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Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration

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SUMMARY

FAK localizes to sites of transmembrane integrin receptor clustering and facilitates intracellular signaling events. FAK-null (FAK⁻) fibroblasts exhibit a rounded morphology, defects in cell migration, and an elevated number of cell-substratum contact sites. Here we show that stable re-expression of epitope-tagged FAK reversed the morphological defects of the FAK⁻ cells through the dynamic regulation of actin structures and focal contact sites in fibronectin (FN) stimulated cells. FAK re-expressing fibroblasts (clones DA2 and DP3) exhibit a characteristic fibrillar shape and display indistinguishable FN receptor-stimulated migration properties compared to normal fibroblasts. Expression of various FAK mutants in the FAK⁻ cells showed that FAK kinase activity, the Tyr-397/SH2 domain binding site, and the first proline-rich SH3 binding region in the FAK C-terminal domain were individually needed to promote full FAK-mediated FAK⁻ cell migration to FN whereas direct paxillin binding to FAK was not required. Expression of the FAK Phe-397 mutant did not promote FAK⁻ cell migration and overexpression of $p50^{csk}$ in DA2 cells inhibited migration to FN suggesting that Src-family PTKs play important roles in FAK-mediated motility events. Expression of the FAK C-terminal domain, FRNK, promoted FAK dephosphorylation at Tyr-397 and potently blocked FAK-mediated cell migration. This dominant-negative effect of FRNK was reversed by a point mutation (Leu-1034 to Ser) which prevented FRNK localization to focal contact sites. Our results show that FAK functions as a key regulator of fibronectin receptor stimulated cell migration events through the recruitment of both SH2 and SH3 domain-containing signaling proteins to sites of integrin receptor clustering.

Key words: FAK, Fibronectin, Cell migration, Signaling

INTRODUCTION

FAK was originally identified as a non-receptor protein-tyrosine kinase (PTK) localized to focal contact structures. FAK functions as part of a cytoskeletally-associated network of intracellular signaling proteins that are activated by transmembrane integrin receptor clustering (reviewed by Hanks and Polte, 1997). FAK has been shown to facilitate the generation of integrin-stimulated signals to downstream targets such as the ERK2 and JNK/mitogen-activated protein kinase cascades (reviewed by Schlaepfer and Hunter, 1998), to transduce survival signals generated by integrins in adherent cells (Frisch et al., 1996; Ilic et al., 1998), and to play a role in the regulation of cell cycle progression (Zhao et al., 1998).

Genetic support for the role of FAK in integrin-stimulated signaling events comes from results showing that either homozygous deletion of the murine fibronectin (George et al., 1993), β1 integrin (Stephens et al., 1995), or FAK (Ilic et al., 1995) genes result in similar early embryonic lethal phenotypes. Comparable abortive gastrulation events (E8.5) due to defective mesodermal development were observed in both the FAK and fibronectin-null mice. Propagation of FAK-null fibroblasts (FAK⁻) from E8.0 embryos has been accomplished and these cells exhibit migration but not proliferative defects in cell culture (Ilic et al., 1995). The expression of the FAK-related PTK, Pyk2, is elevated in the FAK⁻ cells (Sieg et al., 1998) and normal to elevated tyrosine phosphorylation levels of integrin-associated proteins such as $p130^{Cas}$ (Vuori et al., 1996), paxillin, and cortactin are found in the FAK⁻ cells (Ilic et al., 1995). Although Pyk2 functions in combination with Src-family PTKs to facilitate fibronectin-receptor stimulated Shc tyrosine phosphorylation and ERK2 activation in the FAK⁻ cells, transient Pyk2 overexpression did not reverse the migration defects of these cells whereas transient FAK expression promoted FAK⁻ cell motility (Sieg et al., 1998).

Stable FAK overexpression in Chinese hamster ovary (CHO) cells has been shown to cause enhanced cell migration (Cary et al., 1996, 1998). Both expression of a dominant-negative mutant of FAK termed FRNK (for FAK-related non-kinase domain) (Gilmore and Romer, 1996; Richardson and Parsons, 1996) and studies showing strong correlations between

elevated FAK expression and the increased invasive potential of human tumors (Owens et al., 1995) have provided support for a role for FAK in cell migration events. In either growing, integrin-stimulated, or in migrating cells, FAK is highly tyrosine-phosphorylated at a number of different sites in vivo (Calalb et al., 1995; Schlaepfer and Hunter, 1996). These phosphorylation events are important for the ability of FAK to promote migration since overexpression of the protein-tyrosine phosphatase (PTP) PTEN has been shown to directly dephosphorylate FAK and antagonize FAK-enhanced cell migration events (Tamura et al., 1998, 1999). However, cells containing an N-terminally truncated form of the Shp-2 PTP (Yu et al., 1998) or fibroblasts deficient in PTP-PEST (Angers-Loustau et al., 1999) exhibit elevated FAK tyrosine phosphorylation levels and in addition possess reduced migration capabilities. Therefore, it is believed that repeated cycles of phosphorylation and dephosphorylation may be important factors linking FAK to the promotion of cell motility (reviewed by Schlaepfer et al., 1999).

FAK binds to a number of different signaling proteins via Src homology 2 (SH2) and SH3 recognition sites and FAK-mediated connections with proteins such as paxillin (Richardson et al., 1997), Src-family PTKs (Cary et al., 1996; Fincham and Frame, 1998), $p130^{Cas}$ (Cary et al., 1998; Klemke et al., 1998), Shc (Schlaepfer et al., 1998), the p85 subunit of phosphatidylinositol 3-kinase (Chen et al., 1996), or the Rho GTPase activating protein GRAF (Hildebrand et al., 1996) could link FAK to pro-migratory signaling pathways. However, no clear model has emerged on how FAK functions in combination with these signaling proteins to promote cell migration. The inability of the FAK^- cells to efficiently migrate when presented with a fibronectin (FN) stimulus is believed to be a result of the elevated number and the increased stability of cell-substratum contacts that the FAK^- cells make in culture (Ilic et al., 1995). Here we present results of the stable re-expression of FAK within the FAK^- cells and the rescue of the morphological and integrin-stimulated migration defects of these cells. Our results support a model whereby FAK is involved in the dynamic regulation of actin and focal contact structures and that FAK functions as an integrin-activated 'scaffold' for the recruitment of both SH2 and SH3 domain-containing signaling proteins needed for FN-stimulated fibroblast cell migration events.

MATERIALS AND METHODS

Cells

Primary mouse fibroblasts were isolated from embryonic day 8 $fak^{+/+}$ or $fak^{-/-}$ embryos as described (Ilic et al., 1995). Both the normal $FAK^{+/+}$ and $FAK^{-/-}$ fibroblasts carry mutations in the p53 gene introduced by crossing mice heterozygous for the FAK and p53 alleles as described (Furuta et al., 1995). Clonal populations of early passage $FAK^{-/-}$ or $FAK^{+/+}$ fibroblasts were isolated by dilution plating. $FAK^{-/-}$ clones that grew out of this selection exhibited a rounded morphology as initially characterized for the primary $FAK^{-/-}$ cells (Ilic et al., 1995) whereas $FAK^{+/+}$ clones maintained fibrillar shape. The R6 $FAK^{+/+}$ clone was used as the normal fibroblast control and all cells were maintained on gelatin-coated (0.1% in phosphate-buffered saline, PBS) cell culture dishes in DMEM supplemented with 10% fetal bovine serum (FBS), non-essential amino acids for MEM, sodium pyruvate (1 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), ciprofloxacin (0.02 mg/ml), and G418 (0.5 mg/ml). The $FAK^{-/-}$ clone D cells were transfected with either pcDNA3.1 HA-tagged FAK (Sieg et al., 1998) or the pcDNA3.1 empty vector and selected for growth in hygromycin (200 μg/ml). A pooled population of hygromycin-resistant $FAK^{-/-}$ cells was used as the FAK^- controls. FAK re-expressing hygromycin-resistant cell clones were isolated by light scatter FACS sorting of single cells into 96-well culture dishes. The 293T human kidney epithelial cells were maintained as previously described (Schlaepfer and Hunter, 1997).

Mutagenesis

FAK Ser-1034 site-directed mutagenesis was performed using the QuickChange protocol (Clonetech, La Jolla, CA) with a 5′-GCTGTGGATGCCAAGAATAGTCTCGATGTTATTGATCAAGCA AG-3′ sense primer (antisense not shown) and HA-FAK cloned into pBluescript. Mutants were selected by *Bsm*AI digestion and the integrity of the region was confirmed by DNA sequencing. The cDNA sequence for Ser-1034 FAK was cloned into pcDNA3.1 as a 3.5 kb *Bam*HI/*Xba*I fragment whereas Ser-1034 FRNK was cloned into pcDNA3.1 as a 1.4 kb *Afl*II/*Xba*I fragment. FRNK translation is presumed to start at the Met-691 equivalent site in FAK.

Cell transfection

Triple HA-tagged Phe-397, Arg-454, Ala-712/713 FAK or triple HA-tagged FRNK in pcDNA3 were constructed and used as described (Ilic et al., 1998; Schlaepfer and Hunter, 1996). The pSLX expression vectors for $p50^{csk}$ WT and kinase-inactive $p50^{csk}$ (K222M) were used as described (Sieg et al., 1998). Transient transfections of FAK^- cells (primary passages 8-20) were performed using Lipofectamine Plus (Gibco-BRL, Gaithersberg, MD) as per the manufacturer's instructions. Co-transfection of cells with the addition of pcDNA3-LacZ followed by staining for β-gal activity (after 36 hours) using X-gal as a substrate revealed transfection efficiencies at 30 to 40% for both the FAK^- and FAK^+ cells. Human 293T cells were transfected by standard calcium phosphate precipitation techniques as described (Schlaepfer and Hunter, 1997). Trypan blue exclusion assays demonstrated that all transfected cells utilized were >95% viable.

Antibodies

The clone 12CA5 monoclonal antibody (mAb) to the HA-tag was a generous gift from Jill Meisenhelder (The Salk Institute) and the HA-tag mAb (clone 16B12) was purchased from Babco (Berkeley, CA). Affinity-purified phosphotyrosine-specific polyclonal antibodies to the motifs surrounding the FAK Tyr-397 site (pY397) or the FAK Tyr-577 site (pY577) were kindly provided by Erik Schaefer (QCB Inc., Hopkinton, MA). Anti-P.Tyr (clone 4G10) mAb was purchased from Upstate Biotechnology (Lake Placid, NY) and mAbs to vinculin (clone hVIN-1) and talin (clone 8D4) were purchased from Sigma (St Louis, MO). Monoclonal antibodies to either paxillin (clone 349) or $p130^{Cas}$ (clone 21) were purchased from Transduction Laboratories (Lexington, KY). Affinity-purified polyclonal antibodies to the FAK N-terminal region (A17) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and affinity-purified polyclonal antibodies (#5904) to FAK residues 8 through 27 were produced as described (Sieg et al., 1998).

