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A FAK/Src Chimera With Gain-of-Function Properties Promotes Formation of Large Peripheral Adhesions Associated With Dynamic Actin Assembly

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Abstract

Formation of a complex between the tyrosine kinases FAK and Src is a key integrin-mediated signaling event implicated in cell motility, survival, and proliferation. Past studies indicate that FAK functions in the complex primarily as a “scaffold,” acting to recruit and activate Src within cell/matrix adhesions. To study the cellular impact of FAK-associated Src signaling we developed a novel gain-of-function approach that involves expressing a chimeric protein with the FAK kinase domain replaced by the Src kinase domain. This FAK/Src chimera is subject to adhesion-dependent activation and promotes tyrosine phosphorylation of p130Cas and paxillin to higher steady-state levels than is achieved by wild-type FAK. When expressed in FAK ^{-/-} mouse embryo fibroblasts, the FAK/Src chimera resulted in a striking cellular phenotype characterized by unusual large peripheral adhesions, enhanced adhesive strength, and greatly reduced motility. Live cell imaging of the chimera-expressing FAK ^{-/-} cells provided evidence that the large peripheral adhesions are associated with a dynamic actin assembly process that is sensitive to a Src-selective inhibitor. These findings suggest that FAK-associated Src kinase activity has the capacity to promote adhesion integrity and actin assembly.

Keywords

integrins; phosphotyrosine; FAK; Src; p130CAS; paxillin; podosome

INTRODUCTION

Cell adhesion to the extracellular matrix (ECM) is essential for the normal migratory, proliferative, and survival characteristics of most cell types. Cell-ECM adhesion occurs mainly through the clustering of integrin receptors that link the actin cytoskeleton to the ECM. Integrins also function as signaling receptors, a concept fortified by the discovery of focal adhesion kinase (FAK), a nonreceptor protein-tyrosine kinase that becomes activated in response to cell-ECM adhesion [Hanks et al., 1992; Schaller et al., 1992]. A wealth of data have since implicated FAK as a key signaling protein contributing to integrin control of cell motility, invasion, survival, and proliferation [reviewed in Hanks et al., 2003; Parsons, 2003; Mitra et al., 2005]. For example, FAK ^{-/-} mouse embryo fibroblasts (MEFs) have motility

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defects that can be rescued by reconstitution with WT-FAK but not signaling-deficient FAK mutants [Owen et al., 1999; Sieg et al., 1999; Wang et al., 2001]. Conditional knockout studies demonstrating the importance of FAK for proper formation of neuronal [Beggs et al., 2003, Rico et al., 2004] and vascular [Shen et al., 2005, Braren et al., 2006] networks indicate a widespread role for FAK signaling in development. FAK is overexpressed in a variety of human cancers [reviewed in McLean et al., 2005] and FAK signaling can promote invasive behavior [Hsia et al., 2003], implicating deregulated FAK signaling in tumor onset and progression.

FAK contains an N-terminal 4.1/ezrin/radixin/moesin (FERM) domain, a central tyrosine kinase domain, two proline-rich motifs, and a C-terminal focal adhesion targeting (FAT) domain. FAK is activated by a mechanism involving FAT and FERM domain interactions with other integrin-associated proteins, resulting in adhesion targeting and conformational changes releasing autoinhibition [Cooper et al., 2003; Dunty et al., 2004; Cohen and Guan, 2005]. The activation state of FAK is defined largely by the phosphorylation of Tyr-397, an autophosphorylation site that lies in the linker region between the FERM and kinase domains. Tyr-397 phosphorylation creates a high-affinity binding site for the Src SH2 domain, an interaction that recruits and activates Src in adhesions [Schaller et al., 1994]. Formation of the complex with Src is arguably the most critical event in FAK-associated signaling. Src bound to the Tyr-397 site phosphorylates other FAK residues [Schlaepfer et al., 1997; Ruest et al., 2000] including Tyr-576/Tyr-577 in the kinase domain activation loop and Tyr-861 that are important for maximal FAK autophosphorylation activity [Owen et al., 1999; Leu and Maa, 2002]. Furthermore, the ability of FAK to promote tyrosine phosphorylation of p130Cas, a major adhesion-associated tyrosine kinase substrate that binds to FAK proline-rich motifs, depends on Src activity and the Tyr-397 site [Ruest et al., 2001]. In this sense, FAK acts as a classic “scaffold” protein in recruiting Src to its substrate. The scaffolding function of FAK has also been implicated in directing Src-mediated phosphorylation of paxillin [Schaller et al., 1999] and endophilin A2 [Wu et al., 2005].

In addition to Src, the phosphorylated FAK Tyr-397 site can also mediate interactions with SH2 domains of other signaling proteins including PLC- γ 1, the p85 subunit of PI3K, and adaptors including Shc, Grb7, and Nck-2 [reviewed in Hanks et al., 2003]. While these interactions suggest further complexity in FAK signaling, the formation of the FAK/Src complex and resulting Src-mediated tyrosine phosphorylation of adhesion-associated substrates has been implicated from numerous studies as a key event promoting cell motility [Richardson et al., 1997; Cary et al., 1998; Sieg et al., 1999; Nakamura et al., 2000; Shin et al., 2004; Webb et al., 2004]. However, the impact of FAK-associated Src signaling on cell proliferation/survival, and on the physical steps of cell motility involving the dynamic reorganization of the adhesion-cytoskeletal network, remain poorly understood.

