Characterization of an activated mutant of focal adhesion kinase: ‘SuperFAK’

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Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that plays an important role in normal cellular processes such as adhesion, spreading, migration, proliferation and survival. In addition, FAK is overexpressed in a variety of cancer cells and tumours and may play a role in the development of human cancer. As a prelude to modelling the role of aberrant FAK signalling in the initiation of cancer, the goal of the present study was to engineer point mutations in FAK that would enhance enzymic activity. A number of substitutions that were reported as activating mutations in other tyrosine kinases were introduced into FAK. Glutamic acid substitutions for two lysine residues in the activation loop of FAK, based upon the K650E (Lys$^{650}$–Glu) mutant of fibroblast-growth-factor receptor 3, were made to create ‘SuperFAK’. Two brain-specific exons were engineered into avian FAK to create FAK6.7. SuperFAK and, to a lesser extent, FAK6.7, exhibited increased catalytic activity in vitro compared with wild-type FAK. The expression of SuperFAK and FAK6.7 in fibroblasts led to hyperphosphorylation of FAK substrates. Although the catalytic activity of SuperFAK and FAK6.7 was largely independent of cell adhesion, tyrosine phosphorylation of downstream substrates was adhesion-dependent. Further, since SuperFAK exhibited the same ability as wild-type FAK to recruit Src family kinases, tyrosine phosphorylation of substrates was likely due to direct phosphorylation by FAK. In addition to enhanced biochemical signalling, SuperFAK also increased the motility of epithelial cells. SuperFAK and FAK6.7 may be valuable molecular tools to investigate the potential role of aberrant FAK signalling in human disease.

Key words: integrin, motility, phosphotyrosine, paxillin, Src.

INTRODUCTION

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase, first identified in Src-transformed fibroblasts [1]. FAK localizes to focal adhesions through its focal-adhesion-targeting (FAT) sequence located at the C-terminus [2]. The clustering of integrins at focal adhesions upon engagement of their extracellular-matrix (ECM) protein ligands results in tyrosine phosphorylation and activation of FAK [1]. In addition to adhesion, treatment of cells with a variety of soluble factors, including G-protein-coupled-receptor agonists and receptor protein tyrosine kinase ligands, can also induce FAK activation [1].

Upon activation, FAK autoprophosphorylates on Tyr$^{576}$ [3,4], creating a binding site for SH2-domain-containing proteins. The p85 regulatory subunit of phosphoinositide 3-kinase (PI 3-kinase; ‘phosphatidylinositol 3-kinase’), phospholipase C-γ1 (PLC-γ1), growth-factor-receptor-bound protein 7 (Grb7), and possibly Src-homology (SH)-containing protein (Shc), bind to phosphorylated Tyr$^{576}$ through SH2-mediated interactions [5–8]. In addition, the autophosphorylation site on FAK recruits Src-like kinases via their SH2 domains [4]. Furthermore, FAK contains a proline-rich sequence upstream of Tyr$^{576}$, which facilitates binding to the SH3 domain of Src and stabilization of the FAK–Src complex [9,10]. Once Src binds to FAK, it phosphorylates additional tyrosine residues on FAK, including Tyr$^{727}$, Tyr$^{577}$ and Tyr$^{895}$ [3,11]. Phosphorylation of Tyr$^{576}$ and Tyr$^{577}$, which reside in the activation loop of FAK, lead to maximal activation of FAK [3]. Phosphorylation of Tyr$^{925}$ creates a binding site for the SH2 domain of Grb2, which may link FAK to mitogen-activated protein kinase (MAPK) signalling ([11,12], but see [12a]). In addition to SH2-binding sites, FAK also contains proline-rich regions that serve as docking sites for SH3-containing proteins, including the crk-associated protein p130cas [13,14]. FAK can thus recruit a variety of signalling proteins to form an intricate signalling complex.

FAK is implicated in controlling a variety of integrin-mediated biological processes. FAK regulates turnover of focal adhesions, apparently by regulating the activity of Rho ([15], but see [15a]; [16,17]). FAK also regulates cell motility. FAK-null fibroblasts and cells expressing a dominant-negative form of FAK show decreased migration [16,18]. Conversely, the overexpression of FAK in Chinese-hamster ovary (CHO) cells increases cell motility [19]. The autophosphorylation site of FAK is required for the regulation of cell motility [19] and two effectors, PI 3-kinase and Src kinases, have been shown to function in the FAK-dependent regulation of cell motility [6,19]. Furthermore, p130cas has been implicated as a downstream component of the FAK-mediated signalling pathway controlling motility [20]. In addition to migration, FAK also plays a role in mediating cell survival. Inhibition of FAK signalling causes cells to undergo apoptosis [21,22]. Furthermore, a constitutively activated FAK-containing chimaeric protein, CD2FAK, is able to rescue cells held in suspension from undergoing anoikis [23]. Recently FAK overexpression was shown to inhibit apoptosis.

Abbreviations used: CE, chicken embryo; CHO, Chinese-hamster ovary; ECM, extracellular matrix; EGFR, epidermal-growth-factor receptor; ERK, extracellular-signal-regulated kinase; FAK, focal adhesion kinase; FAT, focal adhesion targeting (sequence); FGFR3, fibroblast-growth-factor receptor type 3; Grb, growth-factor-receptor-bound; GST, glutathione S-transferase; K650E, Lys$^{650}$–Glu; MAPK, mitogen-activated protein kinase; p130cas, p130 crk-associated substrate; PI 3-kinase, phosphoinositide 3-kinase (‘phosphatidylinositol 3-kinase’); PLC-γ1, phospholipase C-γ1; PP2, 4-aminophenol-3-(4-chlorophenyl)-7-(1-tbutyl)pyrazolo[3,4-d]pyrimidine; PY, phosphotyrosine; RIPA, radioimmunoprecipitation; SH, Src homology; Shc, Src-homology-containing protein; SuperFAK, an activated K578E/K581E mutant of FAK; TBS, Tris-buffered saline.

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induced by other stimuli [24,25]. Roles for PI 3-kinase, p130Cas and Grb2 in FAK-mediated cell survival have been proposed [25,26]. In addition to regulating cell survival, FAK may also function in the positive regulation of the cell cycle by controlling the levels of cyclin D and the cyclin-dependent kinase inhibitor p21 [27]. Although some of the mechanisms of action are not fully understood, FAK transduces important biological signals following integrin-dependent cell adhesion.

Constitutively activated FAK variants have been described previously [28,29]. However, activation has been achieved by targeting FAK constitutively to the cell membrane. Since FAK is not membrane-bound, these variants may have acquired novel properties and may not fully mimic FAK. Therefore the creation of an activated mutant of FAK exhibiting proper cellular localization is of special interest. A number of reports in the literature describe activating point mutations in tyrosine kinases. These include a valine-to-isoleucine mutation in the ATP-binding pocket of the epidermal-growth-factor receptor (EGF-R) [30], a methionine-to-threonine mutation in the C-terminal lobe of the catalytic domain of RET, Met/HGF/SFR, Ron/RMSH, and Kit kinases [31–34], and substitution of a glutamic acid for a lysine residue in the activation loop of the fibroblast-growth-factor receptor type 3 (FGF-R3) [35]. In the present study we describe the construction and characterization of activated mutants of FAK. Point mutations shown to activate other tyrosine kinases were engineered into FAK. In addition, the regulatory tyrosine residues in the activation loop of FAK, Tyr576 and Tyr577, were mutated to glutamic acid to potentially mimic their phosphorylation. Finally, an avian version of FAK6.7, a neuronal FAK variant with two inserts flanking the autophosphorylation site of FAK that exhibits high autophosphorylation activity, was also engineered [36]. Two of the mutants, SuperFAK (with glutamic acid for lysine substitutions in the activation loop) and FAK6.7, exhibited elevated catalytic activity compared with wild-type FAK. Furthermore, expression of these mutants led to the hyperphosphorylation of the FAK substrates tensin and paxillin as well as FAK itself. Strikingly, upon loss of adhesion, substrate phosphorylation disappeared in SuperFAK and FAK6.7 overexpressors, despite the fact that the kinase activity of the mutants remained high. The increased signalling capacity of SuperFAK and FAK6.7 occurred without affecting FAK–Src complex formation or Src activation, implicating FAK activity itself in the augmentation of downstream signalling. In addition to enhanced biochemical signalling, the activated mutant SuperFAK was also able to enhance biological signals, since expression of SuperFAK increased cell motility in T47D breast epithelial cells. These activated FAK mutants may be powerful molecular tools for investigating the potential role of FAK signalling in the pathology of human disease, including cancer.

EXPERIMENTAL

Cloning and mutagenesis

In order to make an avian version of FAK6.7, the codons for the 6 $^{\text{DEISGD}}$ and 7 $^{\text{KYGIDE}}$ amino acid inserts were introduced into the avian FAK cDNA by site-directed mutagenesis using the Altered Sites Mutagenesis Kit (Promega, Madison, WI, U.S.A.). Mutants were identified by PCR amplification and nucleotide sequencing. The MscI–SalI fragment of the mutated cDNA (extending from nucleotide 1178 in FAK to the multiple cloning sequence of the vector, downstream of the FAK stop codon) was excised from the mutagenesis vector, pALTER, and substituted for the corresponding fragment of wild-type FAK in pBluescript-FAK [37]. Point mutations were engineered into the full-length avian FAK cDNA in pBluescript-FAK [37] by oligonucleotide-directed PCR mutagenesis using the Stratagene QuikChange kit (Stratagene, La Jolla, CA, U.S.A.). Some mutants were initially identified by restriction digestion. To verify the presence of the intended mutations and that no unintended mutations were introduced during the mutagenesis procedures, each construct was completely sequenced at the UNC-CH Automated DNA Sequencing Facility on a model 377 DNA Sequencer (PerkinElmer, Applied Biosystems Division) using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (PerkinElmer, Applied Biosystems Division). The full-length mutant FAK cDNAs were subcloned into the replication-competent avian retroviral vector RCAS type A. RCAS A-FAK and RCAS B-c-Src constructs have already been described [2,38,39].

Cells and viruses

Chicken embryo (CE) cells were harvested from 9-day-old embryos and grown as previously described [40]. T47D breast epithelial cells and the T47D/Tva derivatives were maintained in RPMI 1640 (Gibco BRL, Rockville, MD, U.S.A.) supplemented with 10% (v/v) fetal-bovine serum (Gibco BRL), 0.2 unit/ml insulin (Gibco BRL), penicillin, streptomycin, genamycin and kanamycin (Sigma, St. Louis, MO, U.S.A.). CE cells were transfected with RCAS plasmid DNA using the LIPOFECT-AMINE PLUS™ reagent (Gibco BRL) following the manufacturer’s recommended protocol. At 7 days after transfection, cells were lysed and FAK expression was analysed. T47D cells were transfected with the avian retroviral receptor Tva800 cDNA (generously given by Dr P. Bates, Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, PA, U.S.A.) [41] using the Superfect reagent (Qiagen, Valencia, CA, U.S.A.). Cells were selected in G418-containing growth medium and expanded as stably transfected cells. Expression of constructs was determined by fluorescent immunolabelling using a polyclonal antibody to the Tva receptor (generously given by Dr P. Bates), and a secondary FITC-conjugated anti-rabbit antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA, U.S.A.), followed by fluorescence analysis through flow cytometry.

Viral stocks were made from subconfluent cultures of CE cells 10 days after transfection. The culture medium was removed, 4 ml of fresh culture medium was added, and the cells were incubated overnight. The culture medium was collected, cells and debris were pelleted by centrifugation, and virus-containing supernatants were divided into portions and stored at $-70^\circ$C. Upon passaging T47D cells, 1 ml of virus stock was added to the T47D cultures. At 10–14 days after infection, cells were lysed and FAK expression was analysed.

For adhesion experiments, cells were trypsinized and washed twice in PBS containing 0.5 mg/ml soybean trypsin inhibitor (Sigma). Cells were resuspended in serum-free medium and kept in suspension for 45 min at 37°C. Suspended cells were then collected and lysed or plated at a concentration of $2.5 \times 10^5$ cells/ml on fibronectin-coated dishes (50 $\mu$g/ml) for the indicated times prior to lysis [41a].

Protein analysis

Cells were lysed in modified radioimmunoprecipitation (RIPA) buffer containing protease and phosphatase inhibitors as
previously described [42]. The protein concentration of the lysates was determined using the bicinchoninic acid (‘BCA’) assay (Pierce, Rockford, IL, U.S.A.).

For immunoprecipitations, 0.3–1 mg of cell lysate was incubated with primary antibody on ice for 1 h. The polyclonal FAK antibody BC4, monoclonal tnsin antibody 5B9 (generously given by Dr Tom Parsons, Department of Microbiology, University of Virginia Health Sciences Center, Charlottesville, VA, U.S.A.), Fyn antiserum (kindly given by Dr André Veillette, Laboratory of Molecular Oncology, Institut de Recherches, Cliniques de Montreal, Montreal, PQ, Canada), the monoclonal Srsr antibody EC10 (generously given by Dr Sally Parsons, Department of Microbiology, University of Virginia Health Sciences Center, Charlottesville, VA, U.S.A.), paxillin and p130*8 antigens (Transduction Laboratories, Lexington, KY, U.S.A.) were used for immunoprecipitations. Immune complexes were precipitated with Protein A–Sepharose beads (Sigma) or rabbit anti-mouse IgG (Jackson Immunoresearch Laboratories) pre-bound to Protein A–Sepharose beads (Sigma) at 4 °C for 1 h. The immune complexes were then washed twice with modified RIPA buffer, and twice with Tris-buffered saline (TBS; 10 mM Tris/150 mM NaCl, pH 7.0). Immune complexes were denatured and dissociated from beads by boiling in Laemmli sample buffer [43]. The samples were then resolved by SDS/PAGE on an 8 % gel, and analysed by Western blotting. Nitrocellulose membranes were blocked with TBS-T (10 mM Tris/150 mM NaCl, pH 7.0, containing 0.1 % Tween 20) containing 5 % (w/v) powdered milk or with TBS-T alone when using the RC20 phosphotyrosine antibody or with TBS-T containing 2 % fish gelatin (Sigma) at 4 °C overnight when using the [PTyr397]FAK phosphospecific antibody. Membranes were incubated with primary antibody in blocking solution for 1 h at room temperature. The antibodies described above were used for Western blotting. For detection of phosphotyrosine, horseradish peroxidase-conjugated RC20 (Transduction Laboratories) or polyclonal [PTyr397]FAK, [PTyr116]Src and [PTyr416]Srs phosphospecific antibodies (BioSource International, Camarillo, CA, U.S.A.) were used. Membranes were incubated overnight at 4 °C when using the [PTyr397]FAK phosphospecific antibody. Primary antibodies were detected using horseradish peroxidase conjugated to Protein A or anti-mouse IgG and enhanced chemiluminescence (ECL*; Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.).