Cell stimulation with FN or adherence to poly-L-lysine

Replating assays were performed on serum-starved (0.5% FBS for 18 hours) cells harvested by limited trypsin/EDTA treatment as previously described (Sieg et al., 1998). Cells were held in suspension for 1 hour in DMEM containing 0.1% BSA and then plated onto either FN (10 μg/ml) or poly-L-lysine (100 μg/ml) coated plates for 20 minutes prior to cell lysis.

Cell lysis, immunoprecipitation, and immunoblotting

Unless otherwise indicated, cell lysates were made in modified RIPA buffer containing 1% Triton X-100, 1% sodium deoxycholate, and

0.1% SDS as described (Schlaepfer and Hunter, 1996). Antibodies were incubated in the lysates for 4 hours at 4°C, and either collected on Protein A (Repligen, Cambridge, MA) or Protein G-plus (Calbiochem, La Jolla, CA) agarose beads. The precipitated protein complexes were washed at 4°C in Triton-only lysis buffer (modified RIPA without sodium deoxycholate and SDS) followed by washing in HNTG buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) prior to direct analysis by SDS-PAGE. For immunoblotting, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). Blots were incubated with either 1 μg/ml monoclonal, 1:1000 dilution of polyclonal antibodies, or with FAK phosphotyrosine-specific antibodies at 0.5 μg/ml for 2 to 4 hours at RT. Bound primary antibody was visualized by enhanced chemiluminescent detection and subsequent reprobing of membranes was performed as described (Schlaepfer et al., 1998).

Immunofluorescence staining

Serum-starved cells were either plated (DMEM with 0.1% BSA) onto FN-coated (10 μg/ml) glass coverslips for 2 hours or grown (DMEM containing 5% FBS) on FN-coated coverslips overnight prior to fixation with 3.7% paraformaldehyde. Samples were permeabilized with ice-cold acetone for 10 minutes, washed in PBS, and blocked with the avidin-biotin-kit (Vector, Burlingame, CA) for HA-tag-staining (clone 16B12) or for 10 minutes with blocking buffer (PBS, 0.5% BSA, 200 μg/ml ChromaPure goat IgG; Jackson ImmunoResearch Laboratories, West Grove, PA) for vinculin, paxillin, and talin staining. Primary antibodies were diluted in blocking buffer and applied for 1 hour at RT. After three washes in PBS, coverslips were incubated for 45 minutes in blocking buffer containing either biotin-conjugated horse anti-mouse (Vector) for HA-tag staining or FITC-conjugated goat anti-mouse antibodies (Jackson). In some cases, actin was visualized by the addition of rhodamine-phalloidin (Molecular Probes, Eugene, OR). After washing, vinculin/paxillin/talin stained samples were mounted in Vectashield (Vector), whereas HA-tag stained samples were incubated for 30 minutes with FITC-avidin (Vector) before washing and mounting. Control stainings were performed without either primary or secondary antibodies. Samples were viewed with an Olympus BX60 epifluorescence microscope equipped with appropriate filters and images were photographed with Kodak TMAX 400 film, scanned, and reconstructed using Adobe PhotoShop software.

Wound healing assay

Exponentially growing cells (2×10^6) were plated onto rat tail collagen (Boerhinger Mannheim, Indianapolis, IN) coated (10 μg/ml) cell culture plates in complete growth media. After 8 hours, the monolayer of cells were wounded by manual scratching with a pipet tip, washed with PBS, photographed in phase contrast with a Nikon microscope (0 hour point) and placed into complete growth medium. Matched pair marked wound regions were photographed again after 18 hours.

Cell migration assays

The MilliCell modified Boyden chamber (Millipore, Bedford, MA) migration assays were performed as previously described (Sieg et al., 1998). Cells were used 36 hours after transfection with the indicated constructs following overnight serum starvation (0.5% FBS). Chambers for haptotaxis assays were prepared by pre-coating the under surface of the polycarbonate membrane with FN (10 μg/ml in PBS) for 2 hours at 37°C during which time the upper chamber contained 0.05% BSA in DMEM. To initiate migration assays, chambers were washed in PBS and cells (1×10^5 cells in 0.3 ml DMEM with 0.5% BSA) were added to the upper chamber. After 3 hours at 37°C in the absence of serum, the cells on the upper surface of the membrane were removed by a cotton tip applicator and the migratory cells on the lower membrane surface were fixed by methanol/acid treatment and stained with Crystal Violet (0.1% Crystal Violet, 0.1 M borate pH 9.0, 2% EtOH) or analyzed for β-galactosidase (β-gal) activity using X-gal as a substrate. Cell migration values were determined either by elution of the Crystal Violet stain in 10% acetic acid and measuring absorbance at 600 nM or by counting β-gal stained cells (cells/field using a ×40 objective). Each determination represents the average of three individual wells and error bars represent standard deviation (s.d.). By cell counting methods, background levels of cell migration to 0.5% BSA were <1% of values obtained with FN. Data presented are representative of at least three separate experiments.

Statistical analysis

Ordinary one-way analysis of variance (ANOVA) was used to determine the overall significance within data groups. If a significant result was obtained by ANOVA, the Tukey-Kramer multiple comparisons *t*-test was used to determine significance between individual groups.

RESULTS

Stable re-expression of epitope-tagged FAK in FAK⁻ cells re-establishes wild-type fibroblast phenotype

FAK⁻ cells readily proliferate in culture and they exhibit an elevated expression of the FAK-related Pyk2 PTK compared to normal fibroblasts. In contrast to the focal contact localization of FAK, Pyk2 exhibits a peri-nuclear distribution in the FAK⁻ cells (Sieg et al., 1998). Transient overexpression of Pyk2 only weakly enhanced FAK⁻ cell migration whereas transient FAK expression promoted FAK⁻ cell migration to FN equal to normal fibroblasts (Sieg et al., 1998). To test the hypothesis that FAK and Pyk2 have distinct and nonoverlapping functional properties, hygromycin drug selection was used to facilitate the stable re-expression of FAK. The FAK⁻ cells used for these studies were early passage cells clonally selected for rapid growth and displayed a highly rounded phenotype. Hygromycin resistant pools of FAK-transfected cells exhibited low levels of FAK expression and displayed both rounded and spread morphologies (data not shown). After further passage and expansion of this cell pool, only rounded cells were observed. At the same time, detectable FAK re-expression was lost from the pooled population. Therefore, FAK re-expressing cells were obtained by clonal selection and expansion from the initial hygromycin resistant cell pool (Fig. 1). Clonal-derived FAK re-expressing cells grew slower than the hygromycin empty vector transfected control FAK⁻ cells (D. Sieg and D. Schlaepfer, unpublished results) and all identified FAK re-expressing cells exhibited a morphological reversion to a wild-type spread fibroblast phenotype (see Fig. 1C). No significant changes in the level of Pyk2 mRNA expression were detected in the FAK re-expressing cells compared to the FAK⁻ cell clone used in these analyses (data not shown).

No clones were obtained that overexpressed FAK compared to normal fibroblasts ($FAK^{+/+}$). Instead, FAK re-expression was ~50 to 75% of the level of endogenous FAK in normal fibroblasts (Fig. 1A). The FAK construct used for re-expression in the FAK⁻ cells contained a triple C-terminal hemagglutinin (HA) epitope-tag and identification of HA-tagged FAK which exhibited reduced mobility after SDS-PAGE (~120 kDa) was performed by immunoblotting with antibodies (12CA5) to the HA-epitope tag (Fig. 1A). Since it is possible that the addition of this HA-tag to FAK could influence its activity, standard FN

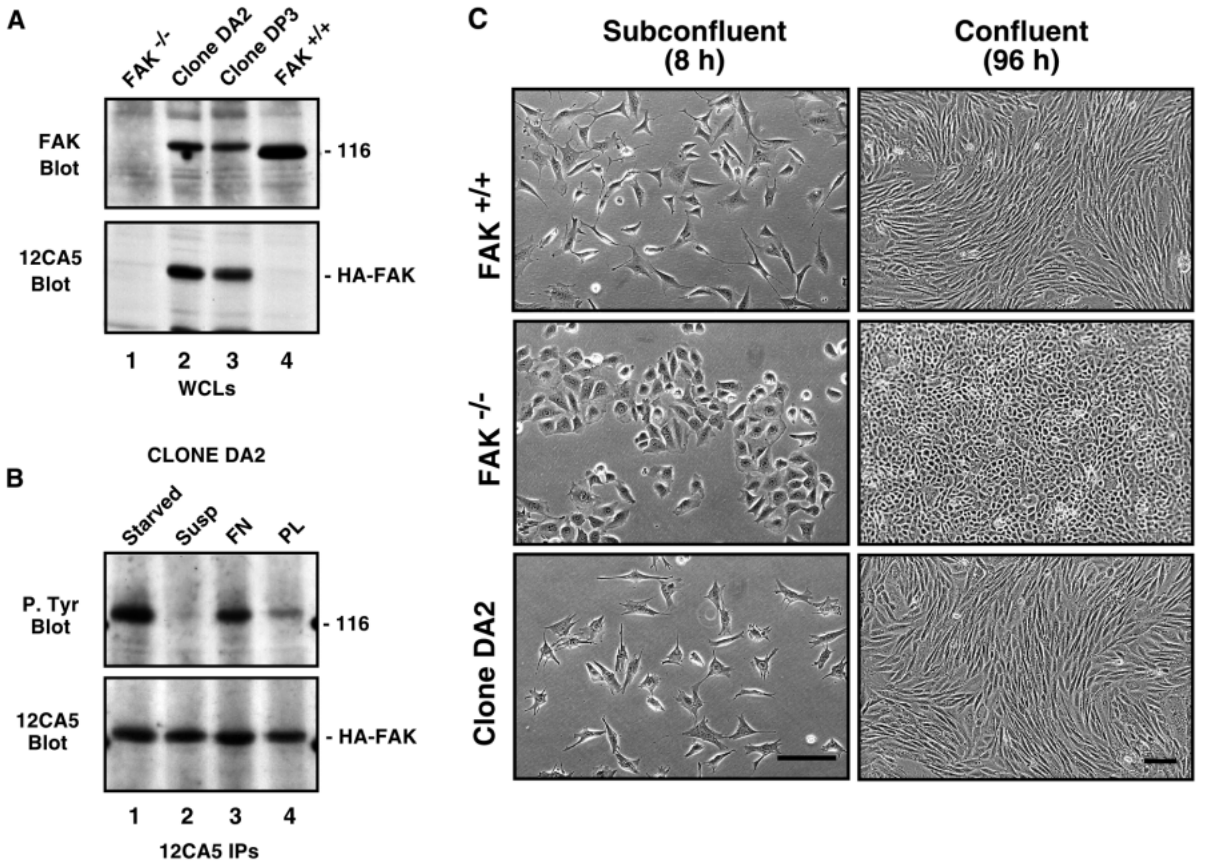

Fig. 1. Stable FAK re-expression promotes FAK$^{-/-}$ fibroblast morphology changes. (A) Whole cell lysates were prepared and 100 μg total cell protein was analyzed by SDS-PAGE from FAK$^{-/-}$ (lane 1), FAK$^{+/+}$ (lane 4), and two FAK re-expressing FAK^{-} clones DA2 (lane 2) and DP3 (lane 3). FAK protein expression was analyzed by blotting with polyclonal anti-FAK (A-17) antibodies and HA-tagged FAK expression was detected by 12CA5 mAb blotting. (B) Lysates were prepared from DA2 cells that were either serum-starved (starved, lane 1), held in suspension for 1 hour (Susp, lane 2), and then replated onto FN-coated (FN, lane 3) or poly-L-lysine-coated (PL, lane 4) dishes for 20 minutes. HA-FAK IPs (12CA5 mAb) were analyzed by anti-P.Tyr blotting and then by anti-HA tag blotting of the same membrane. (C) FAK$^{-/-}$, normal FAK$^{+/+}$, and clone DA2 fibroblasts were plated onto gelatin coated dishes and images were taken by phase contrast microscopy at subconfluent (8 hours) and confluent cell densities (96 hours). Bars, 100 μm.