Given the role for FAK as a molecular scaffold acting to recruit and activate Src, we reasoned that a chimeric protein with the Src kinase domain substituted for the FAK kinase domain may act as a “short-circuited switch” with gain-of-function properties, and thereby provide new insight into the impact of FAK-associated Src signaling. Here we report biochemical and cellular consequences of expressing such a FAK/Src chimera. Our studies suggest a role for FAK-associated Src activity as a positive effector of adhesion integrity and actin assembly, while also emphasizing the importance of proper FAK/Src complex regulation for efficient cell motility.

MATERIALS AND METHODS

Antibodies and Reagents

Monoclonal antibodies against p130Cas (clone 21, here designated “CAS-TL”) and paxillin (clone 349), and horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG

were obtained from BD Transduction Laboratories. Anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology. Anti-FAK polyclonal antibody C-20 was from Santa Cruz Biotechnology. Anti-FAK polyclonal antibody 331 was described previously [Hanks et al., 1992]. Anti-vinculin monoclonal antibody (clone hVIN-1) was from Sigma. Antibodies against cortactin phosphotyrosine 421 and FAK phosphotyrosines 407, 861 and 925 were from Biosource International. Anti-phosphotyrosine antibody PY100, phosphospecific antibodies against p130Cas substrate domain YxxP tyrosines (pCAS-165, pCAS-249, and pCAS-410), paxillin Tyr-118, and Src Tyr-418 were obtained from Cell Signaling Technology. Alexa594-phalloidin was obtained from Molecular Probes. FITC-conjugated and Cy2- and Cy3-conjugated secondary antibodies were from Jackson ImmunoResearch. Plasmids DsRed-SM22 [Gimona et al., 2003] and pLZRS-SrcF529-IRES-EGFP were described previously [Brábek et al., 2004]. Plasmid pEGFP-C1-paxillin- α [Mazaki et al., 1998] was provided by Dr. Hajime Yano, Osaka Bioscience Institute. Src-selective inhibitor AZD0530 [Hennequin et al., 2006] was provided by AstraZeneca.

FAK/Src Chimera cDNA and Retroviral Expression Plasmid Construction

The FAK/Src chimera cDNA was constructed by modification of mouse FAK expression plasmid pRc/CMV-mycFAK(WT) [Zhang et al., 1999]. Using mutagenic PCR, codons for the FAK kinase domain (Arg-421 to Leu-676 of the nonneuronal isoform, UniProt Id. P34152-3) were first replaced with nucleotides agc-tta-aga-cac-tta containing a recognition sequence for restriction enzyme *Bst* 98I. Into this unique site was subcloned the cDNA for the kinase domain of mouse Src (Leu-274 to Leu-523 in the neuronal isoform, UniProt Id. P05480) which was prepared by PCR using primers that introduced flanking *Bst* 98I sites without changing the amino acid sequence. The resulting plasmid was designated pRc/CMV-mycFAK/Src(Y-chimera). Plasmid pRc/CMV-mycFAK/Src(F-chimera), in which Tyr-397 was replaced by phenylalanine, was then constructed by replacing a 1.3 kb *Cla* I - *Eco* 47 III fragment of pRc/CMV-mycFAK(F397) [Zhang et al., 1999], containing essentially the kinase domain, with the same fragment from pRc/CMV-mycFAK/Src(Y-chimera). The same strategy was used to construct plasmid pRc/CMV-myc-FAK/Src(F397/SrcR303-chimera) for expressing the “kinase-dead” FAK/Src F-chimera, with the Src kinase domain amplified from plasmid pRc/CMV-Src-R303 [Polte and Hanks, 1997].

Retroviral vector pLZRS-MS-IRES-GFP was used to stably express the FAK/Src chimeras, WT-FAK, and Src-F529. pLZRS-SrcF529-IRES-GFP was described previously [Brábek et al., 2004]. pLZRS-FAT(WT)-IRES-GFP was constructed by removing the HA-epitope tag-encoding region from plasmid pRcCMV-FAK-HA [Calalb et al., 1995], and then blunt-end subcloning of the FAK cDNA-containing *Bam* HI fragment into the *Sgf* I - *Sfi* I sites of pLZRS-MS-IRES-GFP. Plasmids pLZRS-FAK/Src(F-chimera)-IRES-GFP, pLZRS-FAK/Src(Y-chimera)-IRES-GFP, and pLZRS-FAK/Src(F397/SrcR303-chimera)-IRES-GFP were then constructed by replacing the *Nae* I to *Xho* I fragment from pLZRS-FAT(WT)-IRES-GFP with the corresponding fragment from the appropriate pRc/CMV-constructs. All final plasmid constructions were confirmed by sequencing.

Cells, Cell Culture, Stable Protein Expression, and Fibronectin Replating

FAK^{-/-} MEFs were provided by Dusko Ilic (StemLiefLine, San Carlos, CA), SYF cells were provided by Phil Soriano (Fred Hutchinson Cancer Research Center) and the retroviral packaging cell line Phoenix Eco was provided by Gary Nolan (Stanford). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. pLZRS-IRES-EGFP plasmids expressing WT-FAK, FAK/Src chimeras, or SrcF529 were transfected into Phoenix Eco cells by calcium phosphate transfection, viral supernatants were harvested, and the recipient cells were infected, essentially as we described elsewhere

[Brábek et al., 2004]. Fibronectin-replating was carried out as described in Hanks et al. [1992].

