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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

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The focal adhesion kinase (FAK) protein-tyrosine kinase (PTK) links transmembrane integrin receptors to intracellular signaling pathways. We show that expression of the FAK-related PTK, Pyk2, is elevated in fibroblasts isolated from murine *fak*$^{-/-}$ embryos (FAK⁻) compared with cells from *fak*$^{+/+}$ embryos (FAK⁺). Pyk2 was localized to perinuclear regions in both FAK⁺ and FAK⁻ cells. Pyk2 tyrosine phosphorylation was enhanced by fibronectin (FN) stimulation of FAK⁻ but not FAK⁺ cells. Increased Pyk2 tyrosine phosphorylation paralleled the time-course of Grb2 binding to Shc and activation of ERK2 in FAK⁻ cells. Pyk2 *in vitro* autophosphorylation activity was not enhanced by FN plating of FAK⁻ cells. However, Pyk2 associated with active Src-family PTKs after FN but not poly-L-lysine replating of the FAK⁻ cells. Overexpression of both wild-type (WT) and kinase-inactive (Ala457), but not the autophosphorylation site mutant (Phe402) Pyk2, enhanced endogenous FN-stimulated c-Src *in vitro* kinase activity in FAK⁻ cells, but only WT Pyk2 overexpression enhanced FN-stimulated activation of co-transfected ERK2. Interestingly, Pyk2 overexpression only weakly augmented FAK⁻ cell migration to FN whereas transient FAK expression promoted FAK⁻ cell migration to FN efficiently compared with FAK⁺ cells. Significantly, repression of endogenous Src-family PTK activity by p50^{csk} overexpression inhibited FN-stimulated cell spreading, Pyk2 tyrosine phosphorylation, Grb2 binding to Shc, and ERK2 activation in the FAK⁻ but not in FAK⁺ cells. These studies show that Pyk2 and Src-family PTKs combine to promote FN-stimulated signaling events to ERK2 in the absence of FAK, but that these signaling events are not sufficient to overcome the FAK⁻ cell migration defects.

Keywords: cell migration/c-Src/ERK2/FAK/Pyk2

Introduction

Cell adhesion to the extracellular matrix (ECM) proteins generates signals important for cell growth, survival and migration. In a variety of cell types enhanced tyrosine phosphorylation of signaling proteins is a common response to integrin stimulation by ECM proteins such as fibronectin (FN). In fibroblasts, the focal adhesion kinase (FAK), a non-receptor protein-tyrosine kinase (PTK), co-localizes with transmembrane integrin receptors at cell–substratum contact sites. Since integrin receptors lack catalytic activity and null mutations in both the murine *FN* and *FAK* genes result in similar early embryonic lethal phenotypes, it has been hypothesized that FAK PTK activation is important for both FN-mediated developmental and signal transduction events (for reviews see Ilić *et al*., 1997; Schlaepfer and Hunter, 1998).

FAK and a second PTK, variously called proline-rich tyrosine kinase 2 (Pyk2) (Lev *et al*., 1995), cell adhesion kinase β (CAKβ) (Sasaki *et al*., 1995), related adhesion focal tyrosine kinase (RAFTK) (Avraham *et al*., 1995) or calcium-dependent tyrosine kinase (CADTK) (Yu *et al*., 1996), define a subfamily of non-receptor PTKs (the Pyk2 designation will be used here to avoid nomenclature confusion). Both FAK and Pyk2 have ~45% overall amino acid sequence identity, contain a central kinase domain flanked by large N- and C-terminal domains and lack Src homology 2 or 3 (SH2 and SH3) domains. One distinguishing but undefined difference between FAK and Pyk2, is that Pyk2 can be activated by stimuli that increase intracellular calcium levels in cells (Lev *et al*., 1995; X.Li *et al*., 1997; Raja *et al*., 1997; Zheng *et al*., 1998). Although initial reports suggested that Pyk2 was not activated by FN stimulation of rat fibroblasts (Sasaki *et al*., 1995), subsequent studies have shown that Pyk2 tyrosine phosphorylation is enhanced by integrin stimulation of B cells (Astier *et al*., 1997), megakaryocytes (Li *et al*., 1996), T lymphocytes (Ma *et al*., 1997) and, after exogenous expression, in chicken embryo fibroblasts (Schaller and Sasaki, 1997).