replating assays were performed with both the DA2 and DP3 clones of the FAK re-expressing cells (Fig. 1B). Cells were either serum starved, held in suspension for 1 hour, or replated onto either FN (10 μg/ml) or poly-L-lysine (100 μg/ml) coated plates for 20 minutes and HA-FAK in cell lysates was analyzed by 12CA5 immunoprecipitation and anti-phosphotyrosine (P.Tyr) blotting (Fig. 1B). HA-FAK was highly tyrosine-phosphorylated in serum starved cells, dephosphorylated in suspended cells, and rapidly re-phosphorylated after FN but not poly-lysine replating. This regulation of HA-FAK tyrosine phosphorylation in clones DA2 (Fig. 1B) and DP3 (data not shown) was identical to the regulation of FAK in normal fibroblasts and epithelial cells (Schlaepfer et al., 1998). These results indicate that the addition of a C-terminal epitope tag to FAK did not adversely affect measurable FAK function in the DA2 fibroblasts.

Phase contrast microscopy revealed that the morphology of the DA2 and DP3 FAK re-expressing clones were identical to that of normal fibroblasts (Fig. 1C and data not shown). When grown at low cell densities, DA2 and DP3 fibroblasts proliferated as separate cells whereas the control hygromycin-resistant FAK^{-} fibroblasts tended to grow in clusters. At higher cell densities, the DA2 and DP3 cells formed a fibrillar pattern characteristic of normal mouse embryo fibroblasts whereas the FAK^{-} cells formed a tight monolayer of rounded cells (Fig. 1C). These results show that HA-tagged FAK expression in the FAK^{-} cells re-established the wild-type fibroblast phenotype.

FAK promotes the dynamic regulation of actin structures and focal contact sites in FN-stimulated fibroblasts

When fibroblasts are plated onto extracellular matrix proteins such as FN in the absence of serum co-factors, they undergo rapid cell spreading and assume a polarized morphology within 2 hours. During this early period of rapid cell spreading, FAK exhibits up to a 10-fold elevated level of measurable in vitro kinase activity in NIH3T3 fibroblasts (Schlaepfer et al., 1998). To study these spreading events, normal FAK$^{+/+}$, FAK^{-}, and the DA2 fibroblasts were either plated onto FN glass coverslips for up to 2 hours in the absence of serum prior to immunofluorescence (IF) analyses (Fig. 2). A time course with normal fibroblasts plated onto FN for 20 minutes to 2 hours was established by double-labeling for vinculin and actin (Fig. 2A). After 20 minutes, the cells that had started to spread

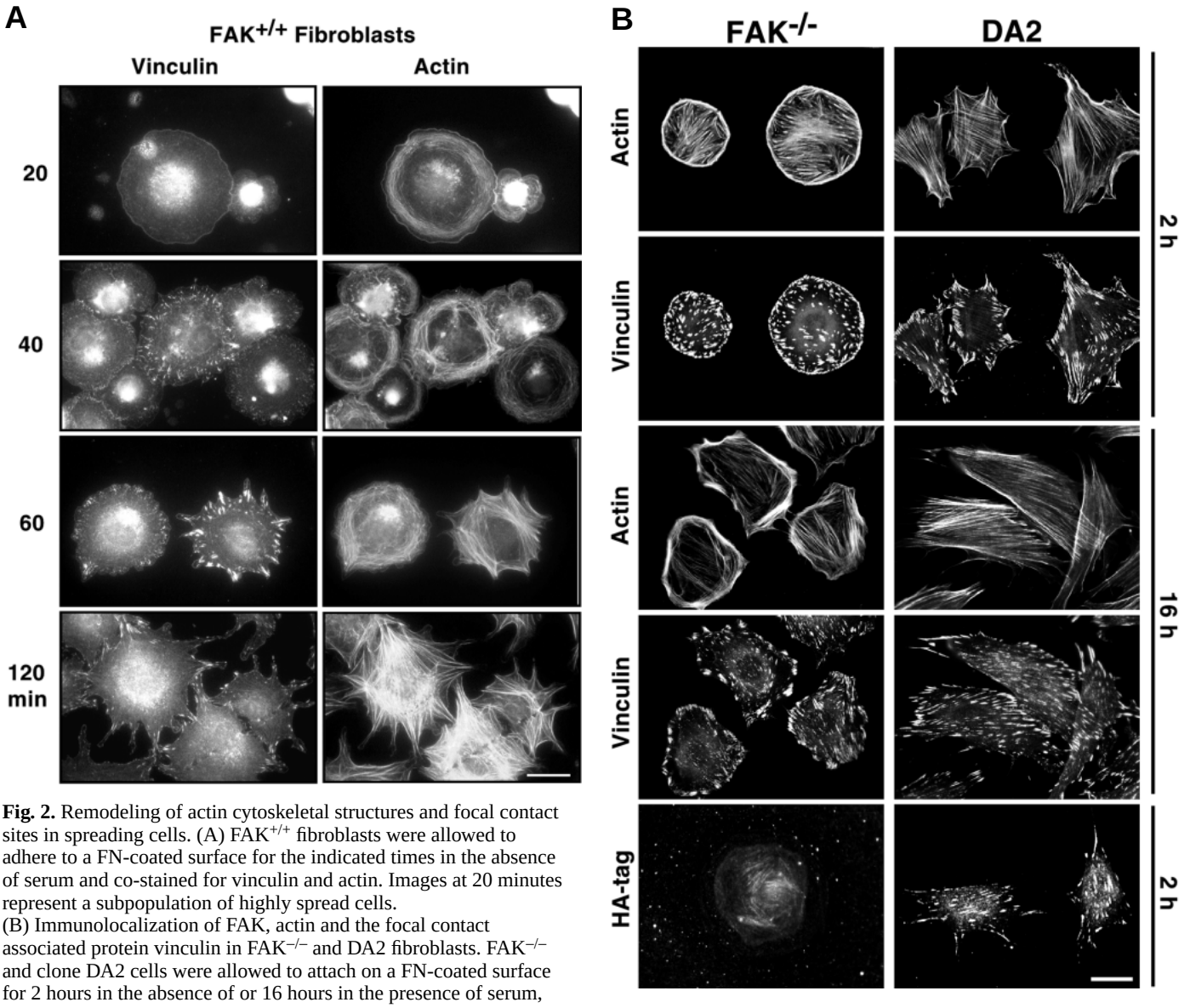

Fig. 2. Remodeling of actin cytoskeletal structures and focal contact sites in spreading cells. (A) $FAK^{+/+}$ fibroblasts were allowed to adhere to a FN-coated surface for the indicated times in the absence of serum and co-stained for vinculin and actin. Images at 20 minutes represent a subpopulation of highly spread cells.
(B) Immunolocalization of FAK, actin and the focal contact associated protein vinculin in $FAK^{-/-}$ and DA2 fibroblasts. $FAK^{-/-}$ and clone DA2 cells were allowed to attach on a FN-coated surface for 2 hours in the absence of or 16 hours in the presence of serum, fixed, permeabilized and stained for vinculin or the HA-tag with monoclonal antibodies and FITC-coupled secondary reagents and for actin with phalloidin-TRITC. The image of $FAK^{-/-}$ cells stained with the HA-tag-antibody (16B12) was taken with an extended exposure time to reveal background staining. Bar, 25 μm.

showed prominent vinculin staining in the center of the cells, whereas cortical actin rings were present around the cell periphery. After 40 to 60 minutes patches of vinculin staining appeared at the cell periphery, whereas vinculin staining in the cell center lessened. By 60 minutes, ~50% of the cells exhibited strong bright patches of vinculin staining in distinct spots at the cell periphery and these sites were associated with the ends of actin stress fibers which had emanated from re-organization of the cortical actin ring structures. The clustered vinculin stained patches most likely represent the formation of focal contact sites. Importantly, it was between 60 minutes and 2 hours that the FN-stimulated FAK^{+} cells exhibited actin stress fiber contractile changes which resulted in a pointed or stellate cell morphology (Fig. 2A).

When replated onto FN-coated slides, the FAK^{-} cells showed similar kinetics of initial cell spreading and actin cortical ring formation (data not shown). Significantly, after 2 hours on FN, the cortical actin ring became very dense around the cell periphery and the cells still exhibited an elevated number of small vinculin-stained patches in the center and in the periphery of the rounded FAK^{-} cells (Fig. 2B). Compared to the DA2 fibroblasts which attained a pointed and elongated morphology after 2 hours on FN, the FAK^{-} cells seemed to display reduced actin stress fiber contractility events and remained in a very static and rounded structure. Since spreading could just be delayed in FAK^{-} cells, cells were stained for actin and vinculin that had been plated onto FN for 16 hours in the presence of 0.5% FBS. After 16 hours, the DA2 cells had spread considerably and occupied a larger surface area than DA2 cells after 2 hours on FN. Actin stress fibers in the DA2 cells were organized in long parallel projections and were equally distributed throughout the cell. In contrast, FAK^{-}

cells still displayed a compact shape after 16 hours and filamentous actin was still enriched around the perimeter of the FAK⁻ cells. After extended incubation on FN, vinculin could be found in distinct spots mainly at the cell periphery co-localizing with the tips of actin stress fibers in both DA2 and FAK⁻ cells (Fig. 2B). Vinculin positive patches in the DA2 cells appeared extended, but showed no gross difference in their distribution compared to the FAK⁻ cells.

IF localization with antibodies to the HA-tag revealed that FAK staining was most prominent at the perimeter regions and at the ends of actin stress fibers or cell extensions in the DA2 cells (Fig. 2B). This distribution of FAK in the DA2 cells is consistent with the observed focal contact association of FAK in normal fibroblasts (Sieg et al., 1998). In contrast, antibodies to the HA-tag did not stain similar structures in FAK⁻ cells (Fig. 2B). These results support the hypothesis that FAK localization to focal contact sites promotes signals leading to actin contractility events and the dynamic regulation of focal contact structures in spreading cells. In the absence of FAK, the cells were unable to readily assume a pointed or elongated morphology.