**In vitro kinase assays**

For in vitro kinase reactions, FAK or Srsr immune complexes were washed twice in modified RIPA buffer, twice with TBS and once with kinase reaction buffer [20 mM Pipes (pH 7.2)/7.5 mM MnCl₂/2.5 mM MgCl₂] or enolase kinase buffer [20 mM Pipes (pH 7.2)/10 mM MgCl₂/1 mM dithiothreitol]. For enolase kinase assays, 5 μg of acid-denatured enolase (Sigma) was added to each reaction mixture. For treatments with 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), either 0.5 μM PP2 (Calbiochem, San Diego, CA, U.S.A.) or vehicle (DMSO; Fisher Scientific, Pittsburgh, PA, U.S.A.) alone were added to the kinase buffer. The immune complexes were then incubated in kinase buffer and 10 μCi of [γ-32P]ATP (DuPont–NEN, Wilmington, DE, U.S.A.) alone were added to the kinase buffer. The immune complexes were then incubated in kinase buffer and 10 μCi of [γ-32P]ATP (DuPont–NEN, Wilmington, DE, U.S.A.) at room temperature for the times indicated. The kinase reactions were stopped by boiling in Laemmli sample buffer [43]. The reactions were subjected to SDS/PAGE. The gels were fixed in 7 % acetic acid and 20 % methanol and dried. 32P incorporation was visualized by autoradiography and PhosphorImager analysis using the Storm860 instrument (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

**In vitro binding assays**

The glutathione S-transferase (GST) fusion proteins were expressed in *Escherichia coli* and purified as described in [43a]. Briefly, expression was induced by the addition of 0.1 mM isopropyl 1-thio-β-β-galactopyranoside and incubation at 37 °C for 2 h. The bacteria were harvested and lysed by sonication in 1 % Triton X-100 in PBS containing protease inhibitors (1 mM PMSE, 0.5 mM EDTA, pH 8.0, 10 μg/ml leupeptin and 10 μg/ml aprotinin). Clarified supernatants were incubated with GSH–agarose beads (Sigma) for 1 h at 4 °C, washed, and finally resuspended in an equal volume of PBS. The fusion proteins were analysed by SDS/PAGE and Coomassie Blue staining.

Approx. 0.5–1 mg of protein lysate was pre-cleared by incubation with GST immobilized on GSH–Sepharose beads for 1 h at 4 °C. For GST–Grb2SH2 pulldowns, cells were treated overnight with 50 μM sodium orthovanadate after lysing. The cleared lysates were then incubated with 2 μg of GST alone or GST–SH2 domain fusion proteins immobilized on GSH–Sepharose beads for 2 h at 4 °C. The beads were washed twice with modified RIPA buffer and twice with TBS. The bound proteins were denatured and eluted from the beads by boiling in Laemmli sample buffer [43] and analysed by Western blotting.

**Motility**

Motility assays were performed as described previously [44]. The underside of 12-mm diameter transwell chambers with a 12-μm pore-size polycarbonate membrane (Costar, Cambridge, MA, U.S.A.) were coated with 0.6 ml of 40 μg/ml rat tail collagen I (Collaborative Biomedical Products, Bedford, MA, U.S.A.) for 6 h at 37 °C. The lower chamber was washed twice and filled with serum-free RPMI 1640 medium. T47D cells were trypsinized, counted, and resuspended in RPMI 1640 medium supplemented with 5 mg/ml BSA (Sigma) to a total concentration of 3 × 10⁶ cells/ml. Then 1.5 × 10⁵ cells were added to the top chamber of the transwell. The T47D cells were allowed to migrate for 20–22 h at 37 °C. Cells remaining on the top of the polycarbonate membrane were removed. Cells that had migrated to the underside of the membrane were stained with DiffQuick (Baxter, Miami, FL, U.S.A.). The cells were counted across two diameters, a total of ten fields, each on duplicate membranes. A mixed model test as well as paired and unpaired Student *t* tests were performed using the SAS® (Cary, NC, U.S.A.) software to identify statistically significant differences in average fold change of motility.

**RESULTS**

A number of mutations known to activate tyrosine kinases were engineered into avian FAK (Figure 1). The Y576E (Tyry76→Glu)/Y577E, V436I and M589T mutations had no effect on the kinase activity of FAK or signalling downstream of FAK (results not shown) and thus were not further characterized. The K578E/K581E mutant, referred to as SuperFAK, and the alternatively spliced neuronal form, FAK6.7, exhibited elevated catalytic activity and were more extensively characterized.

**SuperFAK has increased catalytic activity compared with wild-type FAK**

SuperFAK and FAK6.7 were subcloned into the RCAS A retroviral vector and expressed in CE cells. Western blotting of CE cell lysates with a polyclonal FAK antibody revealed that wild-type FAK, SuperFAK and FAK6.7 were expressed at equal levels (Figure 2A). The altered electrophoretic mobility of FAK6.7...
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Figure 1 Construction of activated mutants of FAK

(A) The sequence of the activation loop of the kinase domain of FAK is shown. In SuperFAK two lysine residues, Lys578 and Lys581 (large Ks with diagonal numbers at the top) were substituted with glutamic acids (E), mimicking the activating K650E mutation in FGFR3 [53]. Y576E/Y577E represents an additional mutant in which the regulatory tyrosine residues (Tyr576 and Tyr577) in the activation loop of FAK (bold Ys with diagonal numbers at the base) were mutated to glutamic acid (E). (B) A schematic diagram of FAK is shown. Proline-rich regions (white Ps on mid-grey background), sites of tyrosine phosphorylation (grey Ys on a white background) and the FAT (white ‘FAT’ on grey) sequence are shown. Two additional FAK mutants were engineered to mimic activating point mutations in EGFR (V436I; vertical white circles) [30] and RET, Met/HGF/SFR, Ron/RMSH, and Kit kinases (M589T; vertical black circles) [31–34]. (C) FAK6.7, an alternatively spliced neuronal form of FAK [36], contains two additional exons (6 and 7; grey boxes with white lettering) flanking Tyr397, the autophosphorylation tyrosine residue on FAK.

was due to the two insertions (Figure 2A, lane 4). Immunofluorescence studies demonstrated that, like wild-type FAK, both SuperFAK and FAK6.7 localized to focal adhesions (results not shown).

