparently altered in cells expressing Y397F-FAK, since FRAP experiments show that Y397F-FAK/YCam has a significantly faster recovery (half-time of 10.3±1.2 seconds, Fig. 4B, n=8 FAs) at FAs than FAK/YCam, whereas no difference was found for cytosolic ROIs (half-time of 5.9±0.6 seconds, Fig. 4B, n=7). The significantly faster dissociation kinetics of Y397F-FAK at FAs compared to wild-type FAK suggest that phosphorylation of FAK at least at Tyr-397 and/or Tyr-576 contributes to regulating the turnover of FAK.

Expression of Y397F-FAK reduces disassembly of FAs induced by nocodazole wash-out

At immobile FAs, the rates of FAK association and dissociation to components of FAs should be the same. Altering one of these rates should consequently have an impact on the disassembly process of FAs. Therefore, experiments were designed to analyse the overall effect of Y397F-FAK expression on disassembly of FAs. Previous studies have demonstrated that treatment of cells with nocodazole, a microtubule-disrupting agent, promotes stabilization of FAs, whereas after nocodazole wash-out, specific microtubule targeting to FAs induced disassembly of FAs (Kaverina et al., 1999; Bhatt et al., 2002). In order to test whether impaired turnover of Y397F-FAK at FAs (Fig. 4) affects disassembly of FAs, nocodazole wash-out studies were done on U87 cells transfected with FAK/YCam

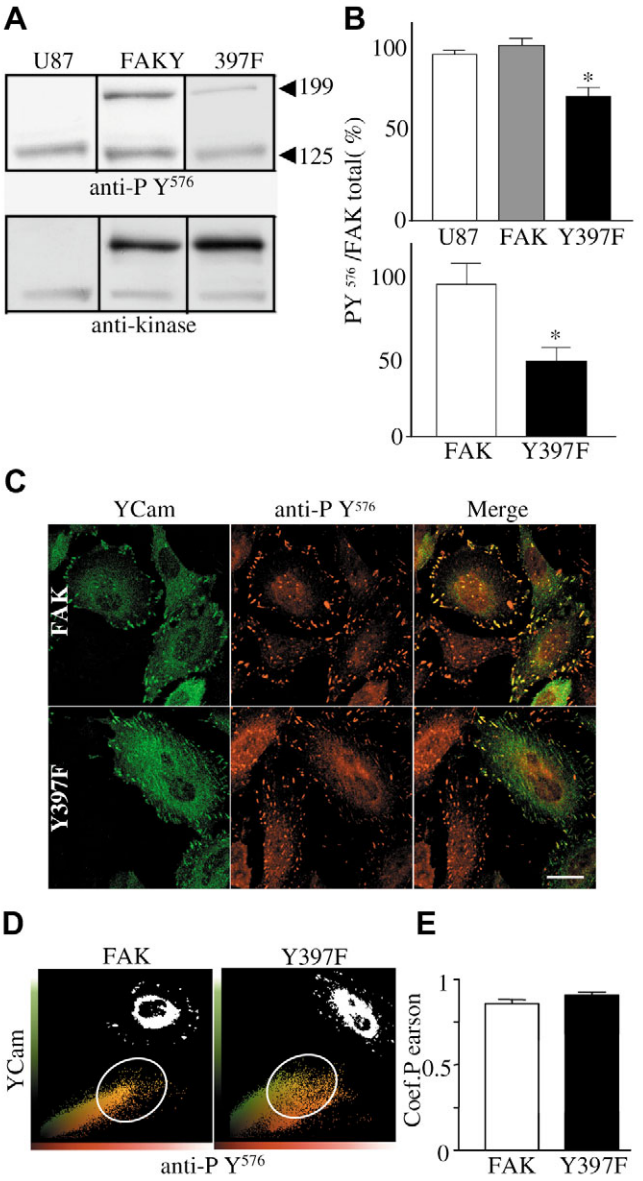

Fig. 3. Expression of Y397F-FAK/YCam decreases phosphorylation of endogenous and exogenous FAK-Tyr-576 at FAs. (A) Cells were lysed and site-specific FAK phosphorylation was analysed in western blots with anti-FAK[P-Tyr-576] antibody. The amounts of proteins were monitored by stripping and reblotting the membranes with anti-FAK antibody. Note the reduction in phosphorylation of endogenous and exogenous FAK at Tyr-576 in Y397F cells. (B) Quantification of the percentage of endogenous (125 kDa; upper panel) and exogenous (199 kDa; lower panel) phosphorylated FAK-Tyr-576/total FAK in control and transfected cells. Data represent mean±s.e.m. (n=5); * P<0.05, paired t-test. (C) FAK/YCam- and Y397F-FAK/YCam-transfected cells plated for 2 days on Matrigel were fixed, permeabilized and FAK-Tyr-576 phosphorylation was analysed by immunocytochemistry using anti-FAK[P-Tyr-576] antibody. Transfected Y397F-FAK/YCam cells (lower panel) have lower FAK Tyr-576 phosphorylation at FAs compared to adjacent non-transfected cells and to transfected FAK/YCam cells (upper panel). Scale bar: 10 μm. (D) Scatter plots show colour and intensity distribution of pixels in a pair of images (YCam, green and P-Tyr-576, red), with lower P-Tyr-576 intensities in Y397F cells. Extracted high intensity pixels (outlined white ROIs) were used to form a new image of the cellular localisation of these pixels (white images in upper right corners). (E) Quantification using Pearson's coefficient reveals no difference in colocalisation of YCam and P-Tyr-576 signals in Y397F-FAK/YCam cells compared to FAK/YCam cells.

or Y397F-FAK/YCam (Fig. 5). In FAK/YCam cells, several FAs underwent disassembly during the 1 hour observation period after nocodazole wash-out (Fig. 5A), accompanied by clear cellular movements. In contrast, in Y397F-FAK/YCam cells, only a few FAs disassembled after nocodazole washout (Fig. 5B), with cells being non-motile. In the same culture dish, adjacent non-Y397F-transfected cells displayed normal motility (Fig. 5C), strongly suggesting that impaired FA turnover in Y397F cells accounts for their impaired motility. Indeed, in FAK/YCam cells, directed movement was observed after nocodazole washout (Fig. 6A), accompanied by FA disassembly at the cell tail (Fig. 6B, black arrow) and formation of new FAs at the front edge (Fig. 6B, white arrow). In contrast, no clear motility was observed in Y397F cells (Fig. 6A,B), very probably because of a decrease in the number of disassembled FAs. Quantification of FA dynamics during recovery from nocodazole revealed that the percentage of constant (immobile) FAs was significantly greater in Y397F-FAK/YCam cells than in FAK/YCam cells, while in contrast, the percentage of disassembled FAs was significantly lower in Y397F cells than in FAK cells (Fig. 6C). Moreover, newly formed FAs were also increased in FAK cells compared with Y397F cells, although this difference was less important. The overall differences in FA dynamics are in favour of a reduced motility of Y397F cells after nocodazole wash-out. These findings are consistent with FAK-Tyr-397 phosphorylation being required for efficient disassembly of FAs and hence, for cell migration. Indeed, we compared the migration speed of Y397F and FAK cells over a 24 hour period using a wound-healing model and found that Y397F cells were significantly less motile than FAK cells (Fig. 6D).

Finally, to ensure that FAK and Y397F-FAK are good markers for FA turnover, immunostaining experiments were done using a paxillin antibody in untreated cells and cells treated with nocodazole. As seen in Fig. 7, paxillin and FAK or Y397F-FAK colocalized at FAs both before addition of nocodazole and also after nocodazole wash-out (not shown). Indeed, using Pearson's analysis, no difference in the degree of colocalisation was detected between FAK or Y397F-FAK and paxillin before addition of nocodazole and after FA disassembly induced by nocodazole wash-out (Fig. 7B). This strongly indicates that both FAK and paxillin turn over with comparable kinetics at FAs.

Discussion

We have assessed the involvement of FAK-Tyr-397 phosphorylation in the disassembly of FAs during migration of human U87 astrocytoma cells. Our data demonstrate that phosphorylation at Tyr-397 and subsequently at Tyr-576 is essential for FA disassembly. Investigation of the molecular dynamics of FAK/YCam and Y397F-FAK/YCam by FRAP indicates that the absence of phosphorylation at specific tyrosine residues decreases the time residency of FAK at FAs, which is probably associated with impaired FA disassembly. We propose a model in which phosphorylation of FAK

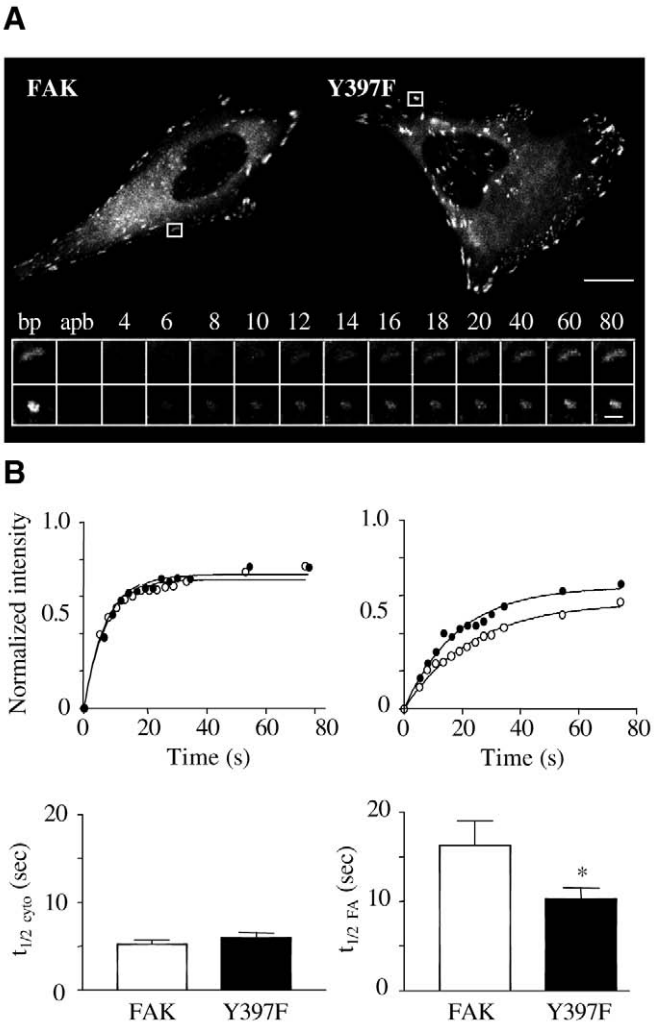

Fig. 4. FRAP experiments at FAs uncover regulation of FAK/YCam molecular dynamics by phosphorylation. (A) U87 cells expressing FAK/YCam (left) or Y397F-FAK/YCam (right) were imaged before bleaching of FA-containing ROIs (white squares). Scale bar: 10 μm. The time lapse sequences (in seconds) below show recovery after bleaching of corresponding FAs for FAK/YCam (upper row) and Y397F-FAK/YCam (lower row). Bpb, before photobleaching; apb, immediately after photobleaching. Scale bar: 2 μm. (B) Kinetics of recovery of FAK/YCam (– μ-) and Y397F-FAK/YCam (–λ-) in cytosolic (top left) and FA compartments (top right) after bleaching. The fluorescence intensity in the bleached region was measured and expressed as the relative recovery. At FAs (bottom right), note the significantly shorter recovery half-time for Y397F-FAK/YCam compared to FAK/YCam.

increases the off rate of FAK from FAs, leading to FA disassembly.

Mechanisms that determine the time residency of FAK at FAs probably involve multiple interactions with FA components, in agreement with the faster recovery half-time of Y397F-FAK/YCam. The identical ability of FAK/YCam and Y397F-FAK/YCam to localize at FAs is based on the C-terminal domain present in both proteins. The C-terminal domain of FAK encloses the FA targeting sequence [FAT (Hildebrand et al., 1993)] and contains binding sites for paxillin and talin, which are considered as the FA components responsible for FA targeting (Tachibana et al., 1995; Hayashi et al., 2002). However, the faster recovery half-time of Y397F-FAK/Cam compared to FAK/YCam indicates that FA association/dissociation kinetics are modified for Y397F-FAK/YCam. This suggests that FA targeting is not uniquely necessary for FAK function, and that regulation of FA association/dissociation kinetics may be an essential aspect of FAK signalling. The fast exchange of FAK/YCam between cytosolic and FA compartments (Fig. 4) (Giannone et al., 2004) contrasts with the immobility of most FAs in our cells, and emphasizes the signalling function of FAK over a structural