To determine whether FAK influences cell morphology by altering the distribution of other focal contact-associated proteins, we performed IF analyses for paxillin, talin, and actin distribution in the DA2 and FAK⁻ cells. Again, FAK⁻ cells plated for 2 hours onto FN showed a dense circumferential actin ring (Fig. 3A and B), where numerous small focal contact sites had accumulated containing both paxillin (Fig. 3A) and talin (Fig. 3B). In contrast, DA2 cells started to elongate at this early time point with paxillin (Fig. 3A) and especially talin (Fig. 3B) enriched in the outermost focal contacts. After 16 hours on FN in the presence of serum, DA2 cells attained an extended and pointed cell shape, whereas FAK⁻ cells retained their compact morphology with strong actin staining remaining in the cell periphery (Fig. 3A). Interestingly, both paxillin (Fig. 3A) as well as talin (Fig. 3B) were organized in well defined patches at the end of actin stress fibers in both the FAK⁻ and the DA2 cells. These results show that FAK localization to focal contact sites does not affect the overall distribution of vinculin or the FAK binding proteins paxillin and talin. Instead, FAK appears to influence cell morphology by regulating the turnover rate of focal contact sites and the re-organization of peripheral actin structures.

Re-expression of FAK rescues the FAK⁻ cell migration defect to FN

Since the DA2 and DP3 FAK re-expressing cells were morphologically identical to normal FAK$^{+/+}$ fibroblasts, wound

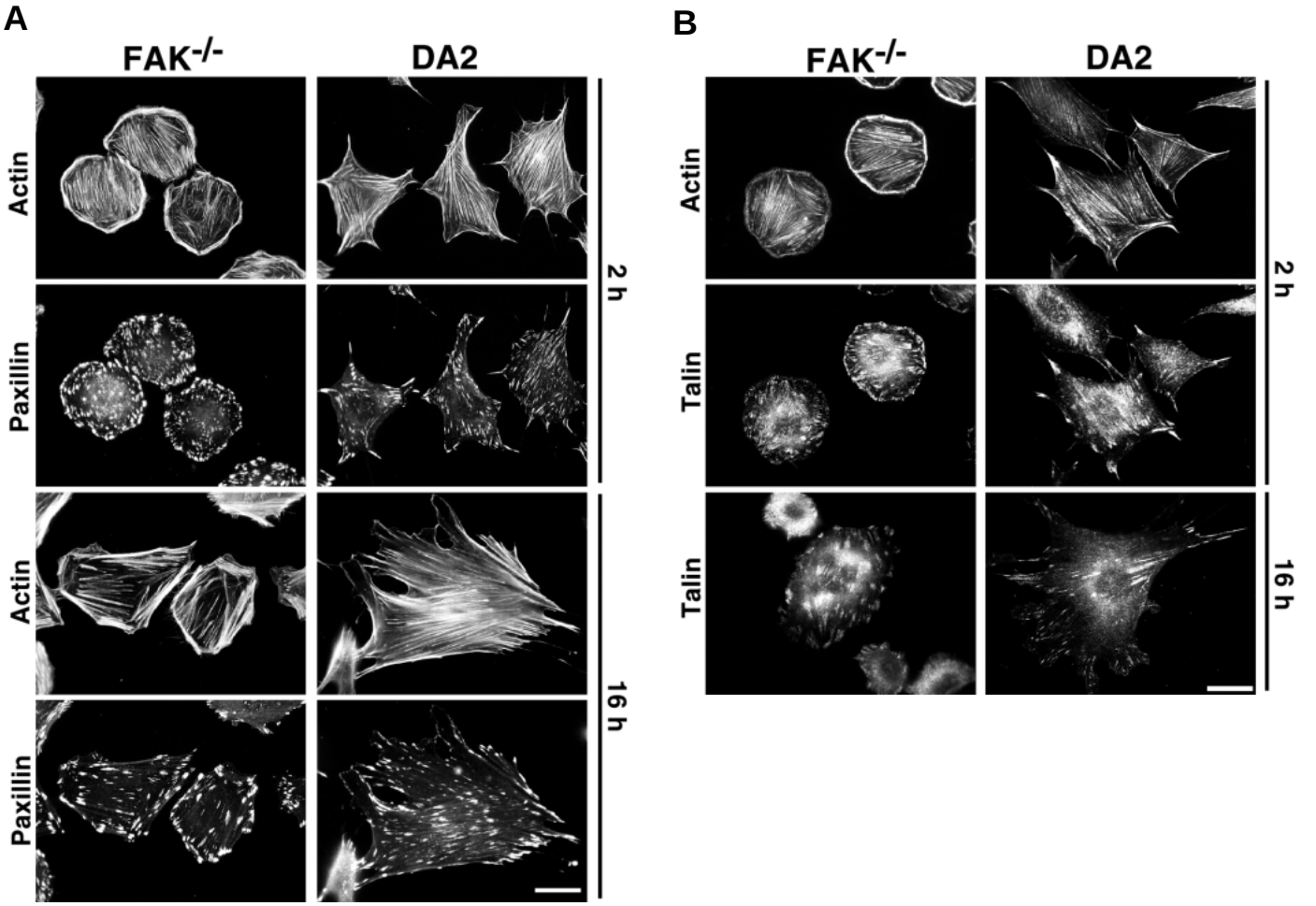

Fig. 3. Paxillin and talin exhibit similar distributions in the FAK$^{-/-}$ and DA2 cells. FAK$^{-/-}$ and DA2 cells were allowed to attach to a FN-coated surface for 2 hours in the absence of or 16 hours in the presence of serum, fixed, permeabilized and stained for paxillin (A) or talin (B) with monoclonal antibodies and FITC-coupled secondary reagents and for actin with phalloidin-TRITC. Bars, 25 μm.

healing scratch assays were performed to determine whether stable HA-FAK expression would also reverse the migration defects of the FAK$^-$ cells (Fig. 4A and data not shown). Equal numbers of growing cells (2×10^6) were plated onto 10 cm dishes and after 8 hours, cells were cleared within a defined area by scratching with a pipet tip, washed with PBS, and allowed to migrate into the cleared area in the presence of serum (Fig. 4A, 0 hours). Eighteen hours after the scratch, the FAK$^-$ cells had become quite dense along the wound edge due to continued cell proliferation, however, they did not significantly migrate into the cleared area or show changes in cell morphology (Fig. 4A, 18 hours). Within the same eighteen hour period, cells from both the normal and DA2 fibroblasts had separated from the monolayer at the wound edges and exhibited intense polarized membrane protrusions characteristic of the leading lamellae of migratory cells (Fig. 4A, 18 hours). In addition, cells within the wound edge of the DA2 fibroblasts also exhibited areas where the cells had become reoriented perpendicular to the scratched region. Significantly, the time course of total wound closure (30 hours) was identical for the normal FAK$^{+/+}$ and DA2 fibroblasts whereas wound closure was observed at ~48 hours for the FAK$^-$ cells and was presumed to be due to continued cell proliferation (data not shown).

To measure quantitatively the effect of FAK on FN-stimulated cell migration events, modified Boyden chamber haptotaxis migration assays were performed with FN (10 μg/ml) for 3 hours in the absence of serum (Fig. 4B). The FAK$^-$, DA2, DP3, and normal fibroblasts equally adhered to the tissue cultured treated membrane and showed only a low level of random cell migration on BSA-coated membranes (Fig. 4B, BSA control). When FN was used to coat (10 μg/ml) the underside of the Boyden chamber membrane, both the FAK$^{+/+}$, DA2, and DP3 fibroblasts readily migrated to the FN surface underside whereas the FAK$^-$ cells did not migrate (Fig. 4B). By cell counting methods, the FN-stimulated level of migration was over 10-fold higher than the BSA controls for both the normal and DA2 fibroblasts (data not shown). Crystal Violet dye elution from the migrating cells yielded increased FAK$^{+/+}$, DA2, and DP3 migration values ~4- to 5-fold higher than controls (Fig. 4B). This fold increase in cell migration obtained for the dye elution method was less than that obtained by cell counting due to non-specific Crystal Violet staining of the BSA-coated control membranes. Importantly, when both sides of the Boyden chamber membrane were coated with FN, neither the normal, DA2, nor DP3 cells migrated to the underside (data not shown). These results show that FAK re-expression did not increase random cell motility. Instead, FAK functions to connect FN receptor integrins to intracellular signaling events promoting cell migration.

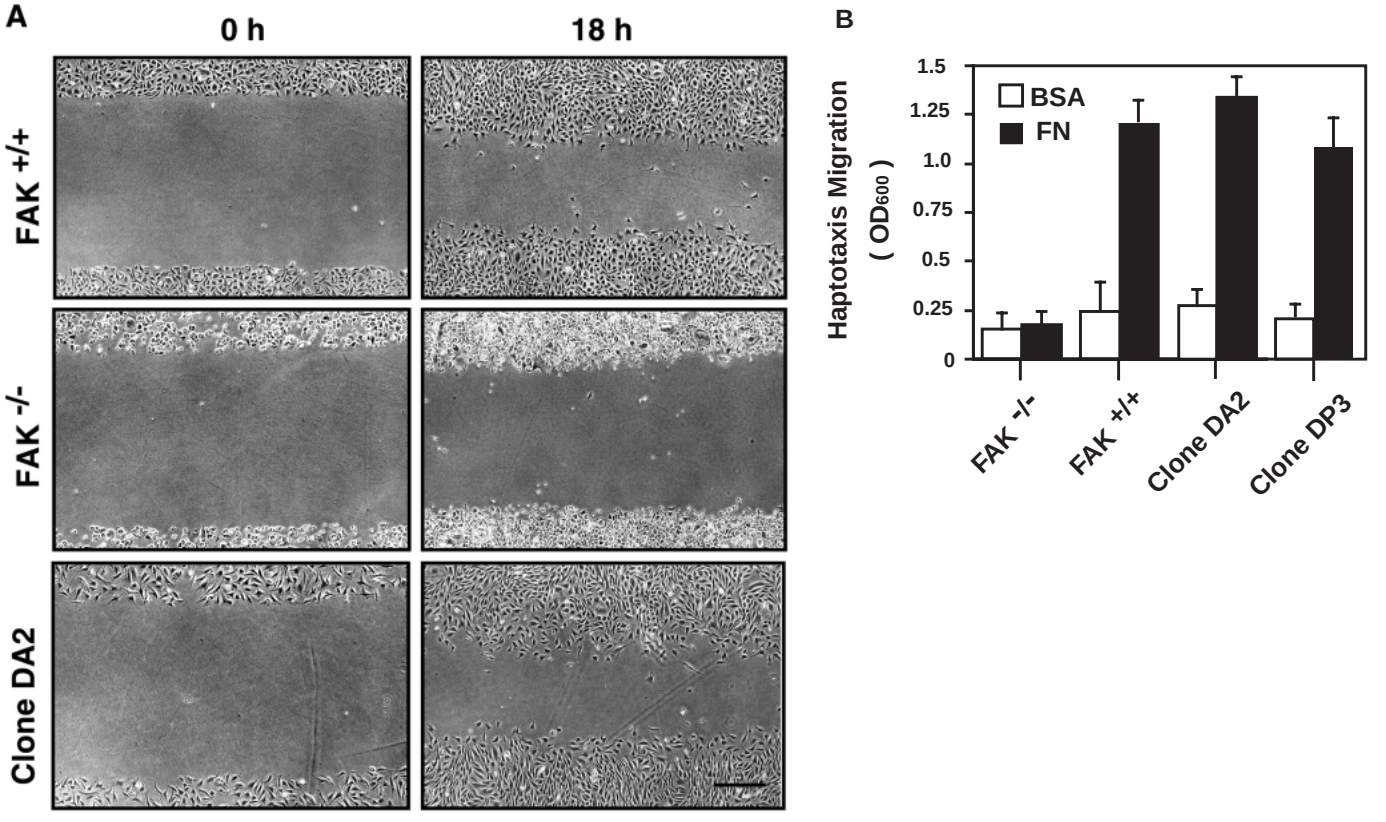

Fig. 4. Stable FAK re-expression rescues FAK$^{-/-}$ cell migration defects. (A) FAK$^{+/+}$, FAK$^{-/-}$ and DA2 fibroblasts were plated onto 10 cm dishes and allowed to grow in the presence of 10% FBS. After 8 hours, a wound was created by scratching with a pipet tip (0 h) and the cells were then allowed to migrate into the wounded area in the presence of 10% FBS. Phase contrast images were taken 18 hours later to assess cell migration. (B) FAK$^{+/+}$, FAK$^{-/-}$, DA2, and DP3 fibroblasts were allowed to migrate for 3 hours towards fibronectin (FN) or BSA (BSA) immobilized on the undersurface of a modified Boyden chamber in a haptotaxis migration assay performed in the absence of serum. The cells that migrated to the underside of the chamber were stained with Crystal Violet, the dye eluted in 10% acetic acid, and the absorbance quantified at 600 nm. Shown is the mean ± standard deviations of triplicates from at least three independent experiments.

FAK kinase activity, the autophosphorylation site, and the first proline-rich motif are all important for cell migration

To determine the mechanism(s) by which FAK re-expression was overcoming the migration defect in the FAK⁻ fibroblasts, FN haptotaxis Boyden chamber migration assays were conducted with FAK⁻ cells transiently transfected with various HA-tagged FAK mutant constructs (Fig. 5). To visualize and score only the FAK-transfected cells in this assay, a β-galactosidase expression vector was co-transfected with the FAK constructs, migratory cells on the membrane underside were stained with X-gal (see Fig. 6B) and only the blue cells were counted (Fig. 5). Wild-type (WT) FAK expression promoted an ~11-fold increase in FN-stimulated cell migration compared to vector control transfected FAK⁻ cells. Expression of the autophosphorylation and SH2 domain binding site mutant of FAK (Phe-397) did not significantly increase FAK⁻ cell

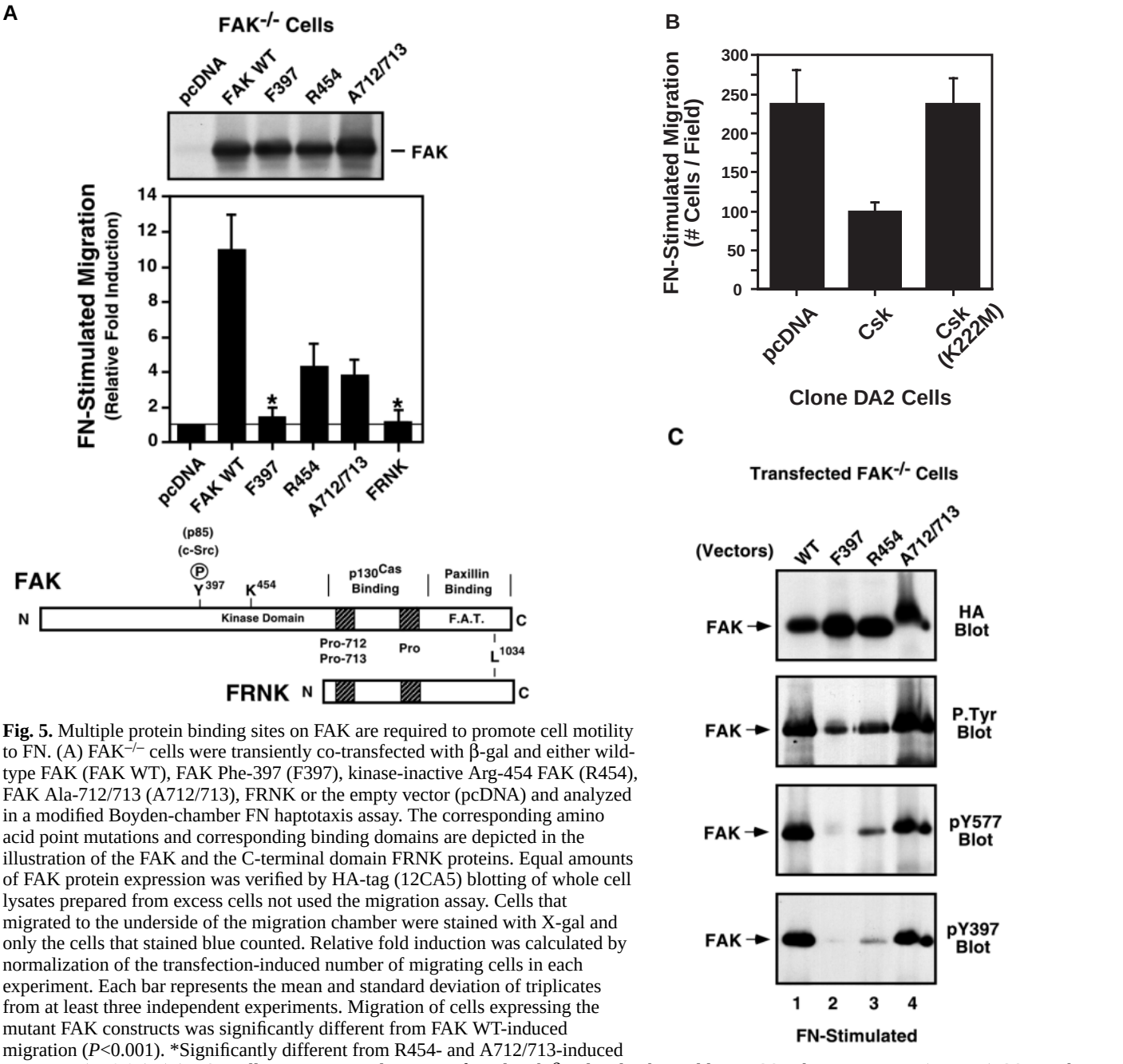

Fig. 5. Multiple protein binding sites on FAK are required to promote cell motility to FN. (A) FAK$^{-/-}$ cells were transiently co-transfected with β-gal and either wild-type FAK (FAK WT), FAK Phe-397 (F397), kinase-inactive Arg-454 FAK (R454), FAK Ala-712/713 (A712/713), FRNK or the empty vector (pcDNA) and analyzed in a modified Boyden-chamber FN haptotaxis assay. The corresponding amino acid point mutations and corresponding binding domains are depicted in the illustration of the FAK and the C-terminal domain FRNK proteins. Equal amounts of FAK protein expression was verified by HA-tag (12CA5) blotting of whole cell lysates prepared from excess cells not used the migration assay. Cells that migrated to the underside of the migration chamber were stained with X-gal and only the cells that stained blue counted. Relative fold induction was calculated by normalization of the transfection-induced number of migrating cells in each experiment. Each bar represents the mean and standard deviation of triplicates from at least three independent experiments. Migration of cells expressing the mutant FAK constructs was significantly different from FAK WT-induced migration (P<0.001). *Significantly different from R454- and A712/713-induced migration (P<0.05). (B) DA2 cells were transiently co-transfected with β-gal and either wild-type CSK, kinase-inactive (K222M) CSK or the empty vector (pcDNA) and analyzed in a modified Boyden-chamber FN haptotaxis assay. Each bar represents the mean and standard deviation of triplicates from at least three independent experiments. (C) FAK$^{-/-}$ cells were transiently transfected with either wild-type FAK (FAK WT), FAK Phe-397 (F397), kinase-inactive Arg-454 FAK (R454) or FAK Ala-712/713 (A712/713). These cells were replated onto FN-coated dishes for 1 hour in the absence of serum. HA-tag IPs were analyzed by blotting with anti-HA tag (16B12), anti-P.Tyr (4G10), or phosphotyrosine-specific antibodies to motifs surrounding FAK Tyr-577 (pY577) or FAK Tyr-397 (pY397).

motility over the low basal level of FN-stimulated FAK$^-$ cell migration (Fig. 5A).

Expression of kinase-inactive FAK (Arg-454) weakly promoted FAK$^-$ cell migration to FN at a level 3-fold less than WT FAK (Fig. 5A), which was a level significantly above Phe-397 FAK. This result differs from those obtained from FAK overexpression studies in CHO cells where kinase-inactive FAK equally enhanced FN-stimulated cell migration compared to WT FAK (Cary et al., 1996). Interestingly, expression of FAK mutated at the first proline-rich site (Ala-712/713), a site previously shown to mediate interactions with SH3 domain-containing proteins such as p130^{Cas} (Polte and Hanks, 1995), also only weakly promoted FAK$^-$ cell migration to FN at a level 3-fold less than WT FAK and at a level significantly above Phe-397 FAK (Fig. 5A). Overexpression of a similar Ala-712/715 FAK mutant in CHO cells did not promote enhanced migration (Cary et al., 1998). The fact that the individual Phe-397, Arg-454, or Ala-712/713 FAK mutations prevented full FAK function in promoting FAK$^-$ cell migration suggests that FN-stimulated increases in FAK kinase activity trigger an important coordination of both SH2 and SH3 domain-containing proteins with FAK. The fact that both Arg-454 and Ala-712/713 FAK promoted significantly more cell migration than Phe-397 FAK suggest that the integrity of the FAK autophosphorylation site is of critical importance for cell motility signaling events.

One important class of signaling proteins which interact with FAK at the Tyr-397 site after FN stimulation of cells are the Src-family PTKs. FN stimulation of fibroblasts has been shown to promote the transient association of c-Src with FAK leading to either Src-mediated phosphorylation of other FAK tyrosine residues creating new SH2 binding sites (Schlaepfer et al., 1994) or Src-mediated phosphorylation of FAK kinase domain residues leading to enhanced FAK catalytic activity (Calalb et al., 1995). To determine the importance of Src-family PTK activity in FAK-mediated cell migration to FN, the DA2 cells were transiently-transfected with p50^{csk} which phosphorylates the C-terminal regulatory site in Src-family PTKs and prevents full Src PTK activity after cell stimulation (Fig. 5B). Compared to the lacZ control vector-transfected DA2 cells, WT p50^{csk} overexpression resulted in a ~50% reduction of FN-stimulated cell migration. This inhibitory effect of p50^{csk} was not observed when the DA2 cells were transfected with a kinase-inactive (K222M) mutant of p50^{csk} (Fig. 5B). These results support the hypothesis that FAK Tyr-397 phosphorylation and recruitment of Src-family PTKs are important components of FN-stimulated and FAK-mediated cell migration events.