FAK and Pyk2 have been proposed to facilitate linkages between integrin receptors and cytoskeletal proteins such as paxillin and Hic-5 (Turner and Miller, 1994; Hildebrand *et al*., 1995; Salgia *et al*., 1996; Matsuya *et al*., 1998). In addition, FAK and Pyk2 may couple integrins to the activation of signaling pathways involving p130^{Cas} (Law *et al*., 1996; Astier *et al*., 1997; Polte and Hanks, 1997), phosphatidylinositol 3′-kinase (Chen *et al*., 1996; Hatch *et al*., 1998), and the JNK/mitogen-activated protein (MAP) kinase (Tokiwa *et al*., 1996; S.Li *et al*., 1997; X.Li *et al*., 1997). Another function common to FAK and Pyk2 is that they are involved in promoting signal transduction events to the extracellular-regulated kinase 2 (ERK2)/MAP kinase. Pyk2 overexpression enhances G-protein-linked signaling to ERK2 (Lev *et al*., 1995), whereas expression of either the kinase-inactive or autophosphorylation site mutants of Pyk2 can attenuate G-protein-stimulated ERK2 activation (Dikic *et al*., 1996;

Della Rocca *et al*., 1997). A linkage between FAK and FN receptor-stimulated ERK2 activation depends upon FAK autophosphorylation at Tyr397 (Schlaepfer and Hunter, 1997; Schlaepfer *et al*., 1998).

The molecular pathways through which FAK and Pyk2 promote ERK2 activation also involve the activity of Src-family non-receptor PTKs. Cell stimulation promotes SH2 domain-dependent binding of Src-family PTKs to the motifs surrounding the autophosphorylation sites of both Pyk2 and FAK. Src-mediated phosphorylation of FAK promotes Grb2 SH2 domain binding to FAK Tyr925 (Schlaepfer *et al*., 1994, 1997; Schlaepfer and Hunter, 1996) and cell stimulation also promotes Grb2 binding to Tyr881 in Pyk2 (Lev *et al*., 1995; Li *et al*., 1996; Ganju *et al*., 1997; Felsch *et al*., 1998). Grb2 SH2 binding to a FN-stimulated signaling complex and association of the Grb2 SH3 domains with the SOS GDP–GTP exchange factor for the small G-protein Ras, is a direct pathway through which integrins can stimulate Ras-dependent ERK2/MAP kinase activation (Clark and Hynes, 1996).

Another common phosphorylation target of Pyk2, FAK and Src-family PTKs is the Shc adaptor protein. Both Pyk2 and FAK overexpression can enhance Shc tyrosine phosphorylation and Grb2 binding to Shc after cell stimulation (Lev *et al*., 1995; Schlaepfer and Hunter, 1997). Direct SH2-mediated binding of Shc to FAK occurs after FN stimulation (Schlaepfer and Hunter, 1997) and FAK can directly phosphorylate Shc at Tyr317 to promote Grb2 binding and signaling to ERK2 (Schlaepfer *et al*., 1998). Although Grb2 binding to Shc Tyr317 has been proposed as essential for integrin signaling to ERK2 (Wary *et al*., 1996; Mainiero *et al*., 1997), Src-family PTK activity can phosphorylate both wild-type and Phe317 Shc (van der Geer *et al*., 1996) to promote FN-stimulated Phe317 Shc tyrosine phosphorylation *in vivo* (Schlaepfer *et al*., 1998). Significantly, co-expression of the Grb2 binding mutants Phe925 FAK with Phe317 Shc attenuates but does not block FN-stimulated ERK2 activation in human 293T cells, whereas signaling is inhibited by co-expression of an SH3 domain-inactivated Grb2 (Schlaepfer *et al*., 1998). Thus, multiple Grb2-mediated pathways can deliver FN-stimulated signals to ERK2.

In addition to promoting FN-receptor-stimulated signaling events, FAK also functions in the processes of cell migration. High levels of FAK protein are expressed in migrating keratinocytes (Gates *et al*., 1994) and in invasive human tumor samples (Owens *et al*., 1995). Mouse fibroblasts derived from *fak*$^{-/-}$ embryos exhibit decreased rates of cell migration *in vitro* (Ilić *et al*., 1995). In Chinese hamster ovary (CHO) cells, stable FAK overexpression enhances FN-stimulated cell motility, and this effect depends upon FAK autophosphorylation at Tyr397 (Cary *et al*., 1996) and linkages to p130^{Cas} tyrosine phosphorylation (Cary *et al*., 1998). Transient overexpression of the non-catalytic C-terminal domain of FAK (FRNK) inhibits FN-stimulated cell spreading, the tyrosine phosphorylation of targets such as paxillin (Richardson and Parsons, 1996; Richardson *et al*., 1997), and reduces cell migration (Gilmore and Romer, 1996) possibly through displacement of cellular FAK from focal contact sites.

As a first step to determining the molecular mechanisms of the FAK-deficient (FAK$^-$) cell migration defect, we present results characterizing the FN-stimulated tyrosine phosphorylation and signaling events in fibroblasts that lack FAK. We report that Pyk2 expression is elevated in the FAK$^-$ cells compared with fibroblasts isolated from *fak*$^{+/+}$ embryos (FAK$^+$). We find that Pyk2 functions in a compensatory manner in combination with Src-family PTKs to facilitate FN-stimulated signaling to ERK2 in the FAK$^-$ cells. However, while Pyk2 and FAK are structurally similar, Pyk2 does not significantly co-localize with vinculin-stained focal contacts in the FAK$^-$ cells and Pyk2 does not efficiently promote migration to FN compared with transient FAK re-expression in the FAK$^-$ cells.

Results

FN-stimulated Pyk2 tyrosine phosphorylation and ERK2 activation in the FAK$^-$ fibroblasts

A null mutation in the murine *FAK* gene leads to an embryonic lethal phenotype at E8.5 due to defective gastrulation events (Ilić *et al*., 1995). FAK$^-$ fibroblasts have been isolated from E8.5 embryos and these cells exhibit a rounded morphology, enhanced cell–substratum contact formation, and decreased rates of migration *in vitro* (Ilić *et al*., 1995). No differences in integrin receptor expression were observed in the FAK$^-$ compared with FAK$^+$ fibroblasts (Ilić *et al*., 1995). In addition, proteins known to associate with FAK such as paxillin, tensin and p130^{Cas} are highly tyrosine-phosphorylated in the FAK$^-$ cells (Ilić *et al*., 1995; Vuori *et al*., 1996; Sakai *et al*., 1997). To determine whether compensatory changes have occurred to promote tyrosine phosphorylation events in the absence of FAK, antibodies to the FAK-related PTK Pyk2 were used to analyze lysates of both FAK$^+$ and FAK$^-$ fibroblasts (Figure 1A).

Antibodies to FAK detected an ~116 kDa protein in immunoprecipitates (IPs) from FAK$^+$ but not FAK$^-$ cells (Figure 1A, lanes 1 and 2), confirming that FAK is not expressed in the FAK$^-$ cells. A monoclonal antibody (mAb) to Pyk2 weakly detected an ~110 kDa band in whole-cell lysates (WCL) of FAK$^+$ cells (Figure 1A, lane 3) and the expression of this 110 kDa protein was greatly elevated in WCLs of FAK$^-$ cells (Figure 1A, lane 4). The identity of this ~110 kDa protein was confirmed as Pyk2 by immunoprecipitation using peptide-specific anti-Pyk2 polyclonal antibodies followed by blotting with the anti-Pyk2 mAb (Figure 1A, lanes 5 and 6). These experiments were performed on pools of early-passage primary FAK$^+$ and FAK$^-$ cells; similar results of enhanced Pyk2 expression were obtained upon analyses of FAK$^-$ clones isolated by dilution plating (data not shown).

Pyk2 was not detectably tyrosine phosphorylated in the FAK$^+$ cells after FN stimulation (Figure 1A, lane 7). This lack of FN-stimulated Pyk2 tyrosine phosphorylation in the FAK$^+$ cells is consistent with studies from other FAK-expressing cells (Zheng *et al*., 1998) and contrasts with the high level of Pyk2 tyrosine phosphorylation after FN stimulation of the FAK$^-$ cells (Figure 1A, lane 8). Since FAK links integrin receptors to the activation of signaling pathways leading to targets such as the ERK2/MAP kinase, FN and control poly-L-lysine (PL) replating assays were performed with the FAK$^-$ cells to determine whether increases in Pyk2 tyrosine phosphorylation correlated with ERK2 activation (Figure 1B and C). These assays were

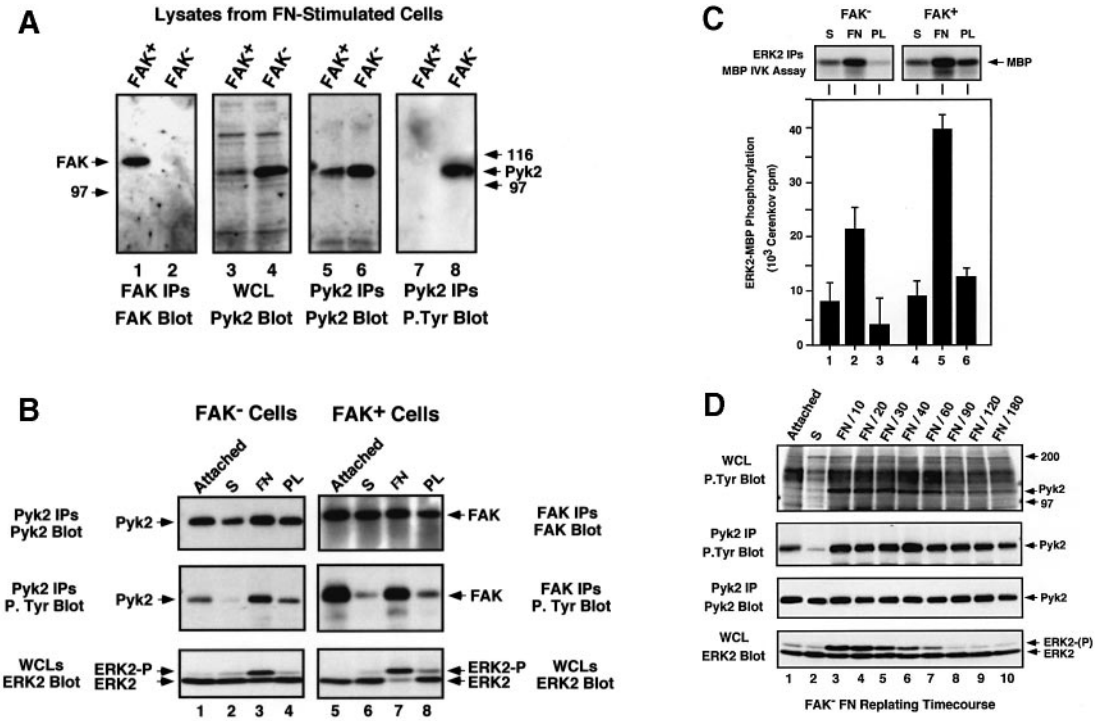


Fig. 1. FN-stimulated Pyk2 tyrosine phosphorylation and ERK2 activation in the FAK$^-$ fibroblasts. (**A**) Lysates of FAK$^+$ or FAK$^-$ cells (as indicated) were prepared after integrin stimulation by FN replating (30 min). Polyclonal FAK IPs were analyzed by anti-FAK blotting (lanes 1 and 2). Equal amounts (~200 μg) of whole-cell lysates (WCL) were resolved by SDS–PAGE and analyzed by blotting with mAb to Pyk2 (lanes 3 and 4). Polyclonal Pyk2 IPs were analyzed by mAb anti-Pyk2 blotting (lanes 5 and 6) followed by anti-P.Tyr blotting (lanes 7 and 8). (**B**) FAK$^-$ cells (lanes 1–4) or FAK$^+$ cells (lanes 5–8) were either serum-starved (attached), held in suspension for 1 h (S), and then replated onto FN-coated (FN) or poly-L-lysine-coated (PL) dishes for 30 min. Pyk2 and FAK IPs were analyzed either by anti-Pyk2 blotting or anti-FAK blotting followed by anti-P.Tyr blotting of the same membrane. Equal amounts (~100 μg) of WCLs were analyzed by anti-ERK2 blotting. ERK2-(P) denotes the phosphorylated and activated form of ERK2. (**C**) Polyclonal ERK2 IPs were made from either suspended, FN- or PL-replated FAK$^-$ or FAK$^+$ cell lysates and ERK2 *in vitro* kinase activity was measured by the phosphorylation of myelin basic protein (MBP). The amount of ^{32}P incorporated into MBP was determined by Cerenkov counting and the values represent the average of three separate experiments. Error bars represent standard deviation. (**D**) Equal amounts of FAK$^-$ WCLs (~100 μg) were resolved by SDS–PAGE and analyzed by anti-P.Tyr blotting and anti-ERK2 blotting as indicated. In parallel, anti-Pyk2 IPs were made from ~500 μg WCL, resolved by SDS–PAGE, and analyzed by anti-P.Tyr followed by anti-Pyk2 blotting.

performed in parallel with FAK$^+$ cells to compare the regulation of Pyk2 in the FAK$^-$ cells with FAK in the FAK$^+$ cells.

In attached and serum-starved FAK$^-$ or FAK$^+$ cells, Pyk2 and FAK respectively exhibited high basal phosphotyrosine (P.Tyr) levels (Figure 1B, lanes 1 and 5) whereas Pyk2 and FAK exhibited only modest levels of associated *in vitro* kinase activity under these conditions (see Figure 3). ERK2 was not activated under serum-starved conditions in either the FAK$^-$ or FAK$^+$ cells as visualized by the lack of ERK2 protein bandshifts (Figure 1B, lanes 1 and 5). Both Pyk2 and FAK P.Tyr levels were reduced upon cell suspension for 45 min, and this procedure did not significantly activate ERK2 in either the FAK$^-$ or FAK$^+$ cells (Figure 1B, lanes 2 and 6). Compared with suspended cells, FN stimulation (30 min) increased both Pyk2 and FAK P.Tyr levels in the FAK$^-$ and FAK$^+$ cell, respectively (Figure 1B, lanes 3 and 7). Control PL-replating (30 min), where the cells adhere to culture dishes independent of integrin receptor activation, did not reproducibly lead to elevated Pyk2 or FAK tyrosine phosphorylation compared with suspended cells (Figure 1B, lanes 4 and 8).

FN stimulation but not PL-replating activated ERK2 in both the FAK$^-$ and FAK$^+$ cells (Figure 1B and C). As visualized by a second immunoreactive ERK2 band corresponding to tyrosine-phosphorylated and activated ERK2 (ERK2-P), FN-stimulated ERK2 activation was greater after 30 min in the FAK$^+$ compared with the FAK$^-$ cells (Figure 1B, lanes 3 and 7). This difference was verified by directly measuring ERK2 *in vitro* kinase activity toward myelin basic protein (Figure 1C). In the FAK$^+$ cells, FN-stimulated ERK2 activity was ~2-fold higher than the level of ERK2 activity in the FN-stimulated FAK$^-$ cells (Figure 1C, lanes 2 and 5). No other consistent differences in ERK2 activity were observed in either the suspended or PL-replated controls between the FAK$^+$ and FAK$^-$ cells (Figure 1C). These results show that FAK is not essential for FN-stimulated signaling to ERK2. However, it is possible that enhanced Pyk2 tyrosine phosphorylation in the FAK$^-$ cells may play a FAK-related role in these FN receptor-stimulated signaling events. Indeed, increased FN-stimulated Pyk2 tyrosine phosphorylation paralleled the time-course of FN-stimulated ERK2 activation from 10 to 60 min in the FAK$^-$ cells (Figure 1D, lanes 3–7).

Perinuclear distribution of Pyk2 in the FAK$^+$ and FAK$^-$ fibroblasts

Since Pyk2 expression was elevated in the FAK$^-$ compared with the FAK$^+$ cells, this difference may represent a compensatory event due to the absence of FAK. To

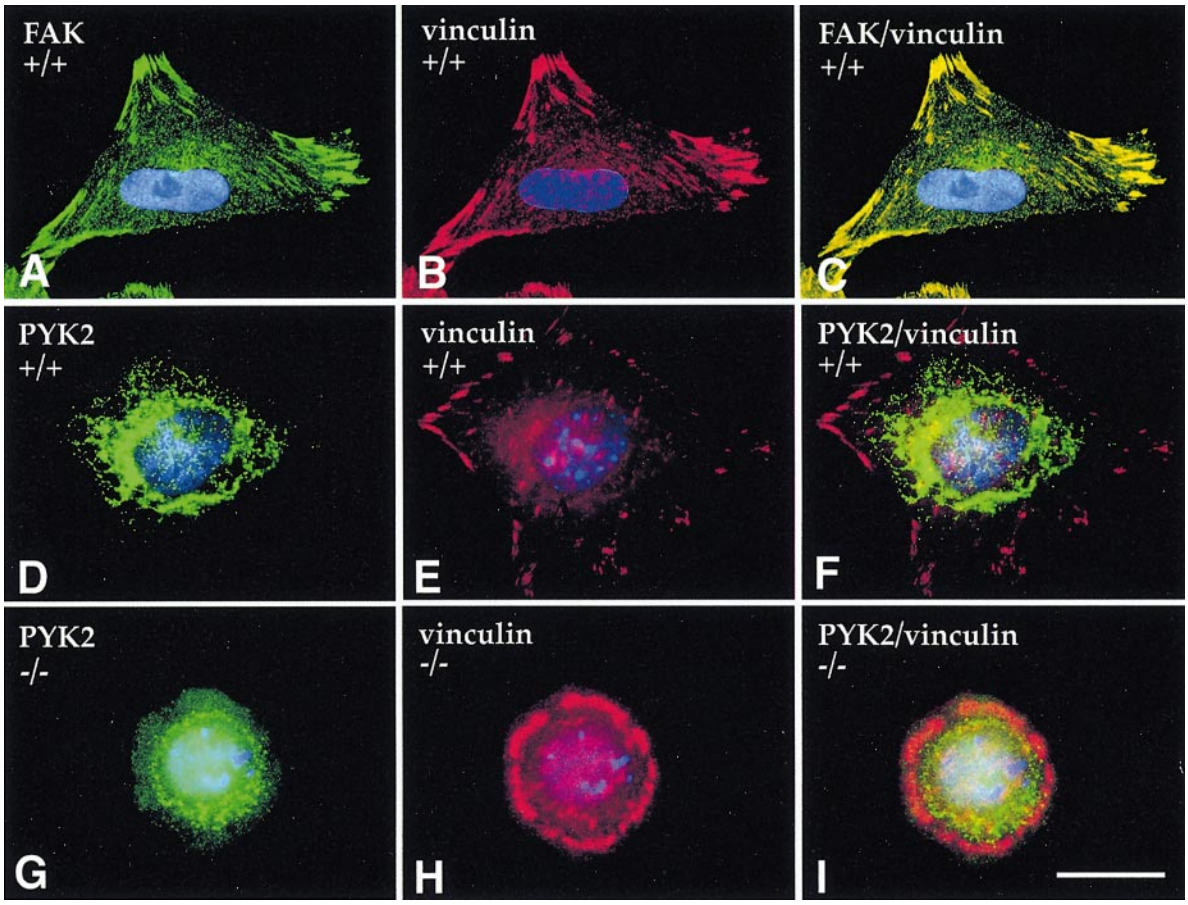


Fig. 2. Localization of FAK and Pyk2 in the FAK$^+$ and FAK$^-$ cells; Pyk2 exhibits a perinuclear distribution. (A–F) Indirect immunofluorescent staining of FN-replated FAK$^+$ cells (+/+) with FITC-antibody conjugates to FAK (**A**) or Pyk2 (**D**). The same cells shown in (A and D) were co-stained with rhodamine–antibody conjugates to vinculin (**B** and **E**). (**C** and **F**) Merged images of (A and B) and (D and E), respectively. Yellow color indicates the overlap in FITC and rhodamine staining. Nuclei were stained with Hoechst. (G–I) Indirect immunofluorescent staining of FN-replated FAK$^-$ cells (–/–) with FITC–antibody conjugates to Pyk2 (**G**) and rhodamine–antibody conjugates to vinculin (**H**). The merged image of (G and H) is shown (**I**). Scale bar is ~50 μM.

determine whether Pyk2 would exhibit a similar subcellular distribution in the FAK$^-$ cells as FAK in the FAK$^+$ cells, indirect immunofluorescence of antibody staining was used to determine the localization of either FAK or Pyk2 in the FAK$^+$ and FAK$^-$ cells (Figure 2). Integrin receptor-associated signaling and structural proteins such as vinculin co-localize with integrin receptors at cell–substratum contact sites termed focal adhesions (Burridge *et al*., 1997). After replating onto FN for 2 h in the absence of serum, the FAK$^+$ cells exhibited a spread morphology and FAK antibody staining exhibited a strong co-localization with antibody staining to vinculin (Figure 2A–C).

Pyk2 polyclonal antibody staining of the FAK$^+$ cells revealed a punctate perinuclear distribution that did not co-localize with vinculin staining at the perimeter of the spread FAK$^+$ cells (Figure 2D–F). This perinuclear staining pattern was observed with a number of different antibodies to Pyk2 (data not shown) and is consistent with the distribution of Pyk2 in rat fibroblasts (Matsuya *et al*., 1998) and smooth muscle cells (Zheng *et al*., 1998). In the FAK$^-$ cells, Pyk2 exhibited a similar perinuclear staining pattern and only a small fraction of Pyk2 co-localized with vinculin in the central region of the FN-stimulated FAK$^-$ cells (Figure 2G–I). Although the level of Pyk2 expression in the FAK$^-$ cells is quite high, only very weak Pyk2 co-staining with vinculin was observed at the FAK$^-$ cell periphery (Figure 2I). Therefore, if Pyk2 is substituting in a compensatory fashion for FAK, the mechanism of Pyk2 action must involve something other than a redistribution to focal contact sites in the FAK$^-$ cells.

Measurements of Pyk2-associated in vitro kinase activity after FN stimulation of FAK$^-$ cells

Since both Pyk2 and FAK exhibited high basal P.Tyr levels in serum-starved FAK$^-$ or FAK$^+$ cells respectively, and since previous studies have shown that P.Tyr immunoblotting analyses of FAK are not an accurate measure of its kinase activation state (Schlaepfer *et al*., 1998), *in vitro* ^{32}P-phosphorylation assays were performed to measure changes in Pyk2 autophosphorylation and Pyk2-associated *in vitro* kinase activity after stimulation of the FAK$^-$ cells (Figure 3). As visualized by autoradiography, the ~110 kDa Pyk2 band exhibited moderate and roughly equivalent levels of *in vitro* phosphorylation from lysates of attached serum-starved, suspended, and FN or PL-plated FAK$^-$ cells (Figure 3A and B). These results for Pyk2 differ from those obtained for FAK from either FN-stimulated NIH 3T3 fibroblasts (Guan and Shalloway, 1992; Hanks *et al*., 1992; Schlaepfer *et al*., 1994) or from FN-stimulated

FAK$^+$ cells where elevated levels of FAK *in vitro* phosphorylation were observed after FN-stimulation compared with serum-starved, suspended or PL-plated FAK$^+$ cells (Figure 3C and D).

FN stimulation promotes FAK autophosphorylation at Tyr397 and the transient SH2 domain-mediated recruitment of Src-family PTKs into a signaling complex. In the FAK$^+$ cells, FN-stimulation promotes the co-immunoprecipitation (co-IP) of Src-family PTKs with FAK (Figure 3C and D, lane 3). Phosphorylation of an equivalent site (Tyr402) in Pyk2 also promotes SH2-dependent Src-family PTK binding to Pyk2 (Dikic *et al*., 1996). A ^{32}P-labeled ~60 kDa protein weakly co-immunoprecipitated with antibodies to Pyk2 from suspended FAK$^-$ cells and strongly associated with Pyk2 from FN-stimulated FAK$^-$ cells (Figure 3A, lanes 2 and 3). This ~60 kDa protein was identified as a Src-family PTK with anti-Src PTK family antibodies (Figure 3B). Subsequent phosphopeptide mapping and further co-IP experiments revealed that both c-Src and Fyn were associated with Pyk2 in the FN-stimulated FAK$^-$ cells (data not shown). Although the mechanism of Src-family PTK association with Pyk2 in the suspended FAK$^-$ cells was not determined, it may involve a phosphotyrosine-independent association of Pyk2, c-Src and p130^{Cas} (data not shown). Similarities clearly exist in the association of Src-family PTKs with Pyk2 and FAK after FN stimulation of the FAK$^-$ or FAK$^+$ cells, respectively.

Since FAK but not Pyk2 *in vitro* phosphorylation was enhanced by FN stimulation, the molecular mechanisms promoting the association of Src-family PTKs with FAK or Pyk2 may differ between the FAK$^+$ and FAK$^-$ cells, respectively. In serum-starved FAK$^-$ cells, Pyk2 exhibited a moderate level of autophosphorylation activity (Figure 3A) and this was also measured as Pyk2-associated *in vitro* kinase (IVK) activity toward poly(Glu–Tyr)(4:1), a known substrate for FAK, Pyk2 and Src-family PTKs (Figure 3E). In addition to FAK$^-$, cell stimulation by FN-replating increases in Pyk2 P.Tyr levels and associated IVK activity were detected after FAK$^-$ cell depolarization with KCl (75 mM), hyperosmolarity shock with sorbitol (300 mM), and intracellular calcium release with thapsigargin (5 μM) treatments (Figure 3E). To determine the importance of intracellular calcium signals on Pyk2 activation events, FAK$^-$ cells were also pretreated with BAPTA-AM (50 μM), a membrane permeable calcium chelator, prior to stimulation. The ~2-fold increase in Pyk2-associated IVK activity stimulated by thapsigargin was inhibited by BAPTA pretreatment of the FAK$^-$ cells (Figure 3E, lanes 9 and 10),

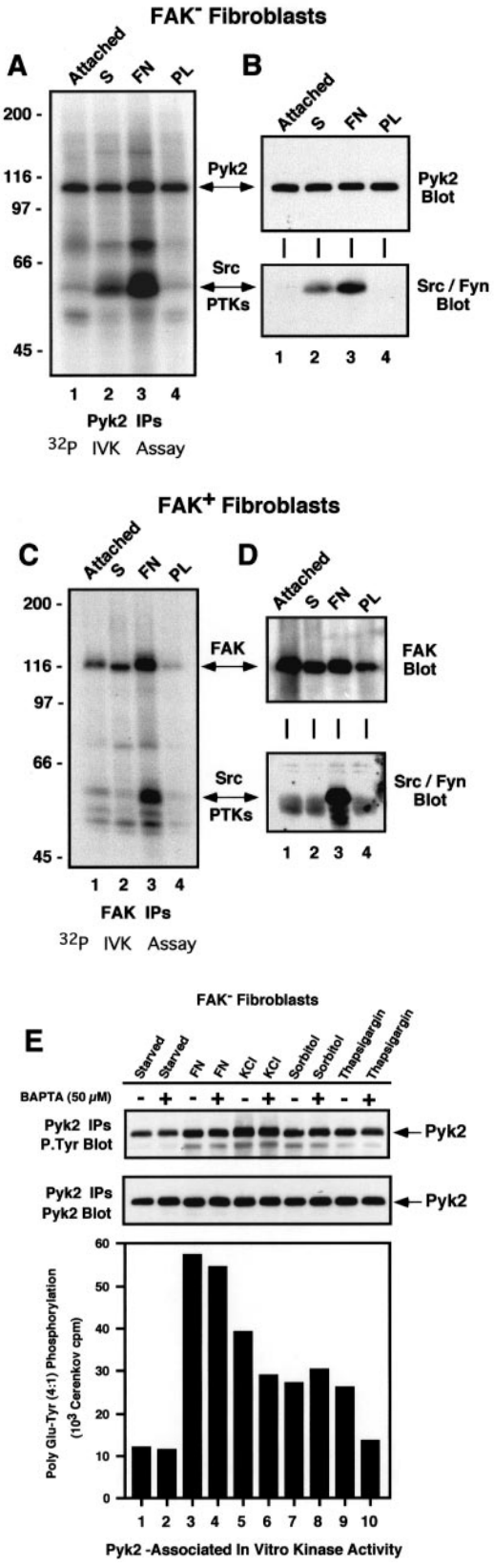
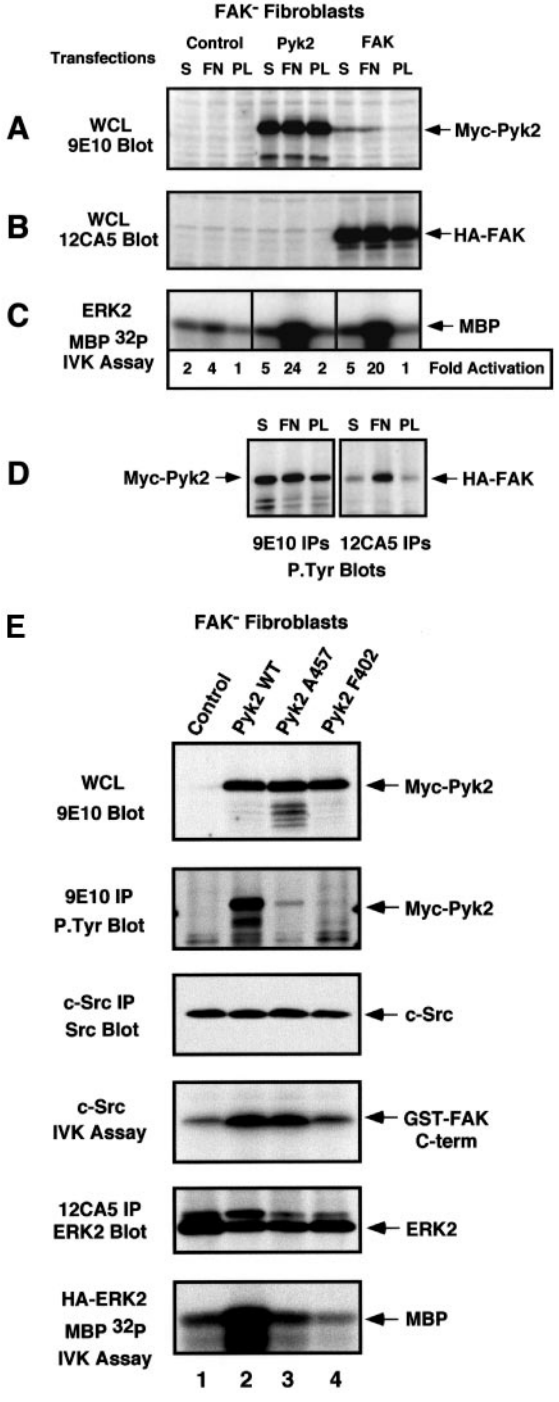


Fig. 3. Measurements of Pyk2 or FAK-associated *in vitro* kinase activity. (A–D) Lysates from either serum-starved (attached), suspended, FN and PL-plated FAK$^-$ cells (A and B) or FAK$^+$ cells (C and D) were prepared and divided in equal aliquots for either Pyk2 IPs or FAK IPs, