Targeting Pyk2 to β1-Integrin–containing Focal Contacts Rescues Fibronectin-stimulated Signaling and Haptotactic Motility Defects of Focal Adhesion Kinase–null Cells

Candice K. Klingbeil, Christof R. Hauck, Datsun A. Hsia, K.C. Jones, Shannon R. Reider, and David D. Schlaepfer

Department of Immunology, The Scripps Research Institute, La Jolla, California 92037

Abstract. Focal adhesion kinase–null (FAK–/–) fibroblasts exhibit morphological and motility defects that are reversed by focal adhesion kinase (FAK) reexpression. The FAK-related kinase, proline-rich tyrosine kinase 2 (Pyk2), is expressed in FAK–/– cells, yet it exhibits a perinuclear distribution and does not functionally substitute for FAK. Chimeric Pyk2/FAK proteins were created and expressed in FAK–/– cells to determine the impact of Pyk2 localization to focal contacts. Whereas an FAK/Pyk2 COOH-terminal (CT) domain chimera was perinuclear distributed, stable expression of a Pyk2 chimera with the FAK-CT domain (Pyk2/FAK-CT) localized to focal contact sites and enhanced fibronectin (FN)-stimulated haptotactic cell migration equal to FAK-reconstituted cells. These gain-of-function studies show that the FAK-CT domain is necessary but not sufficient to mediate the indirect association of FAK or Pyk2/FAK-CT with a β1-integrin–containing complex. Both FAK and Pyk2/FAK-CT but not Pyk2/FAK-CT S-1034 reconstituted FAK–/– cells, exhibit elevated FN-stimulated extracellular signal–regulated kinase 2 (ERK2) and c-Jun NH2-terminal kinase (JNK) kinase activation. FN-stimulated FAK or Pyk2/FAK-CT activation enhanced both the extent and duration of FN-stimulated ERK2 activity which was necessary for cell motility. Transient overexpression of the FAK-CT but not FAK-CT S-1034 domain inhibited both FN-stimulated ERK2 and JNK activation as well as FN-stimulated motility of Pyk2/FAK-CT reconstituted cells. These gain-of-function studies show that the NH2-terminal and kinase domains of Pyk2 can functionally substitute for FAK in promoting FN-stimulated signaling and motility events when localized to β1-integrin–containing focal contact sites via interactions mediated by the FAK-CT domain.

Key words: FAK • Pyk2 • cell migration • integrins • signaling

Introduction

The regulation of integrin-stimulated (haptotactic) cell migration is complex and not well characterized at the molecular level (Horwitz and Parsons, 1999). Haptotaxis differs from chemotaxis-driven cell migration in that no soluble growth factors are required to generate motility signaling events (Vuori and Ruoslahti, 1999). The focal adhesion kinase (FAK),1 protein tyrosine kinase (PTK), colocalizes with integrin receptors at cell substratum contact sites termed focal adhesions and is activated by integrin binding to extracellular matrix proteins (for review see Schlaepfer et al., 1999). Null mutations of either the murine fibronectin (FN) or FAK genes result in similar embryonic lethal phenotypes, and this genetic connection supports a role for FAK in transducing signals downstream of FN–integrin interactions such as those stimulated by the α5β1-integrin heterodimer (Ilie et al., 1995). Proline-rich tyrosine kinase 2 (Pyk2) is a FAK-related PTK variously called cell adhesion kinase β (CAKβ), related adhesion focal tyrosine kinase (RAFTK), or calcium-dependent PTK (CADTK) (for review see Avraham et al., 2000). Pyk2 and FAK share the same overall structure with a highly conserved central catalytic domain, phosphorylation/docking sites for Src homology (SH)2 domain–containing proteins (Schlaepfer et al., 1994; Lev et al., 1995), proline-rich binding sites for SH3 domain–con-
containing proteins (Astier et al., 1997; Polte and Hanks, 1997), and related NH1-terminal (NT) band 4.1, ezrin, radixin, and moesin (FERM) homology regions (Girault et al., 1999). Whereas in vitro results have suggested that the FAK FERM domain may bind to β-integrin cytoplasmic domain sequences (Schaller et al., 1995), recent studies have shown that the FAK COOH-terminal (CT) but not FAK-NT domain forms a complex with β1-integrins in vivo (Chen et al., 2000; Sieg et al., 2000). A region termed the focal adhesion targeting (FAT) motif within the FAK-CT domain promotes the localization of FAK to sites of integrin receptor clustering (Hildebrand et al., 1993) and is also important for binding the β-integrin-associated protein, talin (Chen et al., 1995). Both FAK and Pyk2 contain conserved sites within their CT domains for binding the α-integrin-associated protein, paxillin (Tachibana et al., 1995; Liu et al., 1999). However, Pyk2 does not strongly localize to focal contact sites in fibroblasts or smooth muscle cells (Sieg et al., 1998; Zheng et al., 1998).

FAK-null (FAK−/−) fibroblasts exhibit a rounded morphology, increased numbers of focal contacts, and are refractory to migratory stimuli (Illic et al., 1995). Pyk2 expression is elevated in FAK−/− cells and contributes to FN-stimulated signaling events (Sieg et al., 1998). However, the level of FN- and serum-stimulated extracellular signal–regulated kinase (ERK)2 activation is lower in FAK−/− cells compared with FAK+/+ cells (Sieg et al., 1998; Renshaw et al., 1999). FN stimulation of fibroblasts promotes c-Jun NT kinase (JNK) kinase cascade activation (Oktay et al., 1999) potentially through the phosphorylation of FAK-associated proteins such as p130Cas (Dolfi et al., 1998; Almeida et al., 2000) or through a complex of paxillin-associated proteins (Igishi et al., 1999). FAK−/− cells do not generate signals leading to JNK activation after FN stimulation, and stable FAK reconstitution rescues these signaling defects (Lebrun et al., 2000).

Pyk2 overexpression can potentiate FN-stimulated ERK2 activation in FAK−/− cells (Sieg et al., 1998), but this does not efficiently promote cell migration (Sieg et al., 1998). In contrast, FAK reexpression restores the characteristic fibrillar shape of primary fibroblasts, promotes enhanced focal contact remodeling events (Ren et al., 2000), and reestablishes normal migratory responses of FAK−/− cells (Sieg et al., 1998, 1999, 2000; Owen et al., 1999). Transient expression of various FAK mutants showed that FAK kinase activity, the Tyr-397/SH2 binding site, and the specific COOH-terminal (CT) domain sequences (Schaller et al., 1995), recent studies support the hypothesis that FAK functions to coordinate the formation of migration-promoting signaling complexes localized at focal contact sites.

Materials and Methods

Materials

FN was purchased from Sigma-Aldrich. Antibodies to the hemagglutinin (HA) (mAb 16B12) and Myc (mAb 9E10) epitope tags were purchased from Covance Research; mAbs to vinculin (clone hVIN-1), talin (clone 8d4), and to the flag epitope (clone M2) were purchased from Sigma-Aldrich; and mAbs to paxillin (clone 349) and p130Cas (clone 21) were purchased from BD PharMingen/Transduction Labs. HA epitope mAb (12CA5), and affinity-purified rabbit polyclonal antibodies to the Pyk2-NT domain (# 5906), to the FAK-NT domain (# 5904), and to the FAK-CT domain (# 5952) were used as described (Sieg et al., 1998). Purified antibodies to the β1-integrin cytoplasmic domain were a generous gift from Martin Schwartz (The Scripps Research Institute). Site- and phospho-specific affinity-purified polyclonal antibodies to Pyk2 pTyr-402 and to Pyk2 pTyr-579 were generous gifts from Erik Schaefer (Biosource International, Camarillo, California). Polyclonal antibodies to JNK1/2 (sc-572) and to ERK2 (e-14) were purchased from Santa Cruz Biotechnology, Inc., and antibodies to active phospho-JNK (pTyr183) were purchased from Promega. mAb to ERK2 (B2B9) was obtained from M. Weber (University of Virginia, Charlottesville, VA) and an mAb to active phospho-ERK (9105) was purchased from New England Biolabs, Inc. Human 293 Phoenix-Eco packaging cells were obtained from Gary Nolan (Stanford University, Palo Alto, CA). The PD98059 inhibitor of the mitogen-activated protein kinase (MEK) was purchased from Calbiochem.

Site-directed Mutagenesis and Cloning

Pyk2/FAK chimeras were created using QuikChange (Strategene) site-directed mutagenesis to introduce restriction sites in both Pyk2 and FAK. Using a restriction site–modified Myc-tagged human Pyk2 cDNA template (Sieg et al., 1998), unique ClaI and AsfIi sites were added at nucleotide positions 1225 and 2044, respectively, to the ATG start site. The introduction of the AsfIi site changed Pyk2 residues Tyr-683 to Leu and Gln-684 to Arg whereas the ClaI site addition did not change the coding sequence for Pyk2 residues Gln-404 and Ile-405. The exchange of the CT domain (residues 683-1009) of Pyk2 with the CT domain (residues 680-1052) of murine FAK (Pyk2/FAK-CT) was accomplished by ligating a BamHI–A13II Pyk2 fragment into the same sites within a restriction site–modified FAK cDNA template (Sieg et al., 1998). Pyk2/FAK-CT has an NT 6×-Myc tag, contains the Pyk2 kinase domain, and has a CT 3×-HA tag. The exchange of the NT domain (residues 1-405) of Pyk2 with the NT domain (residues 1-400) of FAK (FAK/Pyk2-CT) was accomplished ligating a ClaI–XbaI Pyk2 fragment into the same sites of a FAK cDNA. A Myc tag was added to FAK/Pyk2-CT by subcloning into pC5-Myc tag (Sieg et al., 1998). FAK/Pyk2-CT has an NT 6×-Myc tag and contains the Pyk2 kinase domain. Both Pyk2/FAK-CT and FAK/Pyk2-CT were subcloned into the pCDNA3.1 expression vector for transient expression studies. Pyk2/FAK-CT (S-1034) was generated by subcloning an A11I–BamHI fragment from S-1034 FAK-related non kinase (FRNK) into the same sites within Pyk2/FAK-CT. BamHI fragments from pCDNA3.1 Pyk2/FAK-CT or Pyk2/FAK-CT (S-1034) were subcloned into the pRetro expression vector (In vitro) for stable expression studies in FAK−/− cells.

Cells and DNA Constructs

Primary mouse fibroblasts were isolated from day 8 fak−/− or fak−/+ embryos and carried mutations in the p53 gene introduced by crossing mice heterozygous for the FAK and p53 alleles as described (Illic et al., 1995). Cells were maintained in DMEM supplemented with 10% FBS as described (Sieg et al., 1998). Both FAK−/− control cells and DA2 HA-tagged FAK-reconstituted cells were selected for growth in hygromycin as described (Sieg et al., 1999). Human 293T cells were cultured as described (Schlaepfer et al., 1998). Mammalian expression vectors (in pCDNA) for HA-tagged wild-type FAK, F-397 FAK, FAK S-1034, FRNK, FRNK S-1034, and Myc-tagged Pyk2 were used as described (Sieg et al., 1998, 1999).

Transfection

Transient transfection of FAK−/− or Pyk2/FAK-CT expressing cells was performed using Lipofectamine Plus (GIBCO BRL) with 2.5 μg of the indicated constructs exactly as described (Sieg et al., 1999). Stable expres-
sion of Pyk2/FAK-CT or Pyk2/FAK-CT (S-1034) in FAK−/− cells was accomplished by retroviral infection and puromycin selection (2–5 μg/ml). In brief, 293 Phoenix-Eco packaging cells were transfected with pRetro Pyk2/FAK-CT and the medium containing retrovirus was collected after 48 h. FAK−/− cells (passage 14) were treated with 5 μg/ml polybrene and infected for 24 h. Stable Pyk2/FAK-CT expression was detected in a subset of the puromycin-resistant cell population. Clonal Pyk2/FAK-CT (clones CA3 and CB4) or Pyk2/FAK-CT S-1034 (clones SE6 and SX4) expressing cells were obtained by FACSS® sorting of single cells into 96-well plates followed by expansion. Transient transfection of human 293T cells was performed by standard calcium phosphate methods as described (Schlaepfer et al., 1998).

**Haptotaxis Cell Migration Assays**

Cells cotransfected with a pCDNA3-1acZ expression vector (2.5 μg) and the construct of interest (2.5 μg) were used 36 h after transfection and serum starvation (0.5% FBS, overnight). MilliCell chambers (8 μm pores; Millipore) were coated on the underside with FN (10 μg/ml in DMEM for transient transfection assays and 2 μg/ml for assays with stable cell lines) for 2 h at room temperature. Membranes were washed with PBS to remove excess ligand and air dried (30 min). Cells were suspended by limited trypsin-EDTA treatment, washed by centrifugation and resuspension in soybean trypsin inhibitor (0.25 mg/ml in DMEM), suspended in Migration Medium (DMEM with 0.5% BSA), and counted. 104 cells in 0.3 ml were added to each MilliCell chamber, and the units were placed into a 24-well cell culture system containing 0.4 ml Migration Medium, and incubated for 3 h at 37°C. Transfected migratory cells were identified by β-galactosidase (β-gal) activity and counted; assays with stable cell lines were processed by Crystal Violet staining as described (Sieg et al., 1999). Mean values were obtained from three individual chambers for each experimental point per assay. The PD98059 pharmacological MEK inhibitor (10–50 μM) was added to suspended cells and included in the migration assay as indicated. FN stimulation for signaling analyses was performed by replating of serum-starved cells (0.5% FBS for 18 h) onto FN-coated dishes (2 μg/ml in PBS) in Migration Medium as described (Sieg et al., 1998).

**In Vitro Kinase Assays**

Immunoprecipitation (IP) in vitro kinase assays were performed with lysates of cells plated onto FN-coated (10 μg/ml) dishes for 30 min as described previously (Sieg et al., 1998). In brief, 1 μCi of [γ-32P]ATP was added to immunoisolated proteins and incubated at 37°C for 20 min in kinase buffer (20 mM Heps, pH 7.4, 10% glycerol, 10 mM MgCl2, and 150 mM NaCl). Labeled proteins were visualized by autoradiography after SDS-PAGE. To measure ERK2 kinase activity, polyclonal ERK2 IPs were made from 500 μg total cell lysate, washed in Triton Lysis buffer, followed by HNTG buffer (50 mM Heps, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol), and then in ERK2 kinase buffer (25 mM Heps, pH 7.4, 10 mM MgCl2). 2.5 μg of myelin basic protein (MBP) was added to each IP as a substrate. Kinase reactions (~35 μl total vol) were initiated by ATP addition (5 μl final concentration 20 μM ATP, 10 μCi/mmol [γ-32P]ATP), incubated at 32°C for 10 min, and stopped by the addition of 2X SDS-PAGE sample buffer. Transfection of 293T cells with a flag-tagged JNK-1 reporter and measurements of JNK in vitro kinase activity towards GST-C-Jun were performed exactly as described (Almeida et al., 2000).

**Immunofluorescence**

Cells in Migration Medium (104 cells/ml) were plated on FN-coated (10 μg/ml) glass slides for 2 h and samples were fixed in 3.7% parafomaldehyde, permeabilized with ice-cold acetone for 10 min, and processed for either vinculin, HA tag, Pyk2, or Myc tag indirect immunofluorescence staining exactly as described (Sieg et al., 1999). Cells were viewed using an Olympus BX60 epifluorescence microscope and photographed with TMAX 400 film (Eastman Kodak Co.).

**Immunoprecipitation and Immunoblotting**

Cells were solubilized in a modified RIPA lysis buffer containing 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS as described (Schlaepfer et al., 1998). For coimmunoprecipitation studies, cells on a 10-cm dish were lysed with 500 μl RIPA buffer, scraped and collected, diluted with 500 μl HNTG buffer, sheared by passage through a 22 gauge needle, and pre-cleared by incubation with agarose beads. Cell lysates for the IPs contained 1–2 mg total cell protein, which varied due to cell density. Antibodies were incubated with lysates for 2.5 h at 4°C and collected with either Protein A or Protein G Plus (Oncogene Research Products) agarose beads. Antibody-complexed proteins were washed at 4°C with Triton-only lysis buffer, followed by washes with HNTG buffer, and analyzed by SDS-PAGE. Immunoblotting of proteins transferred to polyvinylidene difluoride membranes (Millipore) was performed with either 1 μg/ml monoclonal or a 1:1,000 dilution of polyclonal antibodies (in TBS containing 2% BSA and 0.05% Tween 20) for 2 h at room temperature and visualized by enhanced chemiluminescent methods. Sequential reprobing of membranes was performed as described (Schlaepfer et al., 1998).

**Results**

**Pyk2/FAK Chimeras**

Since FAK but not Pyk2 is capable of rescuing the migration defects of FAK−/− cells (Owen et al., 1999; Sieg et al., 1999), chimeric Pyk2/FAK proteins were created to determine whether the kinase domains of these PTKs were interchangeable and whether functional differences existed in the NT or CT domains of FAK and Pyk2, respectively. The Pyk2/FAK-CT chimera consists of the FAK-CT domain (residues 680 to end) added to the NT and kinase domains of Pyk2 (Fig. 1 A). The FAK/Pyk2-CT chimera consists of the FAK-NT domain (residues 1–400) added to the kinase and CT domains of Pyk2. Transient expression of the chimeric proteins in FAK−/− cells showed that FAK/Pyk2-CT and Pyk2/FAK-CT exhibited equivalent in vitro 32P autophosphorylation activity compared with exogenously expressed FAK and Pyk2 (Fig. 1 B).

FAK−/− cells form an increased number of focal contacts, and the enhanced stability of these contacts is associated with motility defects (Sieg et al., 1999; Ren et al., 2000). Expression of Myc-tagged Pyk2 exhibits a perinuclear distribution in FAK−/− cells and does not significantly colocalize with the focal contact–associated protein vinculin (Fig. 2 A). This distribution of exogenous Pyk2 is similar to endogenous Pyk2 in FAK−/− cells and contrasts to the focal contact localization of FAK when expressed in FAK−/− cells (Sieg et al., 1998, 1999). Interestingly, FAK/Pyk2-CT exhibited a perinuclear distribution whereas Pyk2/FAK-CT localized to FAK−/− focal contact sites (Fig. 2 A). Even though previous studies have shown that exogenous expression of the isolated Pyk2-CT domain localized to focal contacts in chicken embryo fibroblasts (Schaller and Sasaki, 1997), our results demonstrate that the Pyk2-CT and FAK-CT domains are not equivalent. Namely, the FAK-CT domain promotes the redistribution of the Pyk2-NT and kinase domains to focal contacts in FAK−/− cells whereas the Pyk2-CT domain does not function in this manner.

**Pyk2/FAK-CT Promotes FN-stimulated FAK−/− Cell Migration**

To test whether Pyk2/FAK-CT can rescue FAK−/− cell motility defects, Boyden chamber haptotaxis cell migration assays were conducted with immobilized FN as the stimulus. FAK/Pyk2-CT expression and Pyk2 overexpression enhanced FAK−/− cell motility approximately three-fold over cells transfected with vector alone (Fig. 2 B). These motility values are consistent with those previously published for Pyk2 overexpression in FAK−/− cells (Sieg et al., 1998). Significantly, cells transfected with Pyk2/FAK-CT exhibited an ~10-fold increase in FN-stimulated migration over FAK−/− cells transfected with vector alone.
FAK\(^{-/-}\) cell migration enhanced by Pyk2/FAK-CT is similar to the level promoted by transient FAK overexpression (Sieg et al., 1998, 1999). Direct blotting analyses of excess cells not used in the migration assay showed that Pyk2, FAK/Pyk2-CT, and Pyk2/FAK-CT were equivalently overexpressed in the transiently transfected FAK\(^{-/-}\) cells (Fig. 2 B). Expression of these constructs did not affect endogenous levels of \(\beta1\)-integrin expression (Fig. 2 B), and the level of overexpression was \(\sim 10\)-fold greater than endogenous Pyk2 (data not shown). These results show a direct correlation between Pyk2/FAK-CT focal contact localization and the enhancement of FN-stimulated cell migration. In addition, these results demonstrate that the Pyk2-NT and kinase domains can functionally substitute for FAK in promoting FN-stimulated cell migration if targeted to focal contact sites.

Figure 1. Creation of Pyk2 and FAK chimeric proteins. (A) Schematic representation of HA-FAK, Myc-Pyk2, Pyk2/FAK-CT, and FAK/Pyk2-CT. Pyk2/FAK-CT consists of Pyk2 residues 1–682 including the Pyk2 kinase domain and FAK residues 680–1052. FAK/Pyk2-CT consists of FAK residues 1–400 including the intact Src SH2 binding site surrounding FAK Tyr-397 and Pyk2 residues 405–1009 encompassing the Pyk2 kinase and CT domains. The inclusion of either six repeated Myc tags at the NT domain or three repeated HA tags at the CT domain is indicated for each construct. (B) Analysis of FN-stimulated \(^{32}\)P in vitro kinase activity as measured in IPs to the transiently expressed epitope-tagged proteins. Immunoprecipitated proteins were transferred to PVDF membranes and visualized by autoradiography, followed by HA and Myc tag blotting. PBS, paxillin binding sequence.

Figure 2. Transient expression of Pyk2/FAK-CT localizes to focal contacts and promotes FN-stimulated FAK\(^{-/-}\) cell motility. (A) FAK\(^{-/-}\) cells transiently transfected with either pCDNA3 control vector, Pyk2, Pyk2/FAK-CT, or FAK/Pyk2-CT were grown on FN-coated glass slides overnight and stained with an antibody to vinculin or to the Myc epitope tag. Arrows indicate transfected cells. (B) FAK\(^{-/-}\) cells were transiently cotransfected with 2.5 \(\mu\)g pCDNA3.1-lacZ and either control vector, Pyk2, FAK/Pyk2-CT, or Pyk2/FAK-CT (2.5 \(\mu\)g each in pCDNA3.1) and were analyzed for FN-stimulated (10 \(\mu\)g/ml) haptotaxis cell migration (3 h). Transfected migratory cells were identified by \(\beta\)-gal staining and counted. Exogenous protein expression was verified in the excess cells not used in the migration assay by Myc tag blotting. Relative fold induction of migration was calculated by normalizing transfection-induced migration to the pCDNA3.1 control transfected FAK\(^{-/-}\) cells. Data represent the mean ± SD of four independent experiments. Bar, \(\sim 20\) \(\mu\)M.
Stable Pyk2/FAK-CT Expression Promotes Changes in FAK−/− Cell Morphology

To better evaluate Pyk2/FAK-CT function without overexpression, retroviral-mediated infection and puromycin selection were employed to achieve stable Pyk2/FAK-CT expression in FAK−/− cells. In addition, a point mutation (S-1034) was introduced in the FAK-CT domain of Pyk2/FAK-CT which disrupts paxillin binding to FAK in vitro and in vivo (Tachibana et al., 1995; Sieg et al., 1999), and this construct was also stably expressed in FAK−/− cells. Puromycin-resistant pools of Pyk2/FAK-CT infected cells displayed both rounded and spread morphologies (data not shown). Immunofluorescence staining showed that Pyk2/FAK-CT expression was detected in a subset of the total population (data not shown) and therefore, clonal cell lines were isolated for both Pyk2/FAK-CT and Pyk2/FAK-CT S-1034 expressing cells.

To compare the stable expression levels of the various constructs, equal total protein from FAK−/−, FAK+/+, DA2 (FAK reconstituted), Pyk2/FAK-CT expressing (clones CA3 and CB4), or Pyk2/FAK-CT S-1034 (clones SE6 and SX4) was resolved by SDS-PAGE and analyzed by blotting with antibodies either to the FAK-CT domain or the Myc and HA tags on the exogenously expressed proteins (Fig. 3 A). By comparisons of the FAK blot signal obtained between FAK+/+ and DA2 cells, stable HA-FAK expression is at ~10% of endogenous FAK levels whereas p130Cas and the β1-integrin were equivalently expressed in these cells (Fig. 3 A, lanes 2 and 3). By comparisons of the FAK, Myc, and HA tag blots of the cell lysates, Pyk2/FAK-CT expression in the CA3 cells was at ~30% the level of FAK in FAK+/+ cells, whereas Pyk2/FAK-CT in the CB4 cells was expressed equivalently to FAK in FAK+/+ cells (Fig. 3 A, lanes 4 and 5). Similar Pyk2/FAK-CT S-1034 expression levels were found in the lysates of SE6 and SX4 cells compared with Pyk2/FAK-CT in the CA3 and CB4 cells, respectively. Importantly, these results show that none of the exogenously expressed constructs were overexpressed compared with the level of endogenous FAK expression in FAK+/+ cells.

The low levels of stable HA-FAK expression in the DA2 cells are sufficient to promote a reversal of the rounded FAK−/− fibroblast morphology to a normal fibroblast shape (Sieg et al., 1999). Cell shape differences between FAK−/− and FAK-reconstituted cells are most evident on FN replating (Fig. 3 B). Whereas no differences in cell attachment to FN were observed (data not shown), FAK−/− cells remain rounded while FAK-reconstituted DA2 cells were well spread after 2 h on FN (Fig. 3 B). By indirect immunofluorescence staining, endogenous Pyk2 was detected in a punctate perinuclear distribution in the most spread FAK−/− cells, whereas HA-FAK was strongly localized to focal contact regions and cell projections in DA2 cells (Fig. 3 B). Stable Pyk2/FAK-CT expression in CA3 and CB4 cells promoted a normal polarized fibroblast morphology and Pyk2/FAK-CT was strongly localized to peripheral focal contact sites and leading projections (Fig. 3 B). In contrast, Pyk2/FAK-CT S-1034 expression in SX4 cells at levels equivalent to Pyk2/FAK-CT in CB4 cells (Fig. 3 A) enhanced cell spreading compared with FAK−/− cells, but did not result in a polarized cell morphology (Fig. 3 B). Pyk2/FAK-CT S-1034 was primarily perinuclear distributed in SX4 cells and not significantly localized to perimeter focal contacts that were visualized by vinculin staining of SX4 cells (Fig. 3 B). Since the S-1034 mutation in the FAK-CT domain disrupts paxillin binding (Tachibana et al., 1995; Sieg et al., 1999), these results support the conclusion that FAK-CT interactions with paxillin play an important role in FAK localization to focal contact sites.

Stable Expression of Pyk2/FAK-CT but Not the S-1034 Mutant Rescues FAK−/− Haptotactic Motility Defects

To determine whether stable Pyk2/FAK-CT expression and localization to focal contact sites also enhanced FN-stimulated cell migration, comparisons were made between FAK−/−, FAK+/+, DA2, CA3, CB4, SE6, and SX4 cells in modified Boyden chamber motility assays (Fig. 4). When assays were performed only in the presence of 0.5% BSA, only low levels of random cell motility were observed for all cells. When stimulated by immobilized FN on the underside of the Boyden chamber membrane, FAK−/−, DA2, CA3, and CB4 cells all showed a three to four fold increase in cell motility compared with the low level of FN-stimulated FAK−/− cell migration (Fig. 4). These fold differences are less than those stimulated by either FAK or Pyk2/FAK-CT transient overexpression (Fig. 2 B), but are identical to those previously measured for comparisons between FAK−/− and FAK-reconstituted clonal cell lines (Sieg et al., 1999). Importantly, although HA-FAK expression in the DA2 cells and Pyk2/FAK-CT expression in the CA3 cells are less than FAK expression in FAK+/+ cells (Fig. 3 A), both HA-FAK and Pyk2/FAK-CT strongly localized to focal contacts (Fig. 3 B) and fully rescued FAK−/− cell haptotactic motility defects (Fig. 4).

Curiously, previous studies showed that transient FAK S-1034 overexpression in FAK−/− cells could localize to focal contacts and partially function to enhance FN-stimulated cell migration (Sieg et al., 1999). Strikingly, introduction of this S-1034 mutation in Pyk2/FAK-CT resulted in the disruption of Pyk2/FAK-CT focal contact localization (Fig. 3 B) and the inhibition of Pyk2/FAK-CT function in promoting FN-stimulated cell motility as analyzed in SE6 and SX4 cells (Fig. 4). These results highlight complications in the interpretation of results obtained from overexpression studies. Namely, results from the stable cell lines support the conclusion that the S-1034 mutation in the FAK-CT domain disrupts binding interactions needed for high affinity focal contact targeting. However, if expressed at high enough levels, FAK S-1034 could be driven to focal contacts by low affinity interactions and could partially function in motility assays (Sieg et al., 1999).

Paxillin Binding Is Necessary but Not Sufficient to Mediate Pyk2/FAK-CT Linkage to β1-Integrins

The inability of stable Pyk2/FAK-CT S-1034 expression to promote FN-stimulated motility supports the importance of paxillin binding for proper focal contact localization and Pyk2/FAK-CT function. Since paxillin has been shown to bind peptides derived from the β1-integrin cytoplasmic domain (Schaller et al., 1995), communoprecipitation experiments were performed to determine whether paxillin binding was sufficient to promote β1-integrin association with either Pyk2, Pyk2/FAK-CT, or FAK (Fig.
5). FAK and Pyk2/FAK-CT associated with a β1-integrin-containing complex (Fig. 5, lanes 3 and 5), whereas FAK S-1034, which does not bind paxillin, did not detectably associate with the β1-integrin (Fig. 5, lane 6). Interestingly, Myc-tagged Pyk2 associated with paxillin, but did not detectably associate with a β1-integrin–containing complex (Fig. 5, lane 2). All constructs were equivalently active, as they associated with p130Cas which binds to a conserved proline-rich motif located in the FAK and Pyk2-CT domains (Fig. 5). Importantly, the FAK S-1034 results support the conclusion that paxillin binding is necessary for β1-integrin association. However, the fact that Pyk2 associates with paxillin but does not localize to focal contacts (Fig. 2 A) or promote FN-stimulated cell migration (Fig. 2 B) shows that singular paxillin binding to the Pyk2-CT domain is not sufficient to mediate a complex formation with the β1-integrin. Only constructs containing the FAK-CT domain localized to focal contacts in FAK−/− cells and associated with the β1-integrin. These results do not support the proposed role of the FAK-NT domain in mediating either direct or functional interactions with the β1-integrin cytoplasmic domain (Schaller et al., 1995).

Another potential intermediary protein is the β1-integrin-associated structural protein, talin (Chen et al., 1995; Calderwood et al., 1999). Co-IP experiments showed that talin associated with Pyk2/FAK-CT, FAK, and FAK S-1034, but not with Pyk2 (Fig. 5). Only those constructs containing the FAK-CT domain associated with talin, and these results support previous conclusions showing the differential binding of talin to the FAK-CT but not Pyk2-CT domains (Zheng et al., 1998). However, the fact that FAK S-1034 bound strongly to talin but did not associate with a β1-integrin–containing complex (Fig. 5, lane 6) shows that singular talin binding to the FAK-CT domain also is not sufficient to promote a linkage to the β1-integrin. Whereas it is possible that a specific, yet unknown protein binds to the FAK-CT domain to facilitate a linkage to the β1-integrin, it is also plausible that the combined binding of proteins such as paxillin and talin to the FAK-CT domain facilitates β1-integrin association, high affinity focal contact localization, and the ability of FAK or Pyk2/FAK-CT to promote FN-stimulated cell motility.

Figure 3. Stable Pyk2/FAK-CT expression promotes fibrillar morphology of FAK−/− cells. (A) Equal total protein from WCLs of FAK−/−, FAK+/+, HA-FAK reconstituted (DA2), Pyk2/FAK-CT reconstituted (clones CA3 and CB4), or Pyk2/FAK-CT S-1034 reconstituted (clones SE6 and SX4) were resolved by SDS-PAGE and sequentially blotted with antibodies to either the FAK-CT domain, Myc tag, HA tag, p130Cas, or to the cytoplasmic domain of the β1-integrin. Panels are a compilation from two gels. The positions of FAK, HA-FAK, Pyk2/FAK-CT, and a non-specific band are indicated in the FAK-CT blot. (B) FAK−/−, DA2, CA3, CB4, and SX4 cells were plated onto FN-coated (10 μg/ml) glass slides for 2 h in the absence of serum and stained with either affinity-purified antibodies to Pyk2, an mAb to the HA tag, or an mAb to vinculin as indicated (inset). Arrows indicate focal contact staining. Bar, ~20 μM.
Kinase Domain Activation of Pyk2/FAK-CT but Not the S-1034 Mutant after FN Stimulation of Cells

Previous studies have shown that FAK kinase activity is required to promote FN-stimulated motility of FAK−/− cells (Sieg et al., 1998, 1999) and that FAK targeting to focal contacts is an important factor promoting its activation (Shen and Schaller, 1999). To determine whether the Pyk2 kinase domain exhibits integrin-specific activation when stably expressed and targeted to focal contacts in CA3 and CB4 cells, signaling comparisons were performed with FAK−/−, DA2, CA3, CB4, SE6, and SX4 cells (Fig. 6). Antibodies directed to the Pyk2-NT domain and to the FAK-CT domain were combined to immunoprecipitate both endogenous Pyk2 or exogenously expressed HA-FAK in DA2 cells, Pyk2/FAK-CT in CA3 and CB4 cells, or Pyk2/FAK-CT S-1034 in SE6 and SX4 cells. Upon FN stimulation, enhanced tyrosine phosphorylation of Pyk2 in FAK−/− cells (Fig. 6, lane 1), HA-FAK (Fig. 6, lane 2), or Pyk2/FAK-CT (Fig. 6, lanes 3 and 4) occurred compared with no detectable tyrosine phosphorylation of these proteins in suspended cells (data not shown).