To elucidate the mechanisms by which the Arg-454 or Ala-712/713 FAK mutants are defective in promoting efficient cell migration, FAK$^-$ cells were transiently transfected, FN-stimulated, and the various HA-tagged FAK constructs were analyzed by blotting with both anti-P.Tyr and phospho-specific antibodies directed to different FAK tyrosine phosphorylation sites (Fig. 5C). HA-blotting of the FAK IPs showed high level expression of all constructs and anti-P.Tyr blotting revealed that WT and Ala-712/713 FAK exhibited strong phosphotyrosine reactivity after FN stimulation. Both Phe-397 and Arg-454 FAK also were tyrosine phosphorylated but to a lesser extent than WT and Ala-712/713 FAK (Fig. 5C). Phospho-specific antibodies directed either to the Tyr-577 site (pY577) within the FAK kinase domain or to the FAK autophosphorylation site at Tyr-397 (pY397) showed strong reactivity to WT and Ala-712/713 FAK, weak reactivity to Arg-454 FAK, and no reactivity to Phe-397 FAK (Fig. 5C).

Since previous studies have shown that Arg-454 FAK weakly associates with c-Src after FN-stimulation of cells (Schlaepfer and Hunter, 1996), it can be concluded that phosphorylation of Arg-454 FAK at the Tyr-397 site may be able to promote cell migration through similar types of interactions in the FAK$^-$ cells. However, Ala-712/713 FAK is strongly phosphorylated at the Tyr-397 site and within the kinase domain at Tyr-577, yet this mutant only promotes a low level of FAK$^-$ cell migration. One of the simplest interpretations of these results is that both SH2-mediated protein interactions at the FAK Tyr-397 site and SH3-mediated interactions at the FAK Pro-712/713 site are both required for FN-stimulated cell migration events. Mutation of either site in FAK disrupts FAK function in promoting cell motility to FN. These results support the hypothesis that FAK kinase activation after FN stimulation promotes the assembly of a multi-protein complex with FAK that is important for focal contact remodeling and cell motility events.

FRNK localization to focal contacts blocks FAK-mediated cell migration events

FRNK is an autonomously expressed protein (see Fig. 5A) in chicken embryo fibroblasts which has been shown to be a negative regulator of FAK function (Schaller et al., 1993). Exogenous expression of FRNK can inhibit both FN-stimulated cell spreading in chicken embryo fibroblasts (Richardson and Parsons, 1996) and endothelial cell migration in wound healing assays (Gilmore and Romer, 1996). FRNK expression in the FAK$^-$ cells did not promote migration (Fig. 5A) and co-expression of FRNK with FAK potently inhibited FN-stimulated and FAK-mediated cell migration in a dose-dependent manner (Fig. 6A). Immunoblotting whole cell lysates of the FRNK and FAK-transfected cells used in the migration assay showed that the inhibitory effects of FRNK were not due to decreases in FAK protein expression or stability (Fig. 6A).

To determine the sites important for the FRNK-mediated inhibition of cell migration, mutagenesis was performed to disrupt known protein binding sites in FRNK. Introduction of the equivalent Ala-712/713 in the first proline-rich motif of FRNK to disrupt SH3 domain-mediated interactions yielded a FRNK construct that when transfected into cells resulted in a toxic phenotype (data not shown). This result is consistent with previous observations that expression of C-terminal domain FAK constructs can generate signals promoting cell apoptosis (Ilic et al., 1998). Mutation of leucine-1034 to serine (Ser-1034) in FRNK (numbering corresponding to FAK residues) has been shown to disrupt the association of paxillin to glutathione-S-transferase (GST) fusion proteins of FRNK in vitro (Tachibana et al., 1995) and has been shown to block the focal contact localization of micro-injected GST-FRNK in fibroblasts (Tachibana et al., 1995). Introduction of Ser-1034 FRNK into FAK$^-$ or DA2 cells yielded a stable protein without toxic side effects when expressed in cells.

By transient transfection, equivalent expression levels of the FRNK or Ser-1034 FRNK were obtained in DA2 fibroblasts

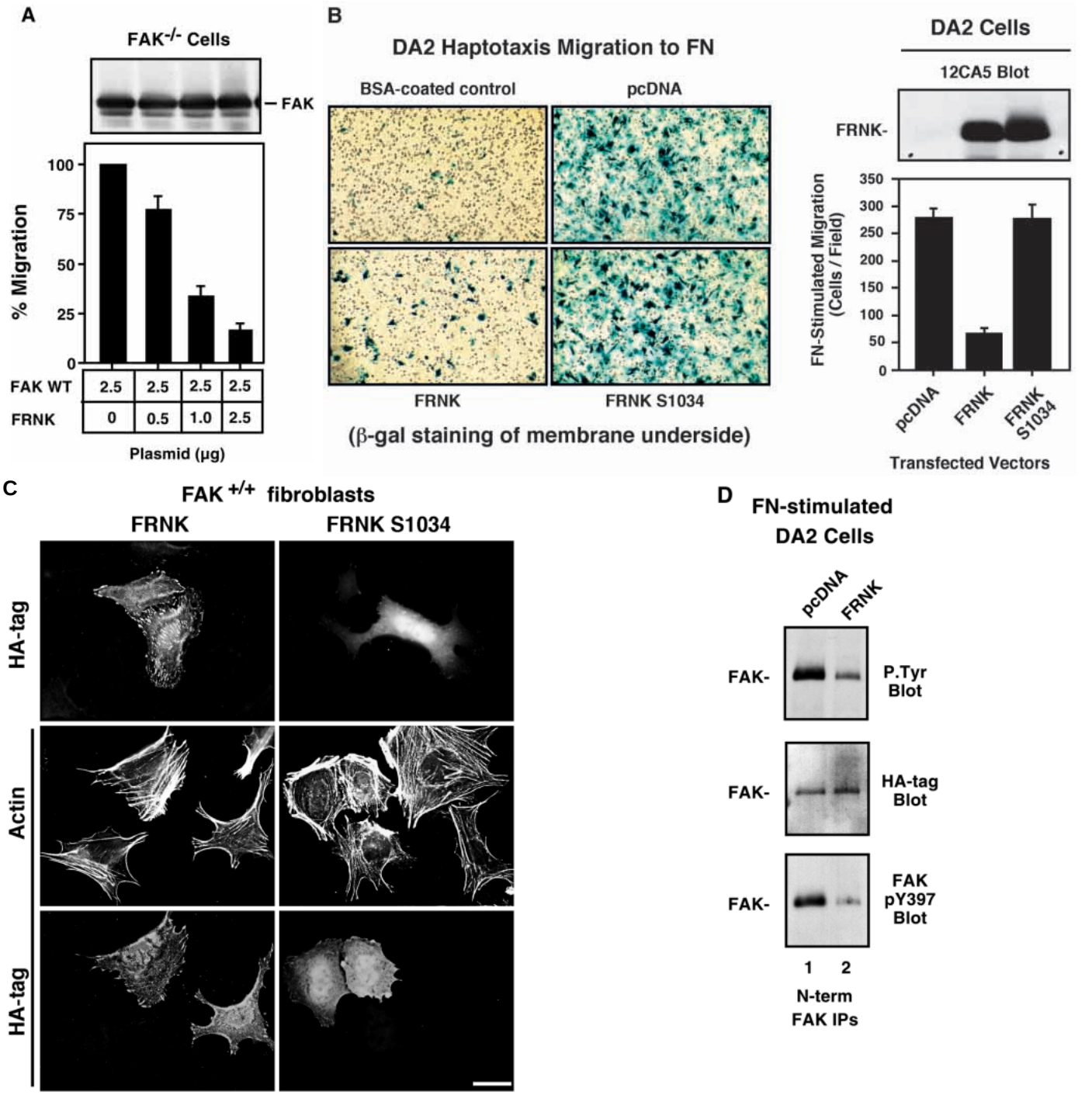

Fig. 6. FRNK expression blocks FAK-enhanced migration and tyrosine phosphorylation. (A) $FAK^{-/-}$ cells were transiently transfected with β-gal, the indicated amounts of FAK and FRNK plasmid DNA and analyzed in a FN haptotaxis Boyden chamber assay. FAK protein expression was verified by HA-tag (12CA5) blotting of whole cell lysates prepared from excess cells not used the migration assay. Shown is the percentage of FAK-promoted migration of the transfected $FAK^{-/-}$ cells towards FN. Each bar represents the mean and standard deviation of triplicates from at least two independent experiments. (B) DA2 cells were transiently transfected with β-gal and either FRNK or Ser-1034 FRNK (S1034) followed by analysis in a FN haptotaxis Boyden chamber assay. FRNK protein expression was verified by HA-tag (12CA5) blotting of whole cell lysates prepared from excess cells not used the migration assay. Images of the X-gal stained cells on the membrane underside of the BSA-coated control or the FN-experimental points are shown. Mean values represent the number of X-gal stained cells that migrated towards FN and error bars are the standard deviation of triplicates from at least two independent experiments. (C) FRNK and Ser-1034 FRNK were detected in transiently transfected $FAK^{+/+}$ cells grown on FN coated coverslips by IF staining with mAb HA-tag antibody (upper panels). Transfected cells were also co-stained with phalloidin-TRITC (middle panels) and HA-tag antibodies (lower panels). Bar, 25 µm. (D) DA2 fibroblasts were transiently transfected with pcDNA control vector (lane 1) or FRNK (lane 2) and replated onto FN-coated dishes for 20 minutes. FAK IPs were made using affinity-purified polyclonal (#5904) antibodies directed to the N-terminal domain of FAK. FAK IPs on the same membrane were sequentially analyzed by anti-P.Tyr blotting, anti-HA-tag blotting, followed by anti-pY397 FAK blotting.

used in a Boyden Chamber FN haptotaxis migration assay (Fig. 6B). Similar to the dose-dependent inhibition of transient FAK-mediated FAK$^-$ cell migration (Fig. 6A), transfection of FRNK into the DA2 fibroblasts potently inhibited cell migration to FN (Fig. 6B). FRNK expression did not noticeably delay either DA2 binding to FN or initial cell spreading events (data not shown). Surprisingly, high levels of Ser-1034 FRNK expression in the DA2 fibroblasts did not effect FAK-mediated cell migration (Fig. 6B). To determine the functional differences between FRNK and Ser-1034 FRNK, IF staining with antibodies to the C-terminal HA-tag antibodies was performed on transiently-transfected normal fibroblasts plated onto FN (Fig. 6C). In FRNK expressing cells, patches of strong HA-staining were detected around the cell perimeter. This staining pattern was similar to that of other focal contact associated proteins.

In contrast to the focal contact distribution of FRNK, Ser-1034 FRNK staining was primarily cytoplasmic and did not strongly co-localize with focal contact sites in the FAK$^{+/+}$ cells (Fig. 6C). Interestingly, in cells co-stained for actin and HA-tag antibodies, high levels of either FRNK or Ser-1034 FRNK did not affect the filamentous actin organization of the normal fibroblasts (Fig. 6C). Thus, while FRNK acts as a potent inhibitor of FAK-mediated fibroblast cell migration, FRNK does not act to inhibit cell binding to FN or act to promote changes in cytoskeletal structures.

The ability of FRNK but not Ser-1034 FRNK to inhibit FAK-mediated cell migration correlated with the strong localization of FRNK to focal contacts. Although technical limitations prevented accurate analyses to determine whether FRNK expression promoted the competitive displacement of FAK from focal contact sites, FRNK expression potently inhibited FAK tyrosine phosphorylation in FN-stimulated DA2 cells (Fig. 6D). In particular, FRNK expression prevented efficient FAK tyrosine phosphorylation at the Tyr-397 autophosphorylation/SH2 binding site (Fig. 6D, pY397). Ser-1034 FRNK expression did not inhibit FAK tyrosine phosphorylation in the DA2 cells (data not shown). These results support the conclusion that the inhibitory effects of FRNK occur through the reduction of FAK tyrosine phosphorylation levels and the prevention of FAK-mediated recruitment of other signaling proteins to focal contact sites.

Paxillin binding to FAK is not required for cell migration

The Ser-1034 mutation in FRNK has been shown to disrupt both paxillin binding and focal contact localization (Tachibana et al., 1995). This point mutation was also introduced into full-length FAK to determine its effects on FAK function in promoting cell motility. Equivalent amounts of either FAK, Ser-1034 FAK, FRNK, or Ser-1034 FRNK were isolated with antibodies to the HA-tag after transient transfection of human 293T cells (Fig. 7A). Immunoblotting with antibodies to paxillin revealed that the Ser-1034 mutation disrupted paxillin association with both FAK and FRNK in vivo (Fig. 7A, lanes 2 and 4). Immunoblotting duplicate HA-tag IPs with antibodies to p130^{Cas} showed that both FAK and FRNK associated with equivalent amounts of p130^{Cas} in vivo (Fig. 7A, lanes 1 and 3). Interestingly, Ser-1034 FAK associated with a greater amount of p130^{Cas} compared to FAK, FRNK and Ser-1034 FRNK (Fig. 7A, lane 2). These results show that FRNK does not act to sequester large amounts of FAK-associated proteins and additionally, that the binding of paxillin and p130^{Cas} to FAK may be mutually exclusive.

Since the Ser-1034 mutation disrupted paxillin binding and blocked the ability of FRNK to inhibit FAK-mediated cell migration events, Ser-1034 FAK was transfected into FAK$^-$ cells and was compared for its ability to promote FN-stimulated cell migration in the haptotaxis Boyden chamber assay (Fig. 7B). Surprisingly, expression of Ser-1034 FAK increased FAK$^-$ cell migration ~6-fold higher than control-transfected cells. Although this level of migration was less than WT FAK, it was greater than the low level of cell migration stimulated by either of the Arg-454 or Ala-712/713 FAK constructs (see Fig. 5). Interestingly, indirect IF analyses of the transfected FAK$^-$ cells revealed that both Ser-1034 FAK and WT FAK localized to focal contact sites (Fig. 7C). In addition to the ability of Ser-1034 FAK to promote migration, FAK$^-$ cells expressing high levels of Ser-1034 FAK showed distinct morphological changes, exhibiting a polarized shape characteristic of the normal and DA2 fibroblasts (Fig. 7C).

These results show that a point mutation at Leu-1034 in FRNK is sufficient to inhibit FRNK binding to paxillin and FRNK localization to focal contacts. This same mutation in FAK disrupts paxillin binding but does not block FAK localization to focal contacts or FAK function in promoting cell migration. Therefore, contrary to conclusions from previous studies (Richardson et al., 1997; Richardson and Parsons, 1996), our results do not support a role for FAK association with paxillin in promoting cell migration events. In addition, although paxillin binding may be necessary for FRNK association with focal contacts (Tachibana et al., 1995), our results with Ser-1034 FAK suggest that paxillin binding to FAK is not an absolute requirement for the focal contact localization of FAK as originally observed (Hildebrand et al., 1995). Instead, increased binding interactions of Ser-1034 FAK with other focal contact-associated proteins such as p130^{Cas} (Fig. 6A), talin (Chen et al., 1995), or FAK N-terminal domain interactions with β-integrin subunits (Schaller et al., 1995) may also promote FAK localization to focal contacts and allow for FAK function in enhancing cell migration.

DISCUSSION

In this study we show that the stable re-expression of epitope-tagged FAK in primary FAK-null fibroblasts promoted the reversion of a rounded FAK$^-$ cell morphology to a elongated and normal fibrillar fibroblast phenotype. The effect of FAK on cell morphology was not due to a detectable redistribution of the focal contact-associated proteins vinculin, paxillin, or talin. Instead, FAK localization to focal contact sites generated signals leading to actin contractility events and the dynamic regulation of focal contact structures as observed by time course vinculin staining studies of FN-stimulated cells. The FAK$^-$ cells exhibited elevated levels of cortical actin structures, a greater number of focal contacts, and were unable to readily assume a pointed or elongated morphology upon FN stimulation. Contrary to initial hypotheses proposing a role for FAK in the formation of focal contacts (Richardson et al., 1997), our results confirm those from the initial knockout studies (Ilic et al., 1995) which concluded that FAK was

involved in the regulation or turnover of focal contact structures during cell migration events.

We show in this study that stable FAK re-expression rescued the migration defects of the FAK⁻ cells. FAK re-expression restored the ability of the FAK⁻ cells to efficiently migrate to a FN stimulus in the absence of serum. In all studies to date, the assessment of the relative contributions of FAK in promoting cytoskeletal changes and cell migration events have been complicated by the presence of endogenous FAK. Our results showing that FAK re-expression in the FAK⁻ cells at levels less than in normal fibroblasts can reverse the morphological defects in these cells and promote cell migration towards FN verifies the role of FAK as a physiological regulator of these events.

Our results with FRNK, showing the competitive inhibition of FAK-mediated cell migration events, do not support a model whereby FRNK may be acting to sequester important FAK-associated proteins. Instead, we show that FRNK localizes to focal contacts and promotes FAK dephosphorylation at the Tyr-397/SH2 binding site. The Ser-1034 mutant of FRNK did not act as an inhibitor of FAK function because it did not strongly localize to focal contacts nor did its expression promote FAK

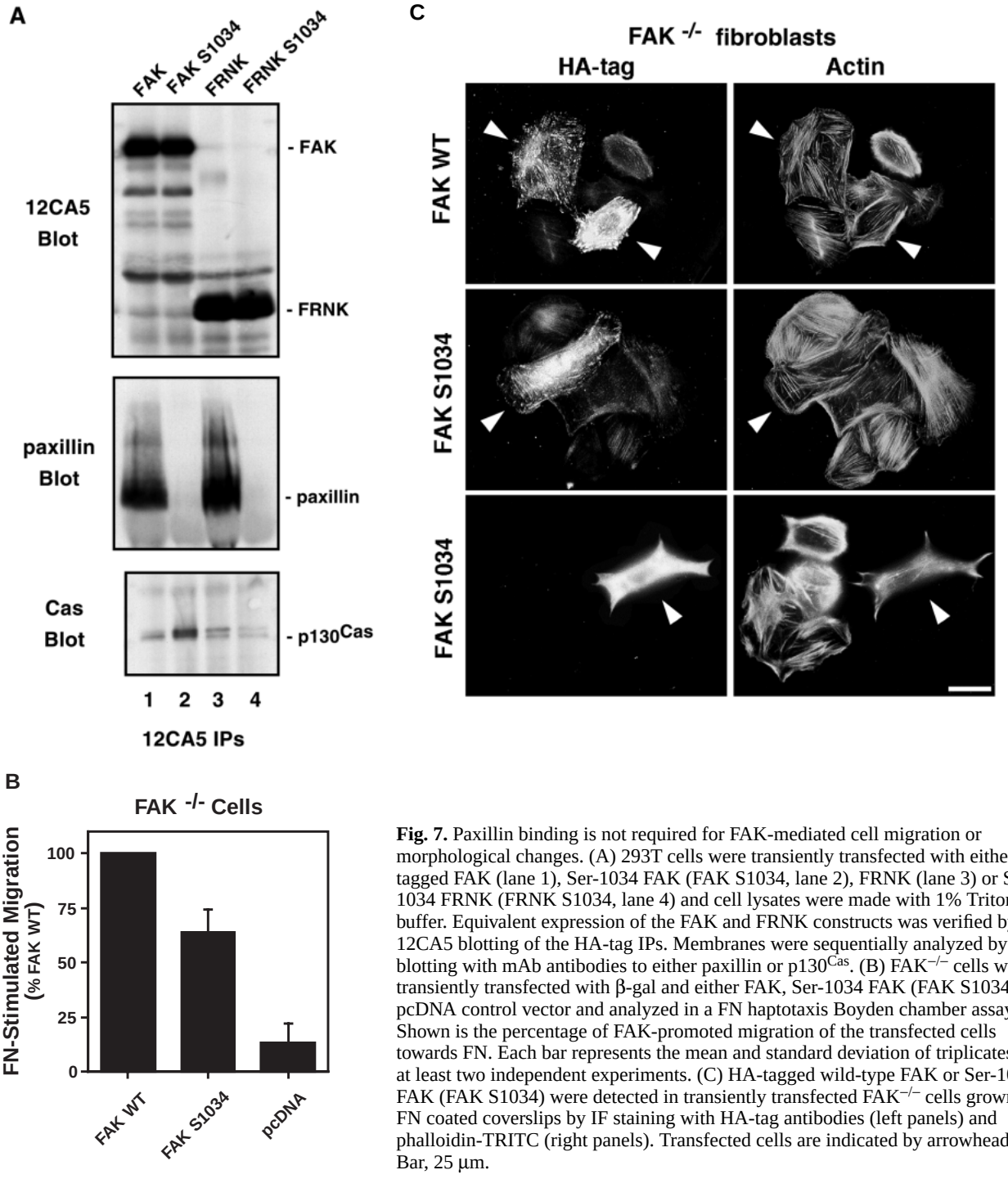

Fig. 7. Paxillin binding is not required for FAK-mediated cell migration or morphological changes. (A) 293T cells were transiently transfected with either HA-tagged FAK (lane 1), Ser-1034 FAK (FAK S1034, lane 2), FRNK (lane 3) or Ser-1034 FRNK (FRNK S1034, lane 4) and cell lysates were made with 1% Triton lysis buffer. Equivalent expression of the FAK and FRNK constructs was verified by 12CA5 blotting of the HA-tag IPs. Membranes were sequentially analyzed by blotting with mAb antibodies to either paxillin or $p130^{Cas}$. (B) $FAK^{-/-}$ cells were transiently transfected with β-gal and either FAK, Ser-1034 FAK (FAK S1034), or pcDNA control vector and analyzed in a FN haptotaxis Boyden chamber assay. Shown is the percentage of FAK-promoted migration of the transfected cells towards FN. Each bar represents the mean and standard deviation of triplicates from at least two independent experiments. (C) HA-tagged wild-type FAK or Ser-1034 FAK (FAK S1034) were detected in transiently transfected $FAK^{-/-}$ cells grown on FN coated coverslips by IF staining with HA-tag antibodies (left panels) and phalloidin-TRITC (right panels). Transfected cells are indicated by arrowheads. Bar, 25 μm.

dephosphorylation. Interestingly, Ser-1034 full-length FAK both localized to focal contacts and promoted cell migration supporting the hypothesis that FAK functions at these sites to initiate cell migration. The fact that Ser-1034 FAK did not detectably associate with paxillin suggests that this interaction is not required for focal contact localization or FAK-mediated cell migration events. Instead, as depicted in the summary model (Fig. 8), FAK localization to sites of FN receptor integrin clustering is essential for the recruitment of other target proteins required to transduce signals for integrin-stimulated cell migration events.