Immunoprecipitation, Immunoblotting, and Cell Staining

Immunoprecipitation and immunoblotting were performed as described previously [Brábek et al., 2004]. To assess p130Cas substrate domain phosphorylation, cells were treated with 500 μ M sodium vanadate for 6 h before lysis. For cell staining, cells were plated on fibronectin-coated coverslips and allowed to attach and spread at 37°C in complete growth medium. To detect WT-FAK or the FAK/Src chimeras, cells were fixed in 50% methanol/50% acetone and immunostaining was carried out [Fonseca et al., 2004] using FAK C-20 antibody followed by incubation with FITC-conjugated anti-rabbit secondary antibody. In other fluorescence costaining experiments cells were fixed in 4% paraformaldehyde and permeabilized as described previously [Brábek et al., 2004]. For costaining with F-actin, cells were incubated overnight at 4°C with antibody (PY100 or cortactin pY421) followed by a 2-h room temperature incubation with Cy2-conjugated secondary antibody, then 3.3 nM Alexa 594-conjugated phalloidin for 45 min. For costaining of phosphotyrosine and vinculin, cells were incubated overnight at 4°C with antibody hVIN-1 followed by Cy3-conjugated secondary antibody, extensive washing in phosphate buffered saline, then incubation with PY100 and Cy2-anti-mouse IgG as above. Stained cells were viewed using a Zeiss Axiophot microscope with Plan-Apochromat 63x/1.40 oil-immersion objective, and captured using Metamorph.

Cell Proliferation Assays

Growth curves under adherent and nonadherent conditions were obtained as described [Brábek et al., 2004]. For adherent growth, cells were initially plated at 30,000 cells per 60-mm dish. For nonadherent proliferation, 100,000 cells were plated per 60-mm dish precoated with polyhydroxymethacrylate (poly-HEMA, Sigma). Cells were counted at 2-day intervals using a Coulter Particle Counter. For assays on adherent cells, growth medium was replaced on the alternate days. The mean cell number for each time point, taken from three independent assays, was plotted.

Cell Adhesion Strength Measurements

Cell adhesion strength measurements were performed using a spinning disk assay [Gallant et al., 2005]. Micropatterned surfaces of adhesive circular islands (5 μ m diameter) in a nonadhesive background were engineered using microcontact printing of alkanethiol self-assembled monolayers (HS—(CH₂)₁₅—CH₃ and HS—(CH₂)₁₁—(CH₂CH₂O)₃—OH). Substrates were coated with fibronectin (20 μ g/ml, 30 min), blocked (1% heat-denatured bovine serum albumin), and soaked in PBS overnight prior to cell seeding. Cell adhesion strength was measured at 24 h after cell seeding. Samples were spun for 5 min, fixed in 3.7% formaldehyde, and stained with ethidium homodimer-2. Cell numbers at different radial positions were quantified using a motorized microscope stage and image analysis system. The profile of adherent cells with respect to the applied shear stress was normalized by the number of cells at the center of the disk, where there is no applied force, and fit with a sigmoid. Mean adhesion strength is defined as the shear stress at which 50% of the cell population detaches.

Live Cell Imaging

Cells were viewed using a Nikon Eclipse TE 2000-E microscope, and images were captured using IpLab software. For DIC microscopy, cells plated overnight on 1 μ g/ml fibronectin were imaged at 15 s intervals using a Cool-Snap HQ camera (Photometrics) and a Nikon Plan-Apo 100X/1.4 oil DIC N2 lens. For studies of adhesion and actin dynamics, cells were cotransfected with expression plasmids for GFP-paxillin and DsRed-Sm22 using Lipofectamine (Invitrogen) and then plated on 10 μ g/ml fibronectin. Images were captured every 60 s for 50 min using a

Cascade 512B camera (Photometrics) and Nikon ApoTIRF 100X/1.49 oil immersion lens. To observe the effects of Src kinase inhibition, the Src-selective inhibitor AZD0530 (0.1-0.25 μ M final concentration in 1:10,000 final dilution of DMSO solvent) was carefully added to the dish after the first 15-20 min of image capture and recording was then allowed to continue for an additional 20-40 min. To quantitate actin in or near the ULPA, IpLab software was used to select regions of interest and the mean (background-subtracted) red fluorescence activity was measured.

RESULTS

A FAK/Src Chimera With Gain-of-Function Properties in Adhesion Signaling

A FAK/Src chimera cDNA was constructed by precisely replacing the FAK kinase domain with the Src kinase domain (Fig. 1A). The chimera retains the FAKFERM and FAT domains and the two proline-rich motifs important for regulation, adhesion targeting, and interactions with other signaling proteins. The chimera retains three of the reported FAK sites of phosphorylation by Src: Tyr-407, -861, and -925. However, Tyr-397 was substituted with phenylalanine (hence designated “F-chimera”) to eliminate other functions resulting from the recruitment of signaling proteins to this phosphorylation site.

The FAK/Src F-chimera, or WT-FAK as a control, was stably expressed in FAK $-/-$ MEFs using a retroviral vector that coexpresses cytoplasmic GFP from a bicistronic transcript. Sorting the cells for equivalent GFP levels resulted in cell populations expressing similar levels of WT-FAK and the F-chimera (Fig. 1B, lanes 1-3). Consistent with gain-of-function properties, F-chimera expression led to enhanced cellular phosphotyrosine levels, relative to WT-FAK, particularly of proteins in the ranges of 120-130 and 65-90 kDa (Fig. 1B, arrows). The 120-130 kDa band includes p130Cas, as shown by immunoblotting with antibodies that recognize tyrosine-phosphorylated sites in the p130Cas substrate domain (Fig. 1C). The broad band in the 65-90 kDa range is indicative of paxillin, and enhanced paxillin tyrosine phosphorylation in the F-chimera expressing cells was confirmed by immunoblotting with a phosphospecific antibody against the major Tyr-118 site (Fig. 1D). Similar results were obtained for a FAK/Src chimera in which the Tyr-397 site was left intact (designated “Y-chimera”) (Figs. 1B-1D, lane 4). The tyrosine phosphorylation profile resulting from FAK/Src chimera expression is more limited than that obtained by expressing oncogenic Src-F529 (Figs. 1B-D, lane 6), indicative of specific subcellular targeting of the chimera. The relative lack of paxillin tyrosine phosphorylation in FAK-null cells expressing oncogenic Src has been noted previously [Roy et al., 2002].