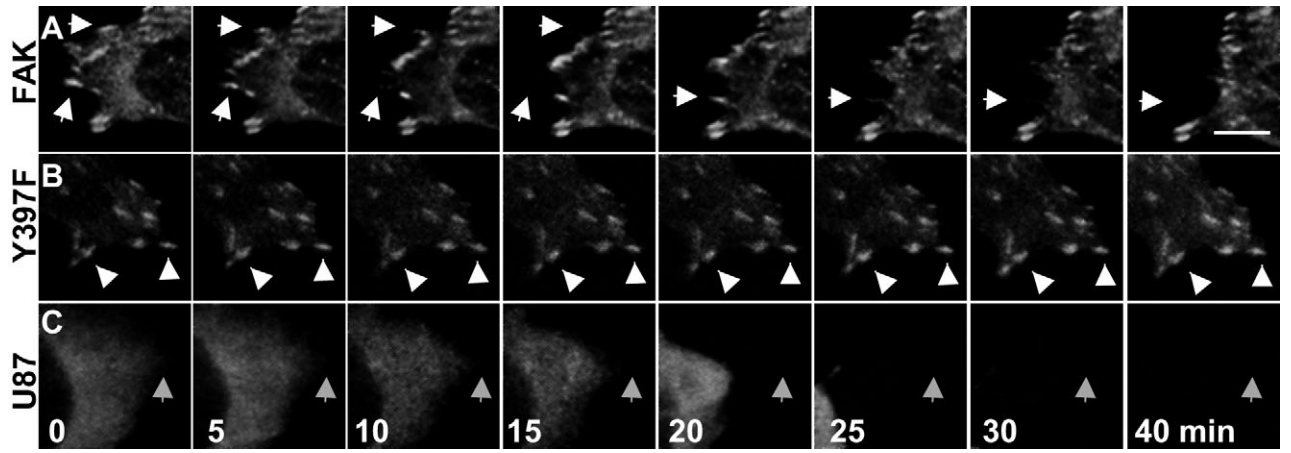

Fig. 5. Effects of mutation at Tyr-397 on the dynamics of FAK/YCam after nocodazole washout observed by confocal imaging during 1 hour of cells transfected with FAK/YCam (A), Y397F-FAK/YCam (B) and non transfected cells (C). White arrows show positions of FAK/YCam-containing FAs that were highly dynamic with clear cell edge movements (A). White arrow heads indicate positions of Y397F-FAK/YCam-containing FAs that were stable (B). A non transfected cell adjacent to the cell transfected with Y397F-FAK/YCam displays normal motility, with cell edge retraction (grey arrow, C). Images are representative of cells from a minimum of for separate experiments. Scale bar: 5 μm.