Model of FAK function in promoting cell migration

Expression of various FAK mutants in the FAK⁻ cells showed that FAK kinase activity, the Tyr-397/SH2 domain binding site, and the first proline-rich SH3 binding region in the FAK C-terminal domain were individually needed to promote FAK⁻ cell migration to FN whereas direct paxillin binding to FAK was not required. The exact mechanism as to how FAK functions to promote cell shape changes and which signaling pathway(s) it uses to promote haptotaxis cell migration will require further analyses. However, the fact that the individual FAK Phe-397, Arg-454, or Ala-712/713 point mutations prevented full FAK function in promoting cell migration suggests that FN-stimulated increases in FAK kinase activity lead to the recruitment of both SH2 and SH3 domain-containing signaling proteins and that a multi-protein complex with FAK is required for efficient cell migration events (Fig. 8).

One important finding in our studies was that FAK kinase activity was required for efficient FN-stimulated cell migration events. This is significant since FAK kinase activity is activated by FN stimulation of cells and gene knockout studies have suggested that FAK is a physiological mediator of both FN and β1 integrin-generated signals (Ilic et al., 1995). Previous studies have shown that overexpression of kinase-inactive FAK in CHO cells functions equally as well as WT FAK in enhancing cell migration (Cary et al., 1996). In this study, we show that kinase-inactive (Arg-454) FAK is weakly tyrosine phosphorylated at Tyr-397 and previous studies have shown that this mutant is weakly associated with Src-family PTKs after FN stimulation of 293T cells (Schlaepfer and Hunter, 1996). Whereas in CHO cells, this presumed adaptor protein function of Arg-454 FAK may be sufficient to promote cell migration (Cary et al., 1996), our studies show that FAK kinase activity is required for maximal Tyr-397 site phosphorylation and full FAK function in promoting FAK⁻ fibroblast migration.

Although elevated levels of FAK kinase activity can directly phosphorylate associated signaling proteins such as $p130^{Cas}$ (Tachibana et al., 1997) and Shc (Schlaepfer et al., 1998), one of the main requirements of FAK kinase activity may be to autophosphorylate the FAK Tyr-397 site and to promote the recruitment of SH2 domain-containing signaling proteins to focal contact sites (Fig. 7). This multi-functional phosphorylation site on FAK is the binding site for the SH2 domains of Src-family PTKs, the p85 subunit of PI 3-kinase, and the Shc adaptor protein. Both PI 3-kinase activation (Keely et al., 1997) and signals downstream of Shc tyrosine phosphorylation leading to ERK2/MAP kinase activation (Klemke et al., 1997) have been shown to be important components of integrin-stimulated cell migration events. Inhibitors of the ERK2/MAP kinase cascade can block FN-stimulated fibroblast cell migration (Anand-Apte et al., 1997) and this connection may be at the level of myosin light chain activation (Klemke et al., 1997). Interestingly, both transient Pyk2 overexpression and FAK re-expression in the FAK⁻ cells potentiated FN-stimulated ERK2 activation; however Pyk2 did not efficiently promote FAK⁻ cell migration to FN compared to FAK (Sieg et al., 1998). Since Pyk2 exhibits a peri-nuclear localization in the FAK⁻ cells, it may be that the differential localization of signaling complexes with FAK to focal contact sites is an important factor for cell motility or that FN-stimulated ERK2 activation is not the rate-limiting step preventing FAK⁻ cell migration.

Our studies showing that $p50^{csk}$ overexpression could inhibit

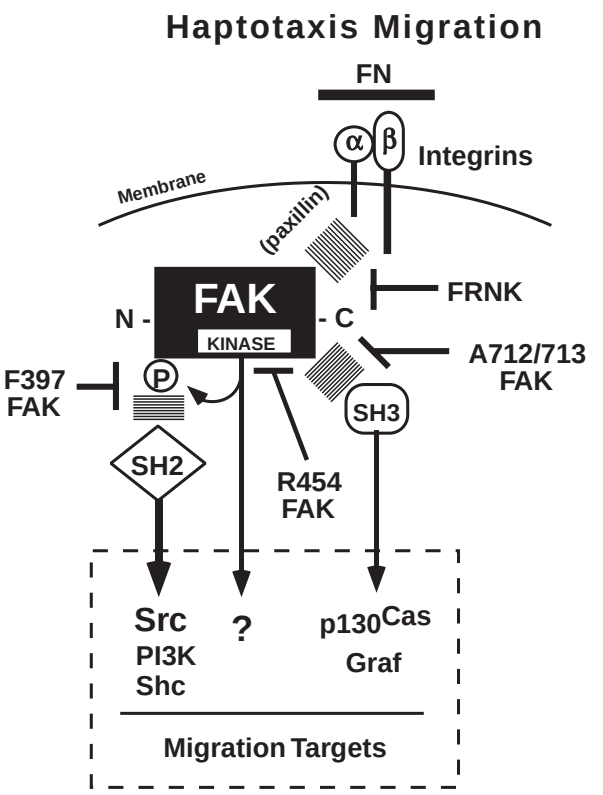

Fig. 8. Model of FAK function in promoting integrin-stimulated cell migration. Fibronectin binding to transmembrane integrin receptors leads to the activation of FAK tyrosine kinase activity. Subsequently, Tyr-397 in FAK becomes phosphorylated by an autophosphorylation mechanism initiating the recruitment of SH2 domain-containing proteins such as Src family PTKs, the p85 subunit of PI3K and Shc. In addition, FAK serves as a scaffold for SH3 domain-containing proteins like $p130^{Cas}$ or Graf which bind to either of two C-terminal proline-rich regions. FAK localization to focal contact sites and regions of integrin clustering occurs independent of paxillin binding to FAK. However, FAK activation by FN stimulation of cells can be blocked by overexpression of the C-terminal domain of FAK termed FRNK which contains the paxillin binding domain. FAK functions to promote FN-stimulated haptotaxis cell migration through the recruitment of other signaling proteins and by promoting changes in actin cytoskeletal structures leading to cell elongation. Mutations at the FAK autophosphorylation site (F397), in the kinase domain (R454), and at the first proline-rich motif (A712/713) impede the formation of a multi-protein signaling complex and thereby inhibit FAK function in promoting cell migration.

FN-stimulated and FAK-mediated cell migration supports the conclusion that the recruitment of active Src-family PTKs to focal contact sites is an important factor promoting cell motility events. Indeed, it has been proposed that the FAK-mediated recruitment of active Src-family PTKs to focal contact sites leads to the disassembly of cell-substratum adhesion sites (Fincham and Frame, 1998) and it has been shown that overexpression of active c-Src can reverse the negative effects of FRNK on chicken embryo fibroblast cell spreading (Richardson et al., 1997). These results are also supported by the studies performed with triple Src-family PTK-null ($Src^{-/-}$, $Yes^{-/-}$, $Fyn^{-/-}$) fibroblasts (SYF cells) (Klinghoffer et al., 1999). In these cells, both FN-stimulated tyrosine phosphorylation events and haptotaxis cell migration to FN were dramatically reduced compared to SYF cells re-expressing c-Src.

Although it is unclear how this Src-FAK complex functions to promote cell migration, our results showing reduced levels of cell migration with the Ala-712/713 FAK mutant also suggest that other SH3-mediated protein binding interactions with FAK are also required for FN-stimulated cell motility events. The fact that Ala-712/713 FAK exhibited high levels of FN-stimulated phosphorylation at the Tyr-397 site but only promoted low levels of cell migration suggest that multiple protein binding interactions with FAK function to coordinate cell migration events. We speculate that FN-stimulated increases in FAK kinase activity promote the recruitment of Src-family PTKs followed by the sequential (Src-mediated) phosphorylation of target proteins bound to the FAK Pro-712/713 site.

One common phosphorylation target of both FAK and Src kinase activities is the adaptor protein, $p130^{Cas}$. Interestingly, $p130^{Cas-/-}$ fibroblasts also exhibit defects in the assembly of actin filaments in the formation of actin stress fibers as do the FAK^- cells (Honda et al., 1998). The Ala-712/713 mutation in FAK disrupts the primary $p130^{Cas}$ SH3 domain binding site (Polte and Hanks, 1995, 1997) and also severely limits the ability of this FAK mutant to promote FAK^- cell migration. Previous studies have shown that overexpression of an Ala-712/715 FAK mutant in CHO cells did not promote elevated cell migration and that co-expression of the SH3 domain of $p130^{Cas}$ can inhibit FAK-enhanced CHO cell motility (Cary et al., 1998). Our findings that Ser-1034 FAK associated with greater amounts of $p130^{Cas}$ by co-immunoprecipitation may reflect the pathway through which this FAK mutant promotes cell migration. It has been proposed that Crk SH2 domain-mediated adaptor protein binding to tyrosine-phosphorylated $p130^{Cas}$ is important for Rac-dependent and Ras-independent cell migration events (Klemke et al., 1998). However, since other adaptor proteins such as Nck bind to $p130^{Cas}$ (Schlaepfer et al., 1997), it is possible that multiple pathways downstream of $p130^{Cas}$ are important for promoting cell migration events.

Another target protein that may be involved in FAK-mediated cell migration events is the Rho GTPase-activating protein, Graf (Hildebrand et al., 1996). Graf binds to FAK through SH3 domain-mediated interactions and overexpression of Graf in fibroblasts affects actin stress fiber formation and cell morphology (Taylor et al., 1999). This connection is interesting since FAK re-expression in the FAK^- cells results in the dynamic rearrangement of cortical and filamentous actin structures. Recent studies have shown that Rho activity is transiently inhibited during the initial period of FN-stimulated cell spreading (Ren et al., 1999) and this transient inactivation of Rho occurs during the same time course of maximal FAK kinase activation (Schlaepfer et al., 1998).

In summary, one of the best analogies to describe the role of FAK in cells is that it functions as an integrin-activated scaffold for the proper localization and assembly of signaling complexes through the coordinated recruitment of Src-family PTKs, GTPase regulatory proteins, and modular adaptor proteins. Future efforts to determine and decipher these FAK-mediated interactions will continue to enhance our knowledge on the regulatory mechanisms governing cell growth, shape, and migration.

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