To determine the effect of the introduced mutations and insertions on catalytic activity, FAK, SuperFAK and FAK6.7 were subjected to in vitro kinase assays. The proteins were immunoprecipitated from CE lysates and then incubated in kinase reaction buffer for the indicated times. The kinase reactions were stopped by the addition of Laemmli sample buffer and analysed by SDS-PAGE and autoradiography [43]. Autophosphorylation of endogenous FAK was not detected at this exposure (Figure 2B; top panel; lane 1), due to the small amount of endogenous FAK that is recovered relative to the exogenously expressed proteins (Figure 2B; bottom panel). However, autophosphorylation of exogenous wild-type FAK was readily detected (Figure 2B; top panel, lanes 2 and 3). A significant increase in autophosphorylation activity was observed in SuperFAK immune complexes compared with wild-type FAK (Figure 2B; top panel, lanes 4 and 5). FAK6.7 also exhibited increased autophosphorylation activity relative to wild-type FAK, but the increase was less dramatic than that of SuperFAK (Figure 2A, top panel, lanes 6 and 7).

Increased phosphorylation of downstream cellular proteins

Since SuperFAK and FAK6.7 had increased catalytic activity in vitro, the ability of these mutants to increase FAK signalling in vivo was investigated. Phosphotyrosine levels in CE cells expressing wild-type FAK or the FAK mutants were used as a measure of FAK signalling. Whole-cell lysates from CE cells were analysed by Western blotting with a phosphotyrosine...
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**Figure 3 Elevated substrate phosphorylation in SuperFAK and FAK6.7 overexpressors**

(A) Lysates (25 µg) of CE cells expressing empty vector (lane 1), FAK (lane 2), SuperFAK (lane 3) or FAK6.7 (lane 4) were Western-blotted with a phosphotyrosine antibody, RC20 (top panel). The positions of the molecular-mass markers are indicated on the left. The same lysates (25 µg) were Western-blotted with a polyclonal FAK antibody, B34, to ensure equal expression of FAK protein (bottom panel). (B–E) FAK (B), tensin (C), paxillin (D) and p130Cas (E) were immunoprecipitated from CE cells (0.5–1 mg) expressing vector alone (lane 1), FAK (lane 2), SuperFAK (lane 3) or FAK6.7 (lane 4). The immune complexes were Western-blotted with a phosphotyrosine antibody, RC20 (B–E, top panels). The nitrocellulose membranes were stripped and re-probed for FAK, tensin, paxillin and p130Cas to ensure equal amounts of protein were being analysed (B–E, bottom panels).

antibody (Figure 3A). As previously described, overexpression of wild-type FAK leads to only a slight increase in cellular phosphotyrosine, and the major tyrosine phosphorylated band corresponds to FAK itself [45] (Figure 3A, top panel, lane 2). However, a striking increase in cellular phosphotyrosine was observed in SuperFAK overexpressors, and to a lesser extent in FAK6.7 overexpressors (Figure 3A, top panel, lanes 3 and 4). The major phosphotyrosine-containing proteins were approx. 200, 125 and 68–75 kDa in size (Figure 3A; arrows). On the basis of these molecular masses, and previous studies of FAK substrate phosphorylation [45], it seemed likely that tensin, FAK and paxillin are the major targets for enhanced phosphorylation in these cells.

In order to verify the identity of the proteins that were hyperphosphorylated, tensin, FAK, and paxillin were immunoprecipitated from lysates of CE cells overexpressing wild-type FAK or the mutant FAK proteins. The immune complexes were analysed by Western blotting for phosphotyrosine (Figures 3B–3D, top panels). FAK immunoprecipitated from CE cells transfected with wild-type FAK had a strong phosphotyrosine signal compared with mock-transfected cells owing to the expression of the exogenous wild-type FAK protein (Figure 3B, lane 2). A modest increase in the phosphorylation of the immunoprecipitated FAK was observed when SuperFAK, and, to a lesser extent, when FAK6.7 was expressed (Figure 3B, top panel, lanes 3 and 4). The phosphorylation of tensin and paxillin was slightly elevated upon expression of wild-type FAK compared with mock-transfected cells, as was previously described [45] (Figures 3C and 3D, top panels, lanes 2). The phosphotyrosine content of both tensin and paxillin was significantly increased in SuperFAK-expressing cells compared with wild-type FAK-expressing cells (Figures 3C and 3D, top panels, lanes 3). Although not so dramatically, FAK6.7 also caused an elevation in the phosphotyrosine content of both tensin and paxillin compared with wild-type FAK (Figures 3C and 3D, top panels, lanes 4). The phosphotyrosine content of an additional FAK substrate, p130Cas, was similarly analysed. In contrast with the observed phosphorylation differences on tensin and paxillin, no significant change in the phosphotyrosine content of p130Cas was observed when either wild-type FAK, SuperFAK or FAK6.7 were expressed (Figure 3C, top panel). Control Western blots verified that equal amounts of protein were being immunoprecipitated in each case (Figures 3B–3E, bottom panels). These observations indicate that the elevated catalytic activity exhibited by SuperFAK and FAK6.7 is sufficient to amplify signalling events immediately downstream of FAK. Interestingly, phosphorylation of some FAK-associated tyrosine-phosphorylated proteins, i.e. paxillin, was increased, whereas phosphorylation

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of others was not, i.e. p130cas. Furthermore, the level of FAK substrate phosphorylation in vivo correlated with the catalytic activity of the kinases in vitro.

**Cell-adhesion-dependent regulation of SuperFAK and FAK6.7 signalling**

Cell adhesion is a major stimulus regulating FAK-mediated signal transduction. Upon cell detachment from the ECM, FAK becomes dephosphorylated, its catalytic activity declines and downstream signals are turned off [1]. To determine whether SuperFAK and FAK6.7 were constitutively active and able to send signals independent of cell adhesion, the cellular phosphotyrosine content was monitored in CE cells expressing wild-type FAK or the FAK mutants. The cells were either kept in culture or held in suspension for 45 min prior to lysis. Lysates were analysed by Western blotting with a phosphotyrosine antibody (Figure 4A, top panel). As described above (Figure 3A), expression of SuperFAK, and, to a lesser extent, FAK6.7, increased cellular phosphotyrosine to a higher level than wild-type FAK in cultured cells (Figure 4A, top panel, lanes 3, 5 and 7). When cells were detached, the cellular phosphotyrosine content was dramatically reduced in every cell type (Figure 4A, top panel, lanes 2, 4, 6 and 8). The level of phosphotyrosine in suspended SuperFAK and FAK6.7 cells was similar to the level of phosphotyrosine in suspended mock-transfected cells (Figure 4A, top panel, lanes 2, 6 and 8). Whole-cell lysates were also Western-blotted for FAK to ensure equal expression of FAK protein (Figure 4A, bottom panel). These observations indicate that the downstream phosphorylation signals mediated by SuperFAK and FAK6.7 are adhesion-dependent.

*In vitro* kinase assays were performed to investigate whether the decreased tyrosine phosphorylation observed upon loss of adhesion in SuperFAK and FAK6.7 overexpressors was due to a reduction in catalytic activity. FAK or the activated FAK mutants were immunoprecipitated from cultured CE cells or cells held in suspension. The immune complexes were incubated in kinase reaction buffer for 5 min at room temperature. The kinase reactions were stopped with the addition of Laemmli sample buffer and the samples analysed by SDS/PAGE and autoradiography. As expected from previous studies [1], the activity of wild-type FAK in suspended cells was decreased compared with the activity of FAK in adherent cells (Figure 4B, top panel, lanes 3 and 4). As shown above (Figure 2B), an elevation of the autophosphorylation activity of SuperFAK, and to a lesser extent FAK6.7, was observed in adherent cells (Figure 4B, top panel, lanes 3, 5 and 7). Although the kinase activity of SuperFAK and FAK6.7 decreased in the absence of an adhesion

**Figure 4 Adhesion-mediated regulation of FAK signalling and kinase activity**

(A) The cellular phosphotyrosine content of CE cells expressing vector alone (lanes 1–2), FAK (lanes 3–4), SuperFAK (lanes 5–6) or FAK6.7 (lanes 7–8) was analysed in cells kept in culture (Cu; lanes 1, 3, 5 and 7) and in cells held in suspension for 45 min (Su; lanes 2, 4, 6 and 8). The lysates (25 μg) were Western-blotted using a phosphotyrosine antibody, RC20 (top panel). The same lysates (25 μg) were Western-blotted with a polyclonal FAK antibody, BC4, to ensure equal expression of FAK protein (bottom panel). (B) The kinase activity of the FAK mutants on loss of cell adhesion was analysed by using an immune-complex kinase assay. FAK was immunoprecipitated from CE cells (0.5–1 mg) expressing empty vector (lanes 1 and 2), FAK (lanes 3 and 4), SuperFAK (lanes 5 and 6) or FAK6.7 (lanes 7 and 8) using a polyclonal FAK antibody, BC4. The immune complexes were incubated in kinase buffer containing (γ-32P)ATP for 5 min. The samples were separated by SDS/8%-PAGE and 32P incorporation was analysed by autoradiography (top panel). The immune complexes were also Western-blotted with a polyclonal FAK antibody, BC4, to ensure equal amounts of protein were being analysed (bottom panel). The positions of the molecular mass markers are indicated on the left.
Phosphorylation status of tyrosine residues in SuperFAK and FAK6.7

One possible mechanism through which the activated mutants might elevate downstream signalling is by recruiting Src family kinases into complex and/or enhancing signalling by Src family kinases. In order to investigate the role of Src in SuperFAK and FAK6.7 enhanced signalling, several lines of investigation were pursued. Lysates of CE cells expressing wild-type FAK, SuperFAK or FAK6.7 were Western-blotted with a phosphotyrosine antibody. As above (Figure 3D), SuperFAK, and to a lesser extent FAK6.7, increased the level of tyrosine phosphorylation on paxillin compared with wild-type FAK in cultured cells (Figure 5, top panel, lanes 4, 7 and 10). Upon loss of adhesion, paxillin phosphorylation was lost regardless of which FAK construct was expressed (Figure 5, top panel, compare Cul versus Su). This result correlates with the previous observation of phosphotyrosine levels in lysates of suspended cells (Figure 4A). Fibronectin-mediated adhesion induced tyrosine phosphorylation of paxillin in all the cells (Figure 5, top panel, lanes 3, 6, 9 and 12). The level of paxillin phosphorylation was significantly higher in cells expressing SuperFAK or FAK6.7 compared with cells expressing wild-type FAK (Figure 5, top panel, lanes 6, 9 and 12). The immune complexes were Western-blotted for paxillin in order to verify that equal amounts of protein were analysed (Figure 5, bottom panel). These observations demonstrate that the activated FAK mutants elevate FAK-mediated signals in response to a physiologically relevant stimulus.

containing the SH2 domain of Src was used. GST fusion proteins were incubated with cell lysates and the bound proteins were Western-blotted to determine the amount of associated FAK. To ensure comparable levels of FAK protein expression, whole-cell lysates were Western-blotted for FAK (Figure 6B, bottom panel). GST alone was used to control for non-specific FAK binding (Figure 6B, top panel, lane 1). Similar amounts of exogenous wild-type FAK and the activated FAK mutants bound to GST–SrcSH2 (Figure 6B, top panel, lanes 3–5). These observations demonstrate that there were no changes in the ability of SuperFAK or FAK6.7 to associate with Src in vitro compared with wild-type FAK and are in agreement with the results of the PY397 Western blot (Figure 6A).

Co-immunoprecipitations were also performed to analyse the association of Fyn with FAK in vivo. Fyn was immunoprecipitated from CE cell lysates that overexpressed wild-type FAK, SuperFAK or FAK6.7. The immune complexes were Western-blotted for FAK (Figure 6C, top panel). The same amount of FAK, SuperFAK and FAK6.7 were co-immunoprecipitated with Fyn (Figure 6C, top panel, lanes 3–5). As a control, secondary antibody was used alone in an immunoprecipitation to demonstrate that the co-immunoprecipitation of FAK was specific (Figure 6C, top panel, lane 1). The blots were also stripped and reprobed for Fyn to ensure that equal amounts of Fyn were immunoprecipitated (Figure 6C, bottom panel).
Figure 6 Phosphorylation of Tyr$^{397}$ and FAK–Src complex formation

(A) The phosphorylation level of Tyr$^{397}$ on FAK was analysed by Western blotting. Lysates (25 μg) from CE cells expressing vector alone (lane 1), FAK (lane 2), Y397F FAK (lane 3), SuperFAK (lane 4) or FAK6.7 (lane 5) were Western-blotted with a [PTyr 397]FAK phosphospecific polyclonal antibody ('Y397F'; top panel). Y397F FAK was used as a negative control for antibody specificity (lane 3). The nitrocellulose membrane was stripped and re-probed with a polyclonal FAK antibody, BC4, to ensure equal loading (bottom panel). (B) The association of the Src SH2 domain with FAK in vitro was analysed. CE cells (0.5 mg) expressing vector alone (lane 2), FAK (lanes 1, 3), SuperFAK (lane 4) or FAK6.7 (lane 5) were pre-cleared with GST (20 μg) for 1 h at 4 °C. The precleared lysates were then incubated with GST–SrcSH2 domain fusion protein (2 μg) for 2 h at 4 °C. GST alone (2 μg) was used to control for binding specificity (lane 1). Bound FAK was detected by Western blotting with a polyclonal FAK antibody, BC4 (top panel). To ensure equal expression of the FAK constructs, the same CE lysates (25 μg), expressing the empty vector (lane 2), FAK (lane 3), SuperFAK (lane 4), or FAK6.7 (lane 5) were Western-blotted with a polyclonal FAK antibody, BC4 (bottom panel). (C) The association between the FAK variants and Fyn in vivo was analysed by co-immunoprecipitation. Fyn was immunoprecipitated using a polyclonal Fyn antibody from CE cell lysates (0.5 mg) expressing vector alone (lane 2), FAK (lane 3), SuperFAK (lane 4), or FAK6.7 (lane 5). As a control for antibody specificity, the secondary antibody was used alone in an immunoprecipitation reaction with lysate of FAK expressing CE cells (lane 1). The immune complexes were Western-blotted with a polyclonal FAK antibody (top panel). The nitrocellulose membrane was stripped and re-probed with a polyclonal Fyn antibody to ensure equal amounts of Fyn were immunoprecipitated (bottom panel).

These observations indicate that SuperFAK, FAK6.7 and wild-type FAK associate with equivalent amounts of Fyn in vitro.

Two approaches were taken to determine whether Src family kinases, in complex with FAK, were responsible for the enhanced catalytic activity of SuperFAK in vitro. First, kinase assays were performed in the presence of the Src inhibitor PP2. FAK was immunoprecipitated from CE lysates expressing vector alone, FAK or SuperFAK. The immune complexes were then incubated in kinase reaction buffer in the presence or absence of PP2 and terminated by the addition of Laemmli sample buffer. The samples were subject to SDS/PAGE and autoradiography. As described above (Figure 2B), SuperFAK had increased autophosphorylation levels compared with wild-type FAK (Figure 7A, lanes 3 and 5). Most importantly, the presence of PP2 had no effect on the autophosphorylation activity of wild-type FAK or SuperFAK (Figure 7A, lanes 3 and 4, and 5 and 6). In the second approach, an enolase substrate kinase assay was used to measure Src activity in FAK complexes. Immune complexes were incubated in reaction buffer with acid-denatured enolase and the samples analysed by SDS-PAGE and autoradiography. As a control, [Phe$^{577}$]Src immune complexes were used and shown to prominently phosphorylate enolase (Figure 7B, lanes 1 and 10). In the presence of 0.5 μM PP2, [Phe$^{577}$]Src autophosphorylation and enolase phosphorylation were significantly inhibited (results not shown and Figure 7, lanes 1 and 2, and 10 and 11). FAK immune complexes weakly phosphorylated enolase and SuperFAK immune complexes induced enhanced enolase phosphorylation (Figure 7B, lanes 6 and 8). However, the presence of PP2 had no effect on the phosphorylation of enolase by FAK or SuperFAK (Figure 7B, lanes 6 and 7, and 8 and 9). This observation suggests that the weak phosphorylation of enolase by FAK and SuperFAK immune complexes was due to FAK activity and not due to co-immunoprecipitating Src family kinase activity. These results demonstrate that the increased activity of SuperFAK in vitro is not due to enhanced activity of co-immunoprecipitating Src kinases.

The results of the phosphospecific-antibody Western blots, in vitro binding assays, Fyn co-immunoprecipitations and in vitro kinase assays indicate that FAK, SuperFAK and FAK6.7 are equally phosphorylated on Tyr$^{397}$ and bind comparable amounts of Src kinases. However, there remains the possibility that these FAK variants might elevate Src activity in vivo. In order to investigate this possibility, the activation state of Src was examined using phosphospecific antibodies. Cell lysates were Western-blotted with phosphospecific antibodies to Tyr$^{416}$...
prominently recognized [Phe\textsuperscript{427}]Src and weakly recognized c-Src, suggesting that the antibody was specific to Tyr\textsuperscript{416} (Figure 8A, top panel). The specificity of PY527 was also verified, since this antibody recognized c-Src, but not the Y527F Src mutant (Figure 8A, middle panel). Upon co-expression with wild-type FAK, an increase in Tyr\textsuperscript{416} phosphorylation was observed on c-Src (Figure 8B, top panel, lanes 1 and 2), indicating that co-expression with FAK can activate Src. The level of Tyr\textsuperscript{416} phosphorylation when SuperFAK or FAK.6.7 was co-expressed with c-Src was the same as in cells co-expressing wild-type FAK and c-Src (Figure 8B, top panel, lanes 2-4). The level of phosphorylation of Tyr\textsuperscript{527} on c-Src remained unchanged, regardless of co-expression of FAK proteins (Figure 8B, second panel). To monitor the amounts of protein being analysed, Src and FAK Western blots were performed (Figure 8A, bottom panel, and Figure 8B, third and bottom panels). The observations indicate that wild-type FAK, SuperFAK and FAK.6.7 have similar effects on the phosphorylation of Src. Therefore the mechanism by which SuperFAK and FAK.6.7 send amplified signals in vivo is not via enhanced activation of Src kinases.

The phosphorylation of Tyr\textsuperscript{925} in the C-terminus of FAK creates a binding site for the SH2 domain of Grb2 ([11,12], but see [12a]). To explore phosphorylation of Tyr\textsuperscript{925} a phosphospecific antibody was initially used. However, under the blotting conditions used, this antibody recognized a FAK mutant with a phenylalanine substitution for Tyr\textsuperscript{925} (results not shown), precluding its use in this analysis. As an alternative approach, Tyr\textsuperscript{925} phosphorylation in SuperFAK and FAK.6.7 was investigated by examining the ability of the FAK mutants to associate with the Grb2SH2 domain in vitro. A GST fusion protein containing the Grb2SH2 domain was incubated with vanadate-treated lysates of CE cells expressing FAK, or the activated FAK variants, and bound proteins analysed by Western blotting for FAK. To ensure comparable levels of FAK protein expression, whole cell lysates were Western-blotted for FAK (Figure 9, bottom panel). FAK bound to the GST-GrbSH2 domain, but failed to associate with GST alone (Figure 9, top panel, lanes 1 and 3). Higher levels of SuperFAK were found associated with the Grb2 SH2 domain compared with wild-type FAK (Figure 9, top panel, lanes 3 and 4). In contrast, similar amounts of FAK6.7 and wild-type FAK associated with the Grb2 SH2 domain (Figure 9, top panel, lanes 3 and 5). These results suggest that SuperFAK has elevated phosphorylation at Tyr\textsuperscript{925}.

**Increased motility of T47D cells expressing SuperFAK**

Since SuperFAK and FAK.6.7 increased FAK signalling biochemically, the ability of these mutants to impinge upon FAK-mediated biological processes was tested. In order to investigate the effects of the activated FAK proteins on cell motility, T47D cells, a breast-cancer epithelial cell line, were utilized. The T47D cells were engineered to stably express the receptor for the avian subgroup A retrovirus, Tva\textsuperscript{800} [41]. Whereas, the parental T47D cells are resistant to infection with avian retroviruses since the cells lack the viral receptor, the derived cell line, T47D/Tva, is susceptible to infection with avian A type retroviruses. The FAK constructs, cloned into RCAS type A, were transfected into CE cells. Virus produced in CE cells was collected and used to infect the T47D/Tva cells to generate populations of cells expressing each of the FAK proteins of interest. Western blots were performed to examine expression of FAK proteins following infection of the T47D/Tva cells. These results demonstrated that FAK, SuperFAK and FAK.6.7 were expressed to high levels in the T47D/Tva cells (Figure 10A).
Figure 8 Phosphorylation status of Src in cells co-expressing FAK and Src

(A) The phosphorylation status of the activation loop tyrosine (Tyr416) and the inhibitory tail tyrosine (Tyr527) in Src were analysed by Western blotting. In order to ensure antibody specificity, CE cell lysates (25 μg) overexpressing c-Src (lane 1) or Src with a tyrosine-527-to-phenylalanine substitution, [Phe 527]Src (‘Src527F’), (lane 2) were Western-blotted with polyclonal Src phosphospecific antibodies against phosphorylated Tyr416 (‘PY416’; top panel) or phosphorylated Tyr527 (‘PY527’; middle panel). The nitrocellulose membrane was stripped and re-probed with a monoclonal Src antibody, EC10, to ensure equal expression (bottom panel).

(B) CE cell lysates (25 μg) co-expressing c-Src and vector alone (lane 1), FAK (lane 2), SuperFAK (lane 3) or FAK6.7 (lane 4) were Western-blotted with a Src activation-loop phosphospecific tyrosine antibody, PY416 (top panel), or a Src inhibitory tail phosphospecific tyrosine antibody, PY527 (second panel). The nitrocellulose membranes were stripped and re-probed with a monoclonal Src antibody, EC10 (third panel), or a polyclonal FAK antibody, BC4 (bottom panel), to ensure equal amounts of protein were being analysed.

Figure 9 Phosphorylation of Tyr925 is increased in cells expressing SuperFAK

The phosphorylation status of Tyr925 on FAK was analysed using an in vitro binding assay. CE cells expressing empty vector (lane 2), FAK (lanes 1, 3), SuperFAK (lane 4) or FAK6.7 (lane 5) were treated overnight with vanadate (50 μM), then lysed. The CE lysates (1 mg) were precleared with GST (20 μg) for 1 h at 4 °C. Pre-cleared lysates were then incubated with a GST–Grb2SH2 fusion protein (lanes 2–5) or GST alone (lane 1). Bound FAK was detected by Western blotting with a polyclonal FAK antibody, BC4 (top panel). The same lysates (25 μg) were Western-blotted with a polyclonal FAK antibody, BC4, to ensure equal expression of the FAK constructs (bottom panel).

Using a transwell motility assay as previously described [44], the haptotactic motility of the cells was analysed. Populations of infected T47D/Tva cells were allowed to migrate for 20–22 h through a porous transwell membrane coated on the underside with collagen I (40 μg/ml). The non-motile cells were removed from the top of the membrane, and the cells that migrated to the underside of the membrane were stained and counted. The average fold change in motility was plotted (Figure 10B). The motility of T47D cells was significantly increased by FAK overexpression, which is consistent with previous findings demonstrating a role for FAK in regulating motility in other cell types [16,18,19]. Expression of SuperFAK further increased the motility of T47D cells compared with cells expressing FAK, whereas FAK6.7 was less efficient than wild-type FAK in enhancing haptotaxis. These observations demonstrate the ability of SuperFAK to amplify a FAK-mediated biological response.

DISCUSSION

In the present study we describe the construction of two activated mutants of FAK: SuperFAK and FAK6.7. SuperFAK, and to a lesser extent FAK6.7, showed increased catalytic activity in vitro compared with wild-type FAK. The catalytic activity of both
For the activation of FAK. Interestingly, although substitutions that the tyrosine-to-glutamic acid substitutions in Y576E enhances the catalytic activity of FAK [3]. However, it appears that higher concentrations of tyrosine phosphorylation signals was observed. It was found that the kinase domain are accessible [46]. In the case of the Y576E mutation, the mechanism of enzyme activation [46] was not clearly understood. The negative charge introduced by the substitution may alter the conformation of the enzyme, leading to enhanced activity. The substitution of basic residues for basic residues in the activation loop results in catalytic activation, as originally reported for FGFR3 [35]. The negatively charged glutamic acid residues may alter the conformation in such a way as to mimic the conformational change that occurs when the regulatory tyrosine residues in the activation loop of FAK are phosphorylated. Alternatively, the activation of SuperFAK could be explained by an increase in the level of phosphorylation of the regulatory tyrosine residues in the kinase domain of FAK, Tyr576 and Tyr577. The double lysine- to-glutamic acid substitution may alter the recognition of FAK by either a tyrosine kinase, likely Src, or a tyrosine phosphatase resulting in elevated phosphorylation of Tyr576 and Tyr577, leading to enhanced activity.

FAK6.7 is an avian version of an alternatively spliced variant of FAK found in rat brain [36]. As previously reported, FAK6.7 has elevated auto-phosphorylation activity in vitro [36,48]. In addition, we have shown that this mutant can also elevate tyrosine phosphorylation of substrates in vitro. The mechanism of activation of the mutant is not clear. It has been speculated that alterations around the autophosphorylation site might alter the level of phosphorylation at that site. It has been reported that FAK6.7 expressed in COS-7 cells does exhibit elevated phosphorylation at Tyr576 [48]. However, our analysis in fibroblasts does not show an elevation of phosphorylation at Tyr576 in vivo. Increased tyrosine phosphorylation of FAK at Tyr576 could result in increased recruitment of Src, but our results suggest that FAK6.7 does not exhibit increased Src binding in vivo. The discrepancy between published results and our results obtained in the present study may be due to differences in cell types and expression systems used or the use of different Tyr576 phosphospecific antibodies. In the light of our observations, the mechanism of activation of FAK6.7 still remains to be solved.

Src plays an important role in biochemical signalling via FAK. Src can bind autophosphorylated FAK and phosphorylate activation-loop residues to further promote the activity of FAK [3]. In addition, recruitment of Src into a complex with FAK may direct phosphorylation of paxillin and tensin in vitro by Src [45]. These observations raise the question of the role Src plays in signalling by SuperFAK and FAK6.7. Experiments using phosphospecific antibodies and GST fusion proteins indicate that there was no significant change in the level of phosphorylation of Tyr576 of the activated FAK mutants compared with wild-type FAK. Further co-immunoprecipitation experiments reveal no difference in the association with Fyn with wild-type FAK or the activated mutants. Similarly, experiments using an Src inhibitor in FAK kinase assays also suggest that the increase in catalytic activity of SuperFAK is not due to increased association with Src kinases. These observations suggest that enhanced signalling by SuperFAK and FAK6.7 is not due to increased recruitment of Src kinases. A FAK-dependent mechanism of Src activation has been proposed [7,10]. However, experiments using Src phosphospecific antibodies demonstrate that SuperFAK and FAK6.7 activate Src in vitro to the same extent as wild-type FAK. Thus the enhanced effects of SuperFAK and FAK6.7 on downstream signalling might be independent of Src. Furthermore, from these studies it seems likely that paxillin and tensin are serving as direct substrates for SuperFAK and FAK6.7. In contrast, expres-

Figure 10 Elevation of T47D/Tva motility in cells expressing FAK or SuperFAK

(A) The expression of the FAK proteins in T47D/Tva cells 10 days after infection was analysed by Western blotting. Lysates (25 µg) of T47D/Tva cells infected with the empty retroviral vector (lane 1) or retrovirus containing the FAK (lane 2), SuperFAK (lane 3), or FAK6.7 (lane 4) were Western-blotted with a polyclonal FAK antibody, BC4. The positions of the molecular-mass markers are indicated on the left. (B) The motility of T47D/Tva cells expressing empty vector (mock), FAK, SuperFAK or FAK6.7 was measured in a transwell system. Cells were allowed to migrate to the underside of a collagen-coated transwell membrane for 20–22 h. The number of cells that reached the underside of the membrane were counted. The average (±S.E.M.) fold change in migration from 11 experiments is shown. The difference in motility between FAK and mock, and between SuperFAK and mock were statistically significant (P < 0.05).