A “kinase-dead” F-chimera in which the conserved ATP-binding lysine in the Src kinase domain was changed to arginine to abolish Src kinase activity [Kamps and Sefton, 1986], was also expressed in the FAK $-/-$ cells as a control (Fig. 1, lane 5). While the kinase-active chimeras were extensively phosphorylated on the Src kinase domain Tyr-418, indicative of auto-phosphorylation activity, this site was only weakly phosphorylated in the kinase-dead F-chimera (Fig. 1E). The residual phosphorylation of Tyr-418 in the kinase-dead F-chimera may be due to endogenous Src-family kinases. Notably, the kinase-dead F-chimera was unable to promote p130Cas tyrosine phosphorylation (Fig. 1C, lane 5). Surprisingly the kinase-dead F-chimera was still capable of stimulating some paxillin Tyr-118 phosphorylation (Fig. 1D, lane 5), suggesting the possible involvement of kinase-independent mechanisms in this response. In further characterizing the cell biological impact of FAK/Src chimera expression we concentrated our analysis on cells expressing the F-chimera, thus focusing on the response to the chimera kinase activity while eliminating other possible functions associated with the Tyr-397 site.

The FAK/Src F-Chimera Retains Adhesion-Dependent Regulation

The F-chimera was found to retain adhesion-dependent regulation, showing much reduced tyrosine phosphorylation in suspended cells compared to attached and fibronectin-replated cells (Fig. 2). Under adherent conditions, F-chimera tyrosine phosphorylation appeared greatly elevated relative to WT-FAK (Fig. 2, top two panels). Specific sites of F-chimera phosphorylation were assessed by immunoblotting with phosphospecific antibodies. The antibody against the Tyr-861 site gave a strong signal that was subject to adhesion regulation (Fig. 2, third panel), while phosphorylated FAK Tyr-407 and -925 sites were not readily detected. The Src kinase domain Tyr-418 site was also found to be phosphorylated and to exhibit adhesion-dependent regulation (Fig. 2, bottom panel). These results indicate that normal adhesion-mediated interactions are maintained to regulate activation of the Src kinase domain within the chimera.

The FAK/Src Chimera Does Not Enhance Cell Proliferation

Oncogenic Src expression enables the FAK $-/-$ cells to proliferate in the absence of anchorage and to overcome contact inhibition, but the F-chimera was unable to confer these properties (Fig. 3).

Expression of the FAK/Src Chimera in FAK $-/-$ Cells Promotes Assembly of Unusual Large Peripheral Adhesions

As anticipated, the F-chimera targeted to focal adhesions as shown by immunostaining using an antibody recognizing the FAK C-terminus (Fig. 4A, left). However, compared to cells expressing WT-FAK (Fig. 4A, center), the adhesions in the F-chimera cell population commonly appeared as unusually large structures, prominently arranged at the cell periphery (Fig. 4A, arrows). We use the acronym ULPA (“unusual large peripheral adhesions”) to conveniently describe this characteristic phenotype. Cells expressing the FAK/Src Y-chimera also exhibited ULPA (data not shown). Adhesions in cells expressing the kinase-dead F-chimera more closely resembled those of the WT-FAK cells (Fig. 4A, right), indicating that assembly of ULPA is a consequence of chimeric Src kinase activity. The ULPA are enriched in phosphotyrosine, while F-actin in the chimera cells was commonly arranged as stress fiber bundles at the cell periphery (Fig. 4B). Immunostaining for vinculin also revealed the ULPA in a manner similar to the phosphotyrosine antibody (Supplemental Fig. S1). While FAK $-/-$ cells are reported to have larger-than-normal focal adhesions [Ilic et al., 1995], the ULPA we observe in association with F-chimera expression clearly represent a distinct adhesion phenotype (Fig. 4B, compare top three panels).

The ULPA are somewhat reminiscent of the large podosome structures observed in Src-transformed cells. Indeed, ULPA stain prominently with cortactin phosphospecific antibody pTyr-421 (Fig. 4C), which strongly labels podosomes but does not recognize normal focal adhesions [Head et al., 2003; Brábek et al., 2004]. Thus the ULPA appear to share some of the properties of invasive podosomes. Yet ULPA are not dramatically enriched in F-actin, which is another distinguishing feature of podosomes (Fig. 4B, compare bottom two panels).

ULPA Form Rapidly and are Associated With Increased Adhesion Strength

To study how F-chimera expression in FAK $-/-$ cells affects adhesion assembly, cells were fixed during the process of attachment and spreading on fibronectin and costained with anti-phosphotyrosine antibody and phalloidin. The ULPA phenotype was readily detected by 30 min and characteristic of the majority of cells by 120 min (Figs. 5A and 5B). ULPA formation appears to arise from earlier states characterized by a prominent network of long adhesions distributed uniformly at the cell periphery, followed by nonuniform clustered adhesions linked

to stress fibers running through the cell (Figs. 5A and 5B). In cells expressing WT-FAK, the long peripheral adhesions characteristic of spreading F-chimera cells are not seen (Fig. 5C).

A hydrodynamic spinning disk assay was employed to determine if ULPA are associated with enhanced adhesion strength. In this assay [Gallant et al., 2005] well-defined detachment forces are applied to adherent cells cultured on fibronectin-coated micropatterned islands in order to control for cell shape and adhesive area. The shear stress (force/area) required to detach 50% of the cell population represents the mean adhesion strength. The mean adhesion strength for FAK $-/-$ cells expressing the F-chimera was 250 dyn/cm², representing a significant twofold enhancement over cells expressing WT-FAK (Fig. 6).