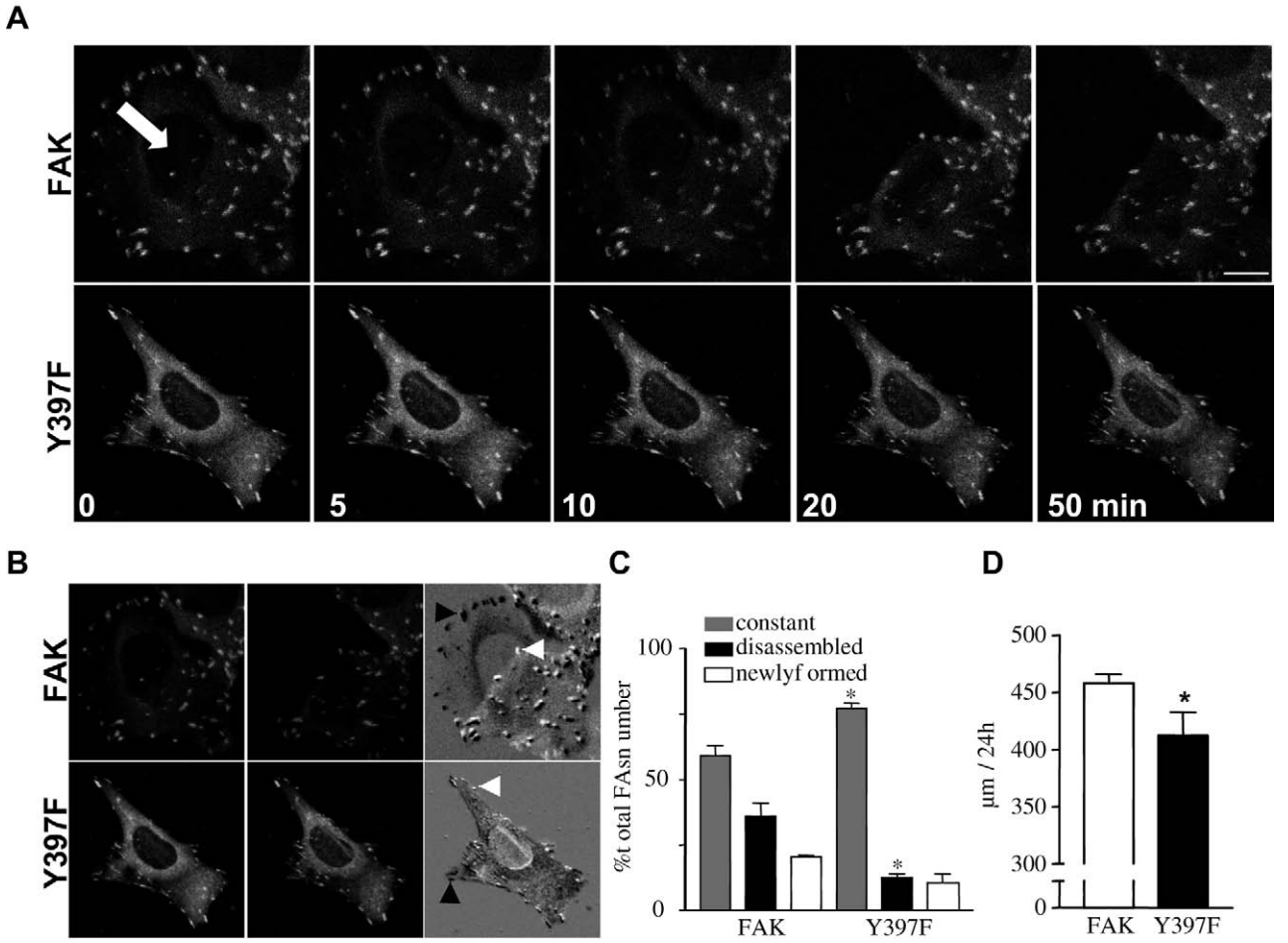

Fig. 6. Quantification of the effects of mutation at Tyr-397 of FAK/YCam on FA disassembly after nocodazole washout. (A) In FAK/YCam-transfected cells, cellular motility was clearly apparent (directions is indicated by the white arrow), during the 1 hour recovery period after nocodazole (see supplementary material Movie 1), unlike Y397F-FAK/YCam-transfected cells (see supplementary material Movie 2). Scale bar: 10 μm. (B) FA turnover was also inhibited in Y397F-FAK/YCam cells compared with FAK/YCam. Left images at t=0, centre images at t=60 minutes; right images are the result of subtraction of the images at t=60 from those at t=0. In the subtracted images, disassembled FAs are black, newly formed FAs are white and constant FAs are grey. (C) Disassembled, newly formed and constant (immobile) FAs plotted as a percentage of the total FAs in FAK/YCam and Y397F-FAK/YCam cells during recovery from nocodazole treatment . Values are the mean of four separate experiments. (D). Migration speed of FAK/YCam and Y397F-FAK/YCam cells was assessed using a wound-healing model (see Materials and Methods). Y397F-FAK/Ycam cells (n=120) had a significantly decreased migration speed compared with FAK/Ycam cells (n=120).

role. Nevertheless, redundant interactions that characterize FA-associated proteins could underlie protein exchange without dissipation of FA architecture and might reconcile fast FAK dynamics with a structural function. In agreement, low affinity interactions between FA components have been described (Goldmann, 2000), which may facilitate protein exchange dynamics. For example, it has been shown that phosphorylation of FAK by Src does not require binding of Src to Tyr-397, indicating that formation of a complex between the Src SH2 domain and FAK is not absolutely required for Src-dependent phosphorylation of FAK (McLean et al., 2000). The difference in FA association time for FAK/YCam and Y397F-FAK/YCam probably results from impaired signalling by Y397F-FAK or may be related to intrinsic binding capabilities. The effects of an absence of phosphorylation at FAK-Tyr-397 support the notion that events linked to FAK tyrosine phosphorylation activity determine the kinetics of FAK localization at FAs. Nevertheless, the difference between signalling and structural functions may be difficult to distinguish in this case. Indeed, in a simple equilibrium model of FA association/dissociation, FAs remain static when the FAK off rate is equal to its on rate, and FA dissociation would result from a FAK off-rate greater to the on-rate (Fig. 8A). This suggests that structural and signalling functions of FAK may be exchangeable in order to gain reactivity to a given stimulus. Because FAK possesses numerous phospho-acceptor sites, FAK when localised at FAs, may function as a molecular switch interacting with both signal transduction and structural proteins (Schlaepfer and Hunter, 1996b; Hanks and Polte, 1997; Parsons, 2003) to control FA disassembly.