In many protein kinases, phosphorylation of residues in the activation loop, analogous to Tyr576 and Tyr577 in FAK, is a mechanism of enzyme activation [46]. The negative charge introduced by the phosphate groups stabilizes the active conformation, in which the ATP and the substrate-binding sites of the kinase domain are accessible [46]. In the case of the Y576E/Y577E FAK mutant, no change in catalytic activity or downstream tyrosine phosphorylation signals was observed. It was expected that the introduction of the charged residues might mimic phosphorylation of the tyrosine residues, which normally enhances the catalytic activity of FAK [3]. However, it appears that the tyrosine-to-glutamic acid substitutions in Y576E/Y577E were not sufficient to cause the conformational change necessary for the activation of FAK. Interestingly, although substitutions of acidic residues for activation loop phosphorylation sites can activate some protein kinases, e.g. MAPK/extracellular-signal-related kinase (ERK) kinase (MEK) [46], there are no reports of mutational activation of tyrosine kinases using this strategy. In the case of SuperFAK, the double substitution of the activation-loop lysine residues (Lys576 and Lys577) with glutamic acid residues did lead to activation of the kinase, whereby catalytic activity was elevated and downstream biochemical and cellular events were augmented. Thus the substitution of acidic residues for basic residues in the activation loop results in catalytic activation, as originally reported for FGFR3 [35]. The negatively charged glutamic acid residues may alter the conformation in such a way as to mimic the conformational change that occurs when the regulatory tyrosine residues in the activation loop of FAK are phosphorylated. Alternatively, the activation of SuperFAK could be explained by an increase in the level of phosphorylation of the regulatory tyrosine residues in the kinase domain of FAK, Tyr576 and Tyr577. The double lysine- to-glutamic acid substitution may alter the recognition of FAK by either a tyrosine kinase, likely Src, or a tyrosine phosphatase resulting in elevated phosphorylation of Tyr576 and Tyr577, leading to enhanced activity.
sion of SuperFAK or FAK6.7 did not result in elevated tyrosine phosphorylation of p130<sup>cas</sup>. This observation is consistent with the hypothesis that Src is the major kinase responsible for tyrosine phosphorylation of p130<sup>cas</sup> [49, 50].

One interesting observation was the apparent increase in phosphorylation of Tyr<sup>992</sup> in SuperFAK relative to wild-type FAK and FAK6.7. Src has been shown to be the kinase responsible for phosphorylation of Tyr<sup>992</sup> ([12], but see [12a]). However, since there is no difference in the association of FAK and SuperFAK with Src family kinases, the mechanism leading to phosphorylation of this site is unclear. Since phosphorylation of Tyr<sup>992</sup> creates a Grb2-binding site linking FAK with the MAPK cascade [51] and SuperFAK exhibits elevated levels of phosphorylation at Tyr<sup>992</sup>, it was anticipated that SuperFAK might induce enhanced activation of ERK. However, no significant effect of SuperFAK upon adhesion-mediated or serum-mediated ERK activation was observed in CE cells (results not shown).

In our studies, the catalytic activity of SuperFAK and FAK6.7 was reduced when cells were held in suspension. However, in the absence of an adhesion signal, SuperFAK and FAK6.7 still displayed much higher kinase activity in vitro compared with wild-type FAK. Thus the kinase activity of SuperFAK and FAK6.7 is only partially regulated by adhesion. These observations suggest that the mutants may not be constitutively active, since they still require a cell-adhesion stimulus for maximal activation. Nevertheless, these mutants exhibit higher enzymic activity compared with wild-type FAK. Although the catalytic activity of SuperFAK and FAK6.7 remained high in the absence of an adhesion signal, tyrosine phosphorylation of downstream effectors did not occur in cells in suspension. These observations indicate that the high catalytic activity of SuperFAK and FAK6.7 is insufficient to induce substrate phosphorylation in suspended cells. There are a number of possible explanations for this observation. The absence of FAK substrate phosphorylation could be attributed to the action of cellular phosphatases, which are more active, or have increased access to substrates, when cells are in suspension. Alternatively, the proximity of the activated FAK mutants to its substrates represents another potential method of regulation. The assembly of proteins into focal adhesions may be required not only for the efficient activation of FAK, but also for the clustering of FAK with its substrates to promote their phosphorylation and the transmission of FAK downstream signals.

Several FAK-binding partners have been implicated in mediating FAK-dependent cell motility, including Src family kinases and PI 3-kinase [6, 19]. Src kinases are likely to phosphorylate downstream substrates to regulate motility, and one important substrate implicated in cell motility is p130<sup>cas</sup> [20, 52]. A FAK mutant that cannot bind p130<sup>cas</sup> or induce its tyrosine phosphorylation is defective for induction of motility, implicating p130<sup>cas</sup> in the regulation of motility by FAK [20]. It may therefore be noteworthy that the FAK and SuperFAK do not induce p130<sup>cas</sup> tyrosine phosphorylation, yet do promote cell motility. FAK may utilize different signalling pathways to control cell motility in different cell types. Further experimentation is required to test this hypothesis.

The characterization of SuperFAK and FAK6.7 has demonstrated the ability of these constructs to increase FAK-mediated signals. Other membrane-bound constitutively activated FAK constructs, CD2FAK and myrFAK, have been described [28, 29]. The best characterized of these FAK variants, CD2FAK, exhibits biochemical properties different from those of SuperFAK. First, its expression does not promote dramatic increases of tyrosine phosphorylation of substrates in adherent cells [23, 50]. Secondly, in suspended cells expressing CD2FAK, the tyrosine phosphorylation of paxillin and p130<sup>cas</sup> are sustained [23, 50]. Presumably, constitutive CD2FAK and MyrFAK signalling can be explained by their constitutive association with the membrane and membrane-bound proteins such as Src. This contrasts with the hyperactive signalling generated by SuperFAK and FAK6.7 in response to normal physiological stimuli. In this regard, SuperFAK may better mimic pathological situations exhibiting overexpression of FAK. In different scenarios, the membrane-bound chimaeras and SuperFAK may be utilized to study the role of aberrant FAK signalling, alone or in combination with other signalling partners, in the development of human disease.

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