FAK $-/-$ Cells Expressing the FAK/Src Chimera Fail to Establish Polarized Cell Movement

Live cell imaging was employed to evaluate how the F-chimera impacts FAK $-/-$ cell movement relative to cells reconstituted with WT-FAK. From analysis by time-lapse differential interference contrast (DIC) microscopy, cells expressing WT-FAK exhibit typical polarized movement associated with a well-defined leading edge and elongated trailing end, while cells expressing the F-chimera had large stable anchorage sites, remain poorly polarized, and showed little or no crawling movement (Fig. 7, Supplemental Movies 1 and 2).

To further view how the F-chimera affects dynamic events associated with cell motility, time-lapse wide-field fluorescence microscopy was performed using cells cotransfected with expression plasmids for GFP-paxillin to visualize adhesions and DsRed-Sm22 to visualize F-actin. Control cells expressing WT-FAK were similarly analyzed. Associated with their polarized movement, cells expressing WT-FAK exhibit a dynamic adhesion/actin network including vibrant actin-rich leading edge lamellipodia and adhesions that form at the periphery, mature into stress fiber-associated stationary adhesions, and disassemble within the cell body (Fig. 8A, Supplemental Movie 3). In contrast, cells expressing the F-chimera are less able to extend lamellipodia and typically appear locked in a rigid state (Fig. 8B, Supplemental Movie 4). Actin in the chimera cells is largely maintained in the form of peripheral stress fibers. ULPA maintain integrity as aggregates of individual adhesion components, although internal dynamics within this larger organization are apparent.

FAK/Src Chimera Signaling Results in Adhesion-Associated Bursts of Actin Polymerization

The time-lapse fluorescence microscopy further indicated an apparent association of the ULPA with a frequent and transient enrichment of F-actin, appearing as flashing red dots visualized by DsRed-Sm22 (Fig. 8D, Supplemental Movie 6). The ULPA also commonly appear to be associated with membrane protrusive (ruffling) activity (Fig. 8D, Supplemental Movie 6) that generally fails to stabilize as a lamellipodium. Another, quite striking, example of the ULPA-associated dynamic actin activity in a cell from the F-chimera-expressing population is shown in Fig. 9A (Supplemental Movie 7). Such bursts of F-actin are not evident in association with adhesions in cells expressing either WT-FAK (Fig. 8C, Supplemental Movie 5) or the kinase-dead FAK/Src chimera (data not shown).

To obtain further evidence as to whether the ULPA-associated actin activity is driven by the kinase activity of the FAK/Src F-chimera, cells were treated with the Src-selective kinase inhibitor AZD0530 (0.1 μ M) during the course of the time-lapse fluorescence recordings. For 17 distinct ULPA (present in 4 different cells) the associated actin activity was measured as DsRed-Sm22 fluorescence intensity for 30 one-minute intervals, 14 preceding and 16 immediately following the treatment. Figure 9B plots the mean actin activity for the 17 ULPA at each time point, normalized as to the mean fluorescence intensity for the pretreatment time points. It is evident from this analysis that AZD0530 addition initiated a steady decline in the ULPA-associated actin activity, stabilizing after $\sim$ 6 min to levels that were significantly

reduced from pretreatment levels. The decline in red fluorescence intensity is also visually apparent from the time-lapse images as a reduction in the number of ULPA-associated flashing red dots (see Supplemental Movie 8, for example). The AZD0530 treatment resulted in retraction of some ULPA (not scored in this analysis), and this response was more apparent at higher doses (e.g. 1 μ M) of the inhibitor (not shown). Eventually after AZD0530 treatment, new adhesions are often seen to reform at the cell periphery and have an associated actin activity (Supplemental Movie 8). Treatment with the DMSO vehicle alone had no apparent effect on ULPA stability or ULPA-associated actin activity (Supplemental Movie 9, and data not shown). Together, these results suggest that FAK/Src chimera signaling is important for maintaining the ULPA organization while also promoting adhesion-associated actin polymerization.

The FAK/Src Chimera Does Not Give Rise to ULPA When Expressed in SYF Cells

To further examine requirements for the ULPA phenotype, the F-chimera was also expressed in SYF cells [Klinghoffer et al., 1999] that lack the three ubiquitously expressed Src family kinases: Src, Yes, and Fyn. In SYF cells, F-chimera expression led to elevated cellular phosphotyrosine content as determined by phosphotyrosine antibody immunoblotting (Supplemental Fig. S2A) and restored phosphotyrosine within focal adhesions (Fig. 10). However, the adhesions seen in the F-chimera-expressing SYF cells were not unusual in their general appearance. Moreover, SYF cells expressing the F-chimera did not exhibit increased adhesion strength or motility defects relative to vector-only cells (Supplemental Figs. S2B and S2C). Thus the ability of the F-chimera to promote the ULPA phenotype may require either the absence of endogenous FAK or the presence of endogenous Src-family kinases.

DISCUSSION

Our studies on a novel FAK/Src chimeric protein with gain-of-function properties provide new insight into the impact of FAK-associated Src kinase activity on the organization of the network of adhesions and associated actin filaments. In a FAK $-/-$ background, FAK/Src chimera signaling gave rise to an “ULPA phenotype” characterized by static strongly-adherent cells with large stable peripheral adhesions that serve as centers for dynamic actin assembly. Thus Src, in association with the adhesion targeting and regulatory properties of FAK, has the capacity to phosphorylate substrate protein(s) that in turn act to promote adhesion assembly/stability and adhesion-associated actin polymerization.