Several studies have reported that phosphorylation of FAK is associated with FA disassembly and thus regulates cell

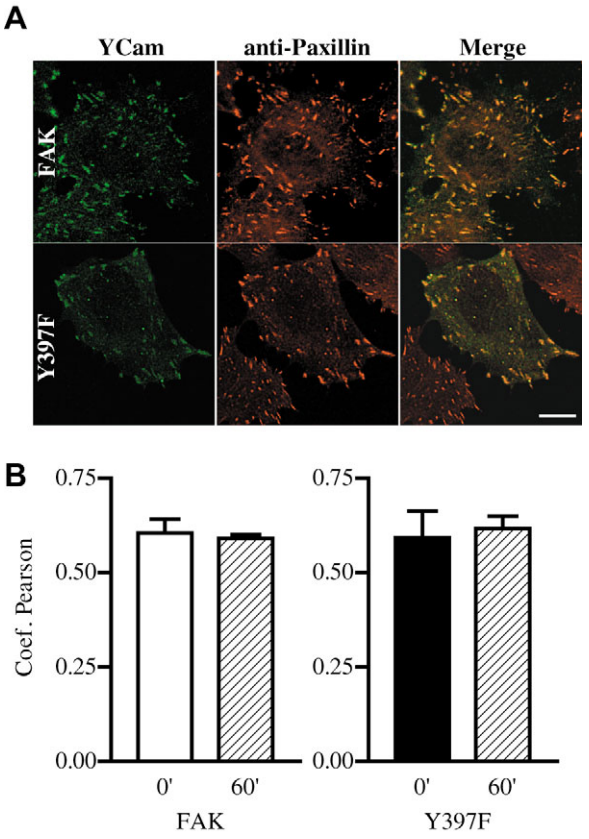

Fig. 7. Analysis of the expression of FAK/YCam or Y397F-FAK/YCam and paxillin in U87 astrocytoma cells before and after FA turnover induced by nocodazole wash-out. FAK/YCam- and Y397F-FAK/YCam-transfected cells plated for 2 days on Matrigel were fixed before addition of nocodazole (0′), or 60 (60′) minutes after nocodazole wash-out, permeabilized and paxillin was revealed by immunocytochemistry. (A) Before addition of nocodazole, FAK/YCam and Y397F-FAK/YCam colocalized with paxillin. (B) Quantification, using Pearson's coefficient, reveals no difference in colocalisation of YCam and paxillin before addition of nocodazole or after FA disassembly induced by nocodazole wash-out in FAK/YCam cells and Y397F-FAK/YCam cells. Scale bar: 10 μm.

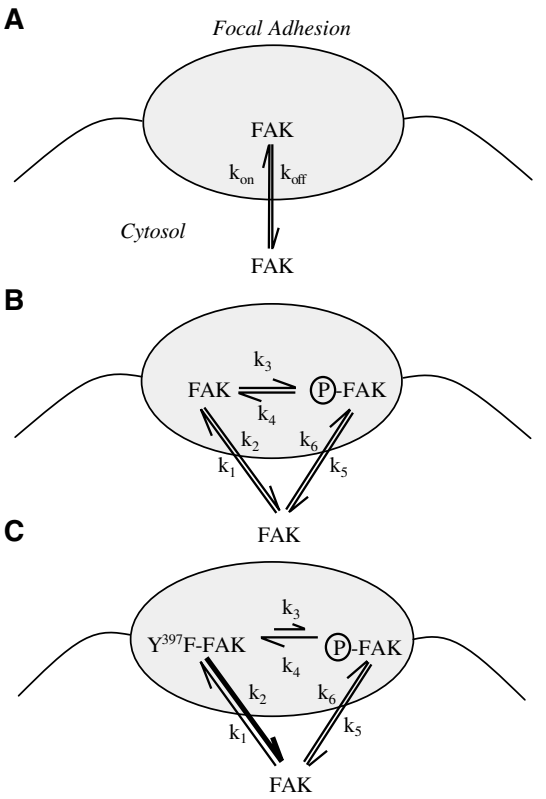

Fig. 8. Model for FAK-mediated FA dynamics. (A) FAK exists in equilibrium between two compartments: in assembled FAs and in cytosol. Increasing k_{on} or k_{off} will either stabilize or disassemble FAs. (B) In FAs, FAK is phosphorylated by cis/trans activation before leaving the FA compartment. (C) Decreasing k_3, for example by expression of mutant Y397F-FAK, will lead to accumulation of non-phosphorylated FAK at FAs and thus a greater k_2 (consistent with the shorter recovery half-time for Y397F-FAK/YCam compared to FAK/YCam), and in turn, to a decrease in FA disassembly.

migration (Sieg et al., 1999; Carragher et al., 2001; Giannone et al., 2002; Westhoff et al., 2004). For example, by assessing the rate constants for disassembly of paxillin, phosphorylation of FAK-Tyr-397 was shown to be essential for FA disassembly (Webb et al., 2004). Others have implicated specific proteins such as Src (Fincham and Frame, 1998) or calpain, a protease that cleaves several FA components such as FAK or talin (Huttenlocher et al., 1997; Bhatt et al., 2002; Franco et al., 2004). In a simple equilibrium model of FAK associated, or not, at FAs (Fig. 8A), FAK cleavage appears not to be necessary for FA disassembly, as an increase in the FAK off-rate would be sufficient to promote FA disassembly. Nevertheless, this does not mean that cleavage of FAK is not implicated in FA disassembly, since cleavage of FAK is associated with increased Src activity, leading to phosphorylation of FAK and activation of calpain (Fincham and Frame, 1998; McLean et al., 2000; Carragher et al., 2001). Cleavage of FAK could therefore be necessary downstream of phosphorylation of FAK to promote FA turnover. We propose that extended docking of FAK at FAs is associated with the phosphorylation of FAK, which is followed by different phosphorylation-dependent processes, and ultimately, FA disassembly (Fig. 8B). In support, our FRAP experiments show that the FA-associated state of FAK/YCam is prolonged and the immobile fraction of FAK/YCam at FAs is increased compared to Y397F-FAK/YCam. In our model, when phosphorylation of Tyr-397 is decreased or absent (as a result of expression of Y397-FAK), k_3 is very small, and non-phosphorylated FAK will accumulate at FAs leading to an increase in k_2 (Fig. 8C). This would account for a more rapid turnover of FAK at FAs, consistent with the shorter time-residency of Y397F-FAK at FAs observed in our FRAP experiments. This implies that decreases in FAK phosphorylation processes would stabilize FAs, consistent with the lower number of dissociated FAs after nocodazole wash-out in Y397F-FAK-transfected cells.

In agreement with this model, we have already reported that rises in calcium trigger FA disassembly, an action correlated with increased phospho-Tyr-397 and enhanced FAK residency at FAs (Giannone et al., 2002; Giannone et al., 2004). Moreover, transfected FRNK (which acts as a dominant-negative of FAK and lacks the kinase domain and the Tyr-397

auto-phosphorylation site) had enhanced turnover at FAs (Giannone et al., 2004), probably because of the increased concentration of non-phosphorylated molecules at FAs. This was also associated with an increase in the size of FAs in FRNK-transfected cells and a decrease in migration speed (Sieg et al., 1999; Giannone et al., 2002), both of which indicate more stable FAs, in accordance with our model. Likewise, plasma membrane targeting of mutant FAK constructs, which results in elevated FAK-Tyr-925 phosphorylation, induced rapid exclusion of the mutant from FAs (Katz et al., 2003). Increased cell motility and decreased cell attachment have been considered to be a consequence of deregulated FA turnover (Schlaepfer et al., 2004). Expression of a FAK mutant (all tyrosine residues whose phosphorylation are Src-dependent were mutated to phenylalanine) in fibroblasts gave rise to a decrease in cell motility associated with a decrease in cell detachment (Westhoff et al., 2004). However, substitution of two lysine residues by glutamic acid in the activation loop of FAK resulted in an activated FAK mutant called 'SuperFAK' having increased catalytic activity and hyperphosphorylation and which confers increased motility to epithelial cells (Gabarra-Niecko et al., 2002).

In conclusion, our results show that FAK molecular dynamics are fast and tuneable by phosphorylation-dependent processes. This may allow the cell to have greater reactivity in response to migration-associated stimuli by inducing rapid changes in phosphorylation of FAK and hence FAK association/dissociation kinetics at FAs, thereby promoting either stabilisation or disassembly of FAs. This regulation would in turn control cellular motility and migration speed as observed in this work.

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