Interestingly, Pyk2/FAK-CT S-1034 was not detectably tyrosine phosphorylated in SE6 cells, whereas at higher levels of expression in SX4 cells, Pyk2/FAK-CT S-1034 was strongly tyrosine phosphorylated after FN stimulation (Fig. 6, lanes 5 and 6). It is known that FN-stimulated phosphorylation of FAK Tyr-397 is a key event in promoting Src-family PTK SH2-mediated binding, and FAK phosphorylation within the kinase domain activation loop at Tyr-576 is required for maximal kinase domain activity (Ruest et al., 2000). In Pyk2, these sites are conserved at Tyr-402 and Tyr-579, respectively. Reprobing the IPs with site and phospho-specific antibodies to Pyk2 pY402 and Pyk2 pY579 revealed that Tyr-402 was phosphorylated in both Pyk2/FAK-CT and Pyk2/FAK-CT S-1034 (Fig. 6). Significantly, Tyr-579 within the Pyk2 kinase domain was phosphorylated only in Pyk2/FAK-CT and not in Pyk2/FAK-CT S-1034 (Fig. 6, lanes 3–6). These results support the hypothesis that FN stimulation of cells promotes Pyk2/FAK-CT kinase domain activation only if it is localized to focal contact sites.

Both Pyk2/FAK-CT S-1034 and endogenous Pyk2 exhibited increased phosphorylation at Tyr-402 but not Tyr-579 after FN stimulation of SX4 and FAK−/− cells, respectively (Fig. 6, lanes 1 and 6). Previous studies have shown that FN stimulation of FAK−/− cells promotes the activation of Src-family PTKs and results in the transphosphorylation but not direct activation of Pyk2 (Sieg et al., 1998). The fact that Pyk2/FAK-CT S-1034 exhibits a perinuclear distribution (Fig. 3 B) and is not detectably phosphorylated at Tyr-579 within the kinase domain (Fig. 6) shows that Pyk2/FAK-CT S-1034 is not appropriately activated after FN stimulation of the SX4 cells. These results demonstrate the importance of functional FAK-CT–mediated localization to sites of integrin receptor clustering as a required event in promoting Pyk2/FAK-CT kinase domain activation after FN stimulation of cells.
from FN-stimulated FAK−/−, DA2, CA3, CB4, SE6, and SX4 cells (Fig. 7 A). Consistent with the IP results in Fig. 6, some of the major phosphotyrosine (P.Tyr)-containing proteins visualized were Pyk2, HA-FAK, and Pyk2/FAK-CT as indicated (Fig. 7 A). In FAK−/− cells, Pyk2 and p130Cas within the 116–130-kD size range as well as p52Shc are tyrosine phosphorylated after FN stimulation (Sieg et al., 1998). The increased FN-stimulated tyrosine phosphorylation of p52Shc as well as unidentified proteins at ~44, ~170, and ~250 kD were visualized in lysates of DA2, CA3, and CB4 cells compared with FAK−/− cells (Fig. 7 A, lanes 2–4). Previous studies have shown that both FAK and Src-family PTKs can phosphorylate Shc after FN stimulation of cells and that this is a major signaling pathway promoting FN-stimulated ERK2 activation (Wary et al., 1996; Schlaepfer et al., 1997, 1998).

To determine whether stable FAK or Pyk2/FAK-CT expression enhanced FN-stimulated ERK activation, lysates from FN-stimulated cells were blotted using phospho-specific antibodies that recognize activated ERK kinases (Fig. 7 B). Low levels of active ERK were detected in FN-stimulated FAK−/− cells, whereas elevated levels of active ERK were detected after FN stimulation of DA2, CA3, and CB4 cells (Fig. 7 B). Reprobing of this blot showed equal ERK2 expression in all samples (Fig. 7 B). Interestingly, an intermediate level of active ERK was detected after FN stimulation of SX4 cells compared with FAK−/− and DA2 cells (Fig. 7 B, lane 6). These findings are consistent with FAK and Pyk2/FAK-CT functioning to promote elevated ERK2 activation in DA2, CA3, and CB4 cells and the partial ability of tyrosine-phosphorylated Pyk2/FAK-CT S-1034 to enhance signaling in SX4 cells.

Since these differences in FN-stimulated ERK activation were analyzed only at one time point, lysates from FAK−/−, DA2, and CA3 cells were analyzed for ERK2 activation at various times (10–180 min) after FN replating (Fig. 7 C). By blotting with an mAb to ERK2 that recognizes both the unphosphorylated as well as activated ERK2 (ERK2-P) which migrates slower in high percentage gels, maximal ERK2 activation occurred between 10 and 20 min in FAK−/− cells (Fig. 7 C, lanes 3 and 4). Whereas low levels of ERK2 activation occurred in suspended DA2 and CA3 cells (Fig. 7 C, lane 2), maximal FN-stimulated ERK2 activation was detected between 10 and 20 min in DA2 cells and between 10 and 30 min in CA3 cells. Notably, both the extent and duration of FN-stimulated ERK2 activation were increased in DA2 and CA3 cells (10 to >180 min) compared with the time course of ERK2 activation (10–90 min) in FAK−/− cells (Fig. 7 C). The FN-stimulated ERK2 activation time course in DA2 and CA3 cells is similar to that previously measured in FN-stimulated NIH3T3 fibroblasts (Schlaepfer et al., 1998). Importantly, time course analyses with SX4 cells showed that Pyk2/FAK-CT S-1034 expression moderately enhanced FN-stimulated ERK2 activation between 10 and 20 min, but did not affect the duration of ERK2 activation compared with FAK−/− cells (Fig. 7 C). These results show that focal contact targeting of Pyk2/FAK-CT in CA3 cells was required to promote maximal FN-stimulated ERK2 activation to the same extent as FAK.

To determine whether ERK2 activation was a required component for FAK-mediated haptotactic cell motility,
Boyden chamber motility assays were performed in the presence of PD98059 MEK inhibitor (Fig. 7 D). High numbers of DA2 cells migrated to the immobilized FN stimulus compared with low levels of random cell migration in the presence of BSA only. Treatment of the DA2 cells with the PD98059 inhibitor revealed a dose-dependent inhibition of FN-stimulated DA2 cell motility (Fig. 7 D). Inhibition of DA2 cell motility was >60% at 50 μM PD98058 and control FN replating assays showed a corresponding inhibition of FN-stimulated ERK2 activation at this concentration but no effect on cell attachment to FN (data not shown). Similar findings were obtained with PD98059 treatment of CA3 cells (data not shown). Additional migration analyses using a pharmacological inhibitor to the p38 kinase (SB203580) at similar concentrations did not reveal significant inhibitory effects on DA2 haptotactic migration (data not shown). These results show that activation of the ERK2 pathway positively contributes to haptotactic cell migration responses.

Pyk2/FAK-CT also Rescues FAK−/− Cell Defects in FN-stimulated JNK Activation

Previous studies have shown that transient Pyk2 overexpression in FAK−/− cells enhanced FN-stimulated ERK2 activation at 20 min to the same extent as FAK, but Pyk2 overexpression did not efficiently promote FAK−/− haptotactic motility (Sieg et al., 1998). Since transient overexpression of activated versions of either the Raf or MEK1 kinases also did not efficiently function to enhance FAK−/− haptotactic motility (Sieg et al., 1998), we conclude from the inhibitory PD98059 results (Fig. 7 D) that FAK-mediated ERK2 activation is necessary but not sufficient to promote haptotactic motility. Another FN-stimulated signaling pathway connected to FAK is the activation of JNK
FN-stimulated signaling events leading to JNK activation. Visualization of FAK or Pyk2/FAK-CT is required to generate results support the hypothesis that the focal contact localization in SE6 and SX4 cells (Fig. 8 A, lanes 5 and 6). These results support the hypothesis that the focal contact localization of FAK or Pyk2/FAK-CT is required to generate FN-stimulated signaling events leading to JNK activation.

**FRNK but Not FRNK S-1034 Expression Inhibits FN-stimulated Signaling and Pyk2/FAK-CT Function in Promoting Haptotactic Motility**

Previous studies have shown that expression of the FAK-CT domain (also termed FRNK) expression promotes the displacement of FAK from focal contact sites (Richardson et al., 1997), dephosphorylation of FAK at Tyr-397 (Sieg et al., 1999), and inhibition of FAK-mediated cell motility (Gilmore and Romer, 1996; Richardson et al., 1997; Sieg et al., 1999). To add support to the conclusion that FAK-CT-mediated localization of Pyk2/FAK-CT to focal contacts was required for cell migration, FRNK expression was tested as a competitive inhibitor of FAK (Figs. 8 B and 9 A) or Pyk2/FAK-CT function (Fig. 9 B). To analyze FRNK effects on FN-stimulated JNK activation, human 293T cells were transiently transfected with a flag-tagged JNK-1 reporter along with FRNK or FRNK S-1034 (Fig. 8 B). Flag-tagged JNK-1 exhibited elevated in vitro kinase activity toward GST-c-Jun in lysates from FN-stimulated compared with lysates from suspended cells. Significantly, coexpression of FRNK but not FRNK S-1034 inhibited the activation of JNK-1 after FN stimulation of cells (Fig. 8 B).

Signaling analyses were also performed to determine the effect of FRNK expression on FN-stimulated ERK2 activation (Fig. 9 A). Transient overexpression of FAK but not F-397 FAK enhanced the activation of a flag-tagged ERK2 reporter construct approximately three- to fourfold after FN stimulation compared with control transfected human 293T cells (Fig. 9 A). Integrin receptor engagement was required as FAK overexpression had no effect in transfected and suspended cells (Fig. 9 A), and these results are consistent with studies showing that FN-stimulated FAK phosphorylation at Tyr-397 is required for FAK signaling functions (Schlaepfer and Hunter, 1997). Cotransfection of FRNK with FAK inhibited both FN-stimulated FAK tyrosine phosphorylation and ERK2 activation (Fig. 9 A). Both FRNK and F-397 FAK overexpression resulted in >50% reduction in FN-stimulated ERK2 activity compared with control transfected cells (Fig. 9 A), consistent with similar assays performed in NIH3T3 fibroblasts (Zhao et al., 1998).

To determine if FRNK expression could specifically inhibit Pyk2/FAK-CT function in promoting FAK<sup>−/−</sup> haptotactic motility, FRNK or S-1034 FRNK was transiently expressed with a lacZ reporter in CA3 cells (Fig. 9 B).

Indeed, FRNK expression potently inhibited haptotaxis of CA3 cells without affecting the expression level of Pyk2/FAK-CT. Control FRNK S-1034 expression did not affect FN-stimulated CA3 cell motility responses (Fig. 9 B). Our combined results show the importance of FAK or Pyk2/FAK-CT localization to focal contact sites for the generation of either distinct (JNK activation) or multiple signals (ERK plus JNK activation) after FN stimulation of cells. FRNK expression specifically inhibits FN-stimulated signaling events promoting ERK2 and JNK activation and is a potent inhibitor of both Pyk2/FAK-CT and FAK-stimulated motility events. Since transient expression of activated Rac to facilitate JNK activation did not rescue FAK<sup>−/−</sup> haptotactic motility defects (data not shown), we conclude that FAK and Pyk2/FAK-CT function to promote haptotactic motility through the coordination of multiple signaling events specifically initiated and localized at focal contact sites.
Discussion

Although many of the proposed molecular mechanisms regarding FAK function in promoting cell motility have been based on results generated either by overexpression or dominant-negative type of experiments (Cary and Guan, 1999), the full rescue of FAK−/− cell morphology and motility defects was accomplished by stable reexpression of epitope-tagged FAK at levels much lower than endogenous FAK expression in FAK+/− fibroblasts (Sieg et al., 1999, 2000). Here, we have addressed the issue as to why Pyk2 expression in FAK−/− cells does not compensate for the loss of FAK. To this end, we stably expressed chimeric Pyk2/FAK proteins in FAK−/− cells at levels either less than or equal to FAK expression in FAK+/− cells. We found that FAK-CT domain–mediated targeting of the Pyk2 kinase and NT domains to β1-integrin–containing focal contact sites rescued morphological, FN-stimulated signaling, and haptotactic motility defects of FAK−/− cells.

Stable Pyk2/FAK-CT Expression Rescues FAK−/− Morphological Defects

Endogenous and exogenously expressed Pyk2 is perinuclear distributed in FAK−/− cells, whereas the Pyk2-NT and kinase domains fused to the FAK-CT domain as a chimeric protein localized to focal contact sites and promoted FN-stimulated cell motility equivalent to FAK. FAK−/− cells exhibit an overabundance of cortical actin structures, a greater number of perimeter focal contact sites, and are unable to readily assume a pointed or elongated morphology (Sieg et al., 1999). Stable FAK reexpression results in the dynamic rearrangement of cortical and filamentous actin structures leading to enhanced cell spreading. One target for these FAK-mediated effects is the small GTPase Rho, the activity of which is transiently inhibited when cells are plated onto FN (Ren et al., 1999). The inhibition of Rho activity may alleviate contractile forces that would otherwise impede cell protrusive activity at the leading edge of migrating cells. Recent studies have shown that integrin engagement suppresses RhoA activity in part through Src-family PTK-mediated phosphorylation and activation of p190RhoGAP (Arthur et al., 2000). However, the proposed mechanism of Rho regulation is complicated by the fact that Src-family PTK activity is elevated upon FN stimulation of FAK−/− cells (Sieg et al., 1998) and these cells fail to transiently inhibit Rho activity when plated onto FN (Ren et al., 2000).

Stable FAK reconstitution restores the normal FN-stimulated regulation of Rho activity and also promotes an enhanced rate of focal contact turnover (Ren et al., 2000). Although the exact mechanism(s) of FAK-mediated Rho inhibition are not known, the targeting of active Src to focal contact sites has been shown to promote the turnover of these structures during cell motility (Fincham and Frame, 1998). It is the FAK-mediated recruitment of Src-family PTKs into a signaling complex that is an important event promoting FN-stimulated cell migration (Sieg et al., 1999). Significantly, we found that the FAK-CT–mediated targeting of the Pyk2 kinase and NT domains (Pyk2/FAK-CT) to focal contact sites in CA3 and CB4 cells is sufficient to promote cell protrusive activity, focal contact turnover, and normal levels of FN-stimulated cell migration equivalent to FAK-reconstituted cells. Importantly, exogenous expression of the FAK-CT domain alone in FAK−/− cells does not promote these changes (Sieg et al., 1999). Whereas other studies have found that Pyk2 and FAK may promote differential downstream signaling events (Schaller and Sasaki, 1997; Xiong and Parsons, 1997; Zhao et al., 2000), our find-
ings show that conserved elements within Pyk2-NT and kinase domains can functionally substitute for FAK in promoting FN-stimulated cell motility and that it is the intracellular localization of Pyk2 or FAK that may determine signaling specificity and biological function.

**FAK-CT Connections to Focal Contacts and to a β1-Integrin–containing Complex**

The FAK-CT domain promotes the redistribution of the Pyk2-NT domain to focal contacts in FAK−/− cells, whereas the Pyk2-CT domain does not share this function. Introduction of a point mutation that disrupted paxillin binding to the FAK-CT domain (S-1034) (Tachibana et al., 1995; Sieg et al., 1999) prevented Pyk2/FAK-CT S-1034 localization to focal contact sites, inhibited the FN-stimulated phosphorylation of Tyr-579 within the Pyk2 kinase domain, and inactivated Pyk2/FAK-CT function in promoting haptotactic cell motility. Since FAK reconstitution of FAK−/− cells does not promote changes in the focal contact distribution of proteins such as vinculin, talin, or paxillin (Sieg et al., 1999), our results support the hypothesis that paxillin binding to the FAK-CT domain functions to link FAK to integrins and to enhance FAK and/or chimeric Pyk2/FAK-CT kinase domain activation after FN stimulation of cells.

This view of a FAK and paxillin linkage differs from the proposed and controversial effector role for paxillin tyrosine phosphorylation in promoting cell motility (Richardson et al., 1997; Petit et al., 2000; Yano et al., 2000). Notwithstanding, our results also do not support paxillin as the sole determinant in mediating focal contact localization and β1-integrin association through the FAK-CT domain. This is due to the fact that paxillin binds to the Pyk2-CT domain (Salgia et al., 1996; Li and Earp, 1997), yet Pyk2 does not strongly localize to focal contact sites and does not detectably associate with a β1-integrin–containing complex in lysates from FAK−/− cells. Although our results with Pyk2/FAK-CT S-1034 showed that paxillin association was necessary for FN-stimulated Pyk2/FAK-CT in vivo biological activity, this singular linkage is not sufficient to account for focal contact targeting and cell motility responses.

What may be the mechanism of FAK-CT domain–mediated targeting to a β1-integrin–containing focal contact complex? It is likely that FAK is targeted to these sites through the stabilization of low affinity interactions. Determinations of the important linkages can be complicated by the fact that transient overexpression can drive paxillin binding mutants of FAK to focal contact sites (Sieg et al., 1999), whereas we found that similar mutants stably expressed in FAK−/− cells at lower levels are perinuclear localized. Previous studies have hypothesized that talin binding provides an integrin-specific link mediating FAK targeting and activation (Zheng et al., 1998). Although we found that constructs containing the FAK-CT but not Pyk2-CT domain associated with talin in vivo, FAK S-1034 also showed strong talin binding yet no detectable association with a β1-integrin–containing complex. Although it is possible that a specific, yet unknown protein binds to the FAK-CT domain to facilitate a linkage to the β1-integrin, it is also plausible that the combined binding of proteins such as paxillin and talin to the FAK-CT domain facilitates β1-integrin association, high affinity focal contact localization, and the ability of FAK or Pyk2/FAK-CT to promote FN-stimulated cell motility. This conclusion is supported by the fact that either the lack of talin association to Pyk2 or the lack of paxillin binding to FAK S-1034 disrupts the linkage to the β1-integrin.

**FAK and Pyk2/FAK-CT Connections to Downstream Signaling Pathways Promoting Cell Motility**

The mechanism(s) through which FAK promotes integrin-stimulated signaling events to targets such as the ERK2 (Zhao et al., 1998; Schlaper et al., 1999) or JNK (Okty et al., 1999; Almeida et al., 2000) is mired in controversy (Giancotti and Ruoslahti, 1999; Zhao et al., 2000). This is due to the fact that many results are based on either overexpression analyses or effects obtained by expression of dominant-negative inhibitors. Importantly, recent studies using primary fibroblasts from knockout mice have shown that neither FAK (Sieg et al., 1998) nor important adaptor proteins such as Shc (Lai and Pawson, 2000) are essential for FN-stimulated signaling promoting ERK2 activation. Nonetheless, FAK−/− cells exhibit reduced FN-stimulated ERK2 activation compared with FAK+/+ cells (Sieg et al., 1998; Renshaw et al., 1999) and FN stimulation of FAK−/− cells does not result in detectable JNK activation (Lebrun et al., 2000). Here we show that stable FAK and Pyk2/FAK-CT expression in FAK−/− cells restored FN-stimulated activation of both ERK and JNK kinase cascades to normal levels. Importantly, these gain-of-function results were obtained from DA2 and CA3 cells where HA-FAK and Pyk2/FAK-CT expression are at levels significantly less than FAK in FAK+/+ cells.

We show that the extent and duration of FN-stimulated ERK2 activation are increased by stable FAK and Pyk2/FAK-CT expression in FAK−/− cells and that pharmacological inhibition of ERK2 activity prevents haptotactic motility. Targets for activated ERK2 involved in promoting cell motility include myosin light chain kinase, and expression of constitutively activated MEK1 can increase random cell motility (Klemke et al., 1997; Gu et al., 1999). However, expression of activated Raf kinase or MEK1 to facilitate signaling to ERK2 did not result in the rescue of FAK−/− haptotactic defects. We conclude from these results that ERK2 activation is necessary but not sufficient to promote FAK−/− haptotactic motility. Accordingly, we speculate that other signaling connections to FAK and Pyk2/FAK-CT are required to initiate cell migration and to this end, we found that FAK and Pyk2/FAK-CT specifically promoted FN-stimulated JNK activation in FAK−/− cells. Signals leading to JNK activation have been connected to enhanced cell motility (Xia et al., 2000). However, expression of activated Rac to facilitate JNK activation in FAK−/− cells did not result in increased haptotactic motility. From these results, we conclude that FAK and Pyk2/FAK-CT activate multiple signaling pathways required for haptotactic motility and/or these PTKs function to coordinate the formation of migration-promoting signaling complexes localized at focal contact sites.

**Localization of Signaling Complexes Is Important for Function**

Importantly, we showed that focal contact–associated Pyk2/FAK-CT but not perinuclear distributed Pyk2/FAK-CT
S-1034 facilitated and rescued FN-stimulated haptotic motility of FAK−/− cells. It is possible that FAK and Pyk2/FAK-CT serve to coordinate and facilitate the formation of motility-promoting signaling complex(es) at focal contact sites that could also contain a fraction of activated ERK2 and activated JNK at these sites (Almeida et al., 2000; Fincham et al., 2000). Since we have been unsuccessful in visualizing the specific recruitment of either activated ERK or JNK to focal contact sites in FN-stimulated DA2 cells (Hauck, C.R., unpublished results), it is also possible that the focal contact–localized complexes coordinated by FAK or Pyk2/FAK-CT increase the efficiency of FN-stimulated ERK and JNK activation. Nonetheless, FRNK overexpression inhibited both FN-stimulated ERK and JNK activation as well as Pyk2/FAK-CT–stimulated haptotic motility, whereas control expression of FRNK S-1034, which does not strongly localize to focal contact sites, did not inhibit either FN-stimulated signaling or motility.

In conclusion, our results suggest that when targeted to focal contacts, common SH2 or SH3 binding proteins to both FAK and Pyk2 function in a complex to enhance FAK−/− cell motility. Recruitment of common interacting proteins such as Src-family PTKs (Sieg et al., 1999, 2000) and p130Cas (Cary et al., 1998; Klemke et al., 1998) may be the first of several events in the generation of a focal contact–associated signaling complex promoting motility signals. As it has been shown that signals generated by p130Cas tyrosine phosphorylation and ERK2 activation can synergize to enhance cell motility (Cheresh et al., 1999), we speculate that FAK functions upstream of p130Cas and ERK2 to coordinate these motility-promoting signaling events. By characterizing the effects of stable and low level expression of various FAK mutants in the FAK−/− cell background, it should be possible in future studies to demonstrate whether FAK-mediated activation of one or several signaling pathways is needed to promote focal contact remodeling events and efficient haptotic cell migration.

We thank D. Ilic for the FAK−/− cells, E. Schaefer for the phospho-specific antibodies to Pyk2, D. Cheresh for activated Raf and MEK kinases, and G. Bossert for activated Rac, and we appreciate the administrative assistance provided by Amanda Moore.

This work was supported by the National Cancer Institute (CA75240) and in part by grants from the American Cancer Society (RPG-98-109-01-TBE) and the American Heart Association (19750822N) to D.D. Schlaepfer. C.R. Hauck was supported by the Deutsche Forschungsgemeinschaft (HA-2856/2).

References

Almeida, E.A.C., D. Ilic, Q. Han, C.R. Hauck, F. Jin, H. Kawakatsu, D.D. Schlaepfer, and C.H. Damsky. 2000. Matrix survival signaling from fibronectin via FAK to JNK. J. Cell Biol. 149:741–754.

Arthur, W.T., L.A. Petch, and K. Burridge. 2000. Integrin engagement suppresses expression of various FAK mutants in the FAK-ERK2 to coordinate these motility-promoting signaling pathways. J. Biol. Chem. 275:24425–24430.

Chen, L.M., D. Bailey, and C. Fernandez-Valle. 2000. Association of beta 1 integrin with focal adhesion kinase and paxillin in differentiating Schwann cells. J. Neurosci. 20:3776–3784.

Cheresh, D.A., J. Leng, and R.C. Klemke. 1999. Regulation of cell contraction and membrane ruffling by distinct signals in migratory cells. J. Cell Biol. 146:1107–1116.

Dolfi, F., M. Garcia-Guzman, M. Ojaniemi, H. Nakamura, M. Matsuda, and K. Vuori. 1998. The adaptor protein Csk connects multiple cellular stimuli to the JNK signaling pathway. Proc. Natl. Acad. Sci. USA. 95:15394–15399.

Fincham, V.J., and M.C. Frame. 1998. The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility. EMBO J. (Eur. Mol. Biol. Organ.) 17:1. 1811–1821.

Fincham, V.J., M. James, M.C. Frame, and S.J. Winder. 2000. Active ERK2 MAP kinase is targeted to newly formed cell-matrix adhesions by integrin engagement and v-Src. EMBO J. (Eur. Mol. Biol. Organ.) 19:2911–2923.

Giancotti, F.G., and E. Ruoslahti. 1999. Integrin signaling. Science. 285:1028–1032.

Gilmore, A.P., and L.H. Romer. 1996. Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decreases cell motility and proliferation. Mol. Biol. Cell. 7:1209–1224.

Girault, J.A., G. Labesse, J.P. Morphon, and I. Callebaut. 1999. The N-terminus of FAK and JAKs contain divergent band 4.1 domains. Trends Biochem. Sci. 24:527–531.

Gu, J., M. Tamura, R. Pankov, E.H. Danen, T. Takino, K. Matsumoto, and K.M. Yamada. 1999. Shc and FAK differentially regulate cell motility and directionality modulated by PTEN. J. Cell Biol. 146:389–404.

Han, D.C., and J.L. Guan. 1999. Association of focal adhesion kinase with grb7 and its role in cell migration. J. Biol. Chem. 274:24425–24430.

Hildebrand, J.D., M.D. Schaller, and J.T. Parsons. 1993. Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions. J. Cell Biol. 123:993–1005.

Horwitz, A.R., and J.T. Parsons. 1999. Cell migration—moving on. Science. 286:1102–1103.

Ishii, T., S. Fukuhara, V. Patel, B.Z. Katz, K.M. Yamada, and J.S. Gutkind. 1999. Divergent signaling pathways link focal adhesion kinase to mitogen-activated protein kinase cascades. Evidence for a role of paxillin in c-Jun NH2-terminal kinase activation. J. Biol. Chem. 274:30738–30746.

Ilic, D., Y. Furuta, S. Kanazawa, N. Takeda, K. Sobue, N. Nakatsuji, S. No-mura, J. Fujimoto, M. Okada, T. Yamamoto, and S. Aizawa. 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature. 377:539–544.

Klemke, R.L., S. Cai, A.L. Giannini, P.J. Gallagher, P. de Lanerolle, and D.A. Cheresh. 1999. Regulation of cell motility by mitogen-activated protein kinase. J. Cell Biol. 137:481–492.

Klemke, R.L., J. Leng, R. Molander, P.C. Brooks, K. Vuori, and D.A. Cheresh. 1998. Cas/Crk coupling serves as a “molecular switch” for induction of cell migration. J. Cell Biol. 140:961–972.

Lai, K.K., and F. Pawson. 2000. The SHa phosphotyrosine docking protein sensitzes cardiovascular signaling in the mouse embryo. Genes Dev. 14:1132–1145.

Lehmann, P., V. Baron, C.R. Hauck, D.D. Schlaepfer, and E. Van Obberghen. 2000. Cell adhesion and focal adhesion kinase regulate insulin receptor substrate-1 expression. J. Biol. Chem. 275:38371–38377.

Lev, S., H. Moreno, R. Martinez, P. Canoll, E. Peles, J.M. Mussacchio, G.D. Plowman, B. Rudy, and J. Schlessinger. 1995. Protein tyrosine kinase PYK2 involved in calcium-induced regulation of ion channel and MAP kinase functions. Nature. 376:737–745.

Li, X., and H.S. Earp. 1997. Paxillin is tyrosine-phosphorylated by and preferentially associates with the calcium-dependent tyrosine kinase in rat liver epithelial cells. J. Biol. Chem. 272:14341–14348.

Liu, S., S.M. Thomas, D.G. Woodside, D.M. Rose, W.B. Kiooss, M. Pfaff, and M.H. Ginsberg. 1999. Binding of paxillin to alpha4 integrins modifies integrin-dependent biological responses. Nature. 402:676–681.

Manes, S., E. Mira, C. Gomez-Mouton, Z.J. Zhao, R.A. Lacalle, and A.C. Martin. 1998. Concerted activity of tyrosine phosphatase SHP-2 and focal adhesion kinase in regulation of cell motility. Mol. Biol. Cell. 19:3125–3135.

Ohtani, M., K.K. Wary, M. Dans, R.B. Beige, and F.G. Giancotti. 1999. Integrin-mediated activation of focal adhesion kinase is required for signaling to Jun NH2-terminal kinase and progression through the G1 phase of the cell cycle. J. Cell Biol. 145:1461–1469.

Owen, J.D., P.J. Raust, D.W. Fry, and S.K. Hanks. 1999. Induced focal adhesion kinase (FAK) expression in FAK-null cells enhances cell spreading and migration requiring both auto- and activation loop phosphorylation sites and inhibits adhesion-dependent tyrosine phosphorylation of Pyk2. Mol. Cell. Biol. 19:4806–4813.

Petit, V., B. Boyer, D. Lentz, C.E. Turner, J.P. Thiery, and A.M. Valles. 2000. Phosphorylation of tyrosine residues 31 and 118 on paxillin regulates cell migration through an association with CRK in NBT-II cells. J. Cell Biol. 148:957–970.

Plecke, P., and S.K. Hanks. 1999. Protein complexes of focal adhesion kinase (FAK) and Csk-associated substrate (p130Cas) are elevated in cytokine-associated fractions following adhesion and Src transformation. Requirements for...
Src kinase activity and FAK proline-rich motifs. *J. Biol. Chem.* 272:5501–5509.

Renshaw, M.W., L.S. Price, and M.A. Schwartz. 1999. Focal adhesion kinase mediates the integrin signaling requirement for growth factor activation of MAP kinase. *J. Cell Biol.* 147:611–618.

Salgia, R., S. Avraham, E. Pisick, J.L. Li, S. Raja, E.A. Greenfield, M. Sattler, R. Richardson, A., R.K. Malik, J.D. Hildebrand, and J.T. Parsons. 1997. Inhibition of cell spreading by expression of the C-terminal domain of focal adhesion kinase (FAK) is rescued by coexpression of Src or catalytically inactive FAK: a role for paxillin tyrosine phosphorylation. *Mol. Cell. Biol.* 17:6906–6914.

Schaller, M.D., C.A. Otey, J.D. Hildebrand, and J.T. Parsons. 1995. Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *J. Cell Sci.* 113:3673–3678.

Sieg, D.J., K.C. Jones, and T. Hunter. 1998. Pyk2 and Src-family protein-tyrosine kinases compensate for the loss of FAK in fibronectin-stimulated signaling events but Pyk2 does not fully function to enhance FAK cell migration. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:5933–5947.

Schaller, M.D., and T. Sasaki. 1997. Differential signaling by the focal adhesion kinase. *J. Biol. Chem.* 272:25319–25325.

Schaller, M.D., C.A. Otey, J.D. Hildebrand, and J.T. Parsons. 1995. Focal adhesion kinase and paxillin bind peptides mimicking β integrin cytoplasmic domains. *J. Cell Biol.* 130:1181–1187.

Schlaepfer, D.D., and T. Hunter. 1997. FAK overexpression enhances Ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. *J. Biol. Chem.* 272:13189–13195.

Schlaepfer, D.D., S.K. Hans, T. Hunter, and P. van der Geer. 1994. Integrin-mediated signal transduction linked to Ras pathway by Grb2 binding to focal adhesion kinase. *Nature.* 372:788–791.

Schlaepfer, D.D., M.A. Broome, and T. Hunter. 1997. Fibronectin-stimulated signaling from a focal adhesion kinase-ε-Src complex: involvement of the Grb2, p130Cas, and Nck adaptor proteins. *Mol. Cell. Biol.* 17:1702–1713.

Schlaepfer, D.D., K.C. Jones, and T. Hunter. 1998. Multiple Grb2-mediated integrin-stimulated signaling pathways to ERK2/mitogen-activated protein kinase: summation of both c-Src and FAK-initiated tyrosine phosphorylation events. *Mol. Cell. Biol.* 18:2571–2585.

Schlaepfer, D.D., C.R. Hauck, and D.J. Sieg. 1999. Signaling through focal adhesion kinase. *Prog. Biophys. Mol. Biol.* 71:435–478.

Shen, Y., and M.D. Schaller. 1999. Focal adhesion targeting: the critical determinant of FAK regulation and substrate phosphorylation. *Mol. Biol. Cell.* 10:2507–2518.

Taylor, J.M., M.M. Macklem, and J.T. Parsons. 1999. Cytoskeletal changes in nascent and mature focal adhesions and requirement for the autophosphorylation site. *Cell Growth Differ.* 11:41–48.

Taylor, J.M., M.M. Macklem, and J.T. Parsons. 1999. Regulation of the cell cycle by focal adhesion kinase. *Proc. Natl. Acad. Sci. USA.* 96:5243–5248.

Xiong, W.C., and T. Parsons. 1997. Induction of apoptosis after expression of Pyk2, a tyrosine kinase structurally related to focal adhesion kinase. *J. Cell Biol.* 139:529–539.

Yano, H., H. Uchida, T. Iwasaki, M. Mukai, H. Akedo, K. Nakamura, S. Hashimoto, and H. Sabe. 2000. Paxillin alpha and Crk-associated substrate exert opposing effects on cell migration and contact inhibition of growth through tyrosine phosphorylation. *Proc. Natl. Acad. Sci. USA.* 97:9076–9081.

Zhao, J.H., H. Reiske, and J.L. Guan. 1998. Regulation of the cell cycle by focal adhesion kinase. *J. Cell Biol.* 143:1997–2008.