Our FAK/Src chimeric protein, made by replacing the FAK kinase domain with the Src kinase domain, has gain-of-function properties in that it greatly enhances the steady-state tyrosine phosphorylation of p130Cas and paxillin two of the known major substrates of the FAK/Src complex. In the normal circumstance, FAK binds to Src by virtue of interactions with Src SH2/SH3 domains and this serves to activate Src while also recruiting Src to p130Cas and paxillin bound to other FAK domains. Our results indicate that the Src kinase domain can still efficiently phosphorylate these two substrates when present in the chimera context. That our FAK/Src chimera promoted greatly enhanced p130Cas tyrosine phosphorylation relative to WT-FAK further indicates the inefficient catalytic activity of the FAK kinase domain toward this exogenous substrate, relative to the much more potent Src kinase domain, which is consistent with the idea that FAK's role in this pathway is primarily as a scaffold [Ruest et al., 2001].

Normally, the interaction of Src with FAK is initiated by adhesion-stimulated FAK Tyr-397 autophosphorylation to create the high-affinity binding site for the Src SH2 domain. Our FAK/Src chimera retains the property of adhesion-dependent activation as indicated by much-elevated phosphorylation of the Src kinase domain Tyr-418 site in adherent cells. In c-Src, Tyr-418 is an autophosphorylation site indicative of the activated state. The adherent cells also exhibited elevated phosphorylation of the FAK Tyr-861 site, a major site of FAK

phosphorylation by Src, but we could detect little or no chimera phosphorylation using phosphospecific antibodies against the Tyr-407 or Tyr-925 sites. Of these two sites, Tyr-925 phosphorylation by Src has been implicated as an important event in adhesion-stimulated FAK signaling by promoting an interaction with the adaptor Grb2 [Schlaepfer et al., 1994; Schlaepfer and Hunter, 1996]. Structural studies showing that Tyr-925 occupies a position in the functional FAK FAT domain where it would be inaccessible indicate that phosphorylation of this site may be of very low stoichiometry in adhesions and occur only in the context of a transient open conformation [Arold et al., 2002; Prutzman et al., 2004]. The mechanism of adhesion-dependent activation of FAK appears to involve the interaction(s) of the FERM domain with clustered integrins and/or integrin-associated proteins in nascent adhesion sites, thereby releasing an autoinhibitory interaction of the FERM domain with the FAK kinase domain [Cooper et al., 2003; Dunty et al., 2004; Cohen and Guan, 2005]. Our results indicate that such negative-regulatory interactions do not require the presence of the FAK kinase domain *per se*, and can also occur in the context of the substituted Src kinase domain. While our FAK/Src chimera retains many of the signaling and regulatory properties of the normal complex of Src associated with FAK *via* SH2/SH3 domain interactions, there is a likely possibility that the full range of substrate phosphorylation by the normal FAK-Src complex is not achieved by the chimera.

The FAK/Src chimera was unable to generate the ULPA phenotype when expressed in SYF cells that are deficient in Src-family kinases. This finding provides some insight into the nature of additional cellular factors necessary for establishing the ULPA phenotype. It is possible, for example, that additional Src substrates that are phosphorylated by endogenous Src-family kinases, but not the FAK/Src chimera, play a role. Alternatively, the endogenous WT FAK expressed in the SYF cells may counteract the appearance of ULPA by promoting adhesion turnover [Webb et al., 2004]. Additional experimentation will be needed to resolve these mechanistic possibilities.

Others have investigated the function of FAK or the FAK/Src complex through study of variant proteins with possible gain-of-function properties. In one study, a chimeric protein termed “Src-FAT” was constructed by fusing full length Src to the FAK FAT domain [Shen and Guan, 2001]. In another study, a FAK mutant with elevated kinase activity, “SuperFAK,” was engineered to mimic constitutive FAK activation loop phosphorylation [Gabarra-Neicko et al., 2002]. Both Src-FAT and Super-FAK were reported to promote tyrosine phosphorylation of paxillin (but not p130Cas) and enhance cell migration. The ULPA phenotype resulting from our FAK/Src chimera could be due, at least in part, to p130Cas-mediated signaling. The kinase-dead F-chimera, unlike the kinase-active chimera, was unable to either promote p130Cas phosphorylation or give rise to the characteristic phenotype of ULPA and ULPA-associated actin polymerization. On the other hand, the kinase-dead F-chimera appeared capable of promoting a partial paxillin tyrosine phosphorylation response. This unexpected observation raises the intriguing possibility that FAK may have an additional noncatalytic role in stimulating paxillin tyrosine phosphorylation. One possibility is that paxillin may undergo a conformational change upon interaction with the chimera FAT domain, such that the Tyr-118 site becomes more accessible to phosphorylation by endogenous Src-family kinases.

In FAK *-/-* cells expressing the FAK/Src chimera, the rapid assembly of large adhesions after fibronectin-plating and ultimate ULPA formation suggests an imbalance of adhesion assembly over disassembly. Other studies have indicated roles for both FAK and Src in promoting focal adhesion disassembly [Webb et al., 2004]. There are several possible explanations for these contrasting views. One is that FAK and Src may promote adhesion turnover due to functions unrelated to their forming a complex. Another possibility is that normal levels and regulation of FAK-associated Src signaling promote adhesion turnover, while enhanced deregulated signaling achieved by the FAK/Src chimera has the opposite effect of promoting assembly. A

third consideration is that ULPA resemble the class of trailing “sliding adhesions” that form at the cell rear and flanks and mature rapidly into larger stress fiber-associated structures [Rid et al., 2005]. Sliding adhesions are distinct from the stationary adhesions studied by Webb et al. and could be subject to different regulation. Enhancement of the RhoA/ROCK pathway driving actomyosin contractility [Burridge and Wennerberg, 2004] is a conceivable mechanism underlying ULPA formation.

In addition to their size and integrity, a striking feature of the ULPA is their apparent association with a dynamic actin assembly process. This activity is not readily seen in cells expressing either WT-FAK or the kinase dead F-chimera, and treatment with the Src-selective inhibitor AZD0530 resulted in a dramatic reduction of the activity. While it should be noted that AZD0530 also exhibits significant activity toward Abl [Hennequin et al., 2006], taken together these findings provide evidence that adhesion-associated actin polymerization can be driven by FAK-associated Src activity. This activity may be due to nucleation by the Arp2/3 complex, which localizes to adhesions through its interaction with vinculin [DeMali et al., 2002]. Src-mediated tyrosine phosphorylation of the p130Cas substrate domain occurs in leading-edge adhesions [Fonseca et al., 2004], which could stimulate Arp2/3-mediated actin polymerization and membrane protrusion *via* the Crk > DOCK180/ELMO > Rac pathway [Chodniewicz and Klemke, 2004]. Indeed the dynamic actin activity occurring in or nearby the ULPA also appeared to be commonly associated with dynamic protrusive activity of the nearby plasma membrane. This ULPA-associated protrusive activity may fail to stabilize as a lamellipodium because the adhesion-associated proteins are “tied-up” within the existing ULPA and thus unavailable to form new adhesions to anchor the protrusion.

The ULPA observed in association with FAK/Src chimera signaling share some common characteristics with the invasive podosome rosettes that arise in response to oncogenic Src. Both result from elevated Src kinase activity, contain tyrosine-phosphorylated cortactin, and are sites of active actin polymerization and membrane protrusive activity. These similarities raise the possibility that FAK-associated Src activity may also have a role in podosome formation. In particular, ULPA-associated actin polymerization involving p130Cas signaling to the Arp2/3 complex could reflect a key step in podosome formation. Both p130Cas [Brábek et al., 2004] and the Arp2/3 complex [Kaverina et al., 2003] are known to have roles in podosome assembly. Since adhesion formation is associated with intensive actin polymerization [Rid et al., 2005], the rapid assembly of new adhesions and ultimate ULPA formation may also be a consequence of FAK/Src signaling driving actin polymerization.

In summary, our studies on a novel FAK/Src chimeric protein with gain of function properties demonstrate the capacity of FAK-associated Src kinase activity to dramatically impact the adhesion/cytoskeletal network by promoting formation of large stable adhesions that are associated with an actin polymerizing activity. The FAK/Src chimera should be a useful tool for future studies aimed at better understanding the Src-mediated tyrosine phosphorylation events that regulate adhesion/cytoskeleton dynamics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. FAK/Src F-chimera structure and signaling activity. **A:** The FAK/Src F-chimera features a substituted Src kinase domain and mutated autophosphorylation site (F397). The remaining major sites of tyrosine phosphorylation are numbered according to their position in the parental proteins. The FAT, FERM, and proline-rich motifs (PR) are also shown. **B-E:** Immunoblot analysis of total cell lysates of cells expressing WT-FAK, FAK/Src chimeras, or oncogenic Src-F529. FAK/Src chimera variants included “F-chimera” described above, “Y-chimera” with intact Tyr-397 site, and “dead F-chimera” with inactivated Src kinase domain. **B:** Expression and phosphotyrosine profiles. (Top) WT-FAK and chimera expression detected using antibody 331 that recognizes the FAK C-terminal region. (Bottom) Tyrosine-phosphorylated proteins detected using antibody 4G10. Arrows indicate regions of elevated tyrosine phosphorylation in cells expressing the FAK/Src chimeras. Numbers indicate size markers (kDa). **C, top:** p130Cas substrate domain tyrosine phosphorylation detected using a mixture of pCAS-165, pCAS-249, and pCAS-410 antibodies. **C, bottom:** Total p130Cas protein. **D, top:** Paxillin Tyr-118 phosphorylation detected using a phosphospecific antibody. **D, bottom:** Total paxillin protein. **E:** Analysis of FAK/Src chimera activity by detection of the phosphorylated Tyr-418

site in the Src kinase domain. Two different regions of the same blot are shown for detecting the FAK/Src chimera proteins (top), or the oncogenic Src-F529 (bottom).

Fig. 2.

Adhesion-dependent regulation of the FAK/Src F-chimera. Cells expressing either WT-FAK or the FAK/Src F-chimera were analyzed under normal adherent growth conditions (Att), after holding in suspension for 30 min post-trypsinization (Sus), or after plating onto fibronectin-coated dishes for 30 (Fn30) or 60 (Fn60) min. (Top panels) The proteins were immunoprecipitated using FAK C-20 antibody and assessed by immunoblotting with either FAK C-20 antibody to show protein recovery or 4G10 antibody to show changes in overall tyrosine phosphorylation. (Bottom panels) Changes in specific phosphotyrosine sites in the F-chimera were revealed by immunoblotting with phosphospecific antibodies against FAK (Tyr-861, Tyr-407, or Tyr-925) or Src kinase domain (Tyr-418).

Fig. 3.

The FAK/Src F-chimera does not enhance cell proliferation. Growth curves in media containing 10% FBS were established for FAK-null MEFs expressing either WT-FAK, the FAK/Src F-chimera, or oncogenic Src-F529. (Top) Nonadherent growth of cells plated on poly-HEMA. (Bottom) Adherent growth, with serum-containing media replaced on odd-numbered days. Data represent averages from three independent experiments, with error bars indicating standard deviations.

Fig. 4.

The FAK/Src chimera promotes formation of unusual large peripheral adhesions (ULPA) associated with thick peripheral actin stress fibers. Cell immunostaining was performed on cells plated overnight on 10 μ g/ml fibronectin. **A:** FAK/Src F-chimera, WT-FAK, and the "kinase-dead" F-chimera were detected using FAK C-20 antibody followed by FITC-conjugated anti-rabbit IgG. Arrows indicate ULPA in FAK/Src F-chimera cells. **B:** Double staining for adhesions and F-actin in cells expressing GFP only (vector), WT-FAK, FAK/Src F-chimera, or oncogenic Src-F529. Adhesions in the WT-FAK, F-chimera, and vector-only cells and podosomes in Src-F529 cells were visualized with antiphosphotyrosine antibody PY100 followed by Cy2-anti-mouse IgG (green). F-actin was visualized using Alexa 594-

phalloidin (red). **C**: Double staining for tyrosine-phosphorylated cortactin and F-actin in cells expressing the FAK/Src F-chimera. Phosphocortactin was visualized with a phosphospecific antibody against the Tyr-421 site (green) and F-actin was visualized using Alexa 594-phalloidin (red). Yellow indicates colocalization in the merged images of panels B and C. Bars indicate 20 μm .

Fig. 5. ULPA assemble rapidly. FAK/Src F-chimera cells were plated on 10 μ g/ml fibronectin for various periods of time, then double-stained to reveal adhesions (green) and F-actin (red) as in Fig. 4B. **A:** Representative cells showing the four categories of scoring: SA, small punctate adhesions; PA, long adhesions distributed uniformly at the cell periphery; CA, clusters of adhesions with evidence of stress fiber linkage; and ULPA, unusual large peripheral adhesions linked by peripheral stress fibers. Bar indicates 20 μ m. **B:** Plot showing the percentage of cells in each of the four categories at 15, 30, 60, and 120 min after plating. For each time point a total of 250 cells were scored. **C:** Example of a spreading cell expressing WT-FAK.

Fig. 6.

FAK^{-/-} cells expressing the FAK/Src F-chimera have enhanced adhesion strength. Adhesion strength was measured using a hydrodynamic adhesion assay after plating cells on 5 μ m diameter fibronectin-coated islands. **A:** Characteristic adherent cell fraction profile for FAK/Src F-chimera cells after spinning. Results from independent experiments are indicated by the grey circles. The adherent cell fraction is fit with a sigmoid to determine shear stress for 50% detachment, which represents the mean adhesion strength (250 dyn/cm²). **B:** Mean adhesion strength plot (+ S.E.M.) showing two-fold enhancement in adhesion strength for FAK/Src F-chimera compared to WT-FAK (* $P = 0.004$, ANOVA test).

Fig. 7. Chimera cells fail to establish motile polarity. DIC images of representative FAK $-/-$ cells expressing either WT-FAK or the FAK/Src F-chimera, after plating overnight on 1 μ g/ml fibronectin-coated dishes. Bar indicates 10 μ m. Time-lapse images of the cells over a 20 min duration are provided as Supplemental Movies 1 and 2, respectively.

Fig. 8.

ULPA have high integrity and may serve as centers for actin polymerization and membrane protrusion. FAK $-/-$ cells expressing either WT-FAK or the FAK/Src F-chimera were transfected with expression plasmids for GFP-paxillin (green) and DSred-SM22 (red) to reveal adhesions and actin cytoskeleton, respectively, then cultured overnight on 10 μ g/ml fibronectin and visualized by time-lapse fluorescence microscopy. **A:** Motile cell expressing WT-FAK with prominent lamellipodium. **B:** Nonmotile cell expressing the FAK/Src F-chimera. **C:** Higher magnification time sequence of an extending edge of the WT-FAK-expressing cell (boxed region in A). **D:** Higher magnification time sequence of a ULPA (boxed region in B) showing associated dynamic actin polymerization (red dots, arrows) and membrane protrusive activity (dotted lines). Scale bar indicates 10 μ m. Time-lapse sequences for cells in A and B and regions in panels C and D are provided as Supplemental Movies 3-6, respectively.

Fig. 9.

ULPA-associated actin assembly is blocked by a Src-selective kinase inhibitor. Cells were prepared for fluorescence microscopy to visualize adhesions (green) and F-actin (red) dynamics as described in Fig. 8. **A:** Time sequence showing the edge of a cell with prominent ULPA and striking associated actin activity (red dots, arrows). Scale bar indicates 10 μm . The full time-lapse sequence is provided as Supplemental Movie 7. **B:** Effect of Src-selective inhibitor AZD0530 on ULPA-associated actin activity. The average of the normalized DsRed-Sm22 fluorescence intensities from 17 distinct ULPA at each time point are plotted ($\pm$ S.E.M) over a 30 min interval, with 0.1 μM AZD0530 added between the indicated time points. The difference in the mean values obtained for the 10 indicated time points preceding AZD0530 treatment and the 10 indicated time points following AZD0530 treatment is highly significant ($P = 1.1 \times 10^{-12}$; Student *T*-test, paired, two tail).

Fig. 10.

The FAK/Src chimera does not promote the ULPA phenotype in SYF cells. SYF cells expressing either the FAK/Src Fchimera or GFP alone (vector) were plated overnight on 10 $\mu\text{g}/\text{ml}$ fibronectin, then double-stained with antiphosphotyrosine antibody to reveal adhesions (green) and phalloidin to reveal F-actin (red) as in Fig. 4B. Bars indicate 20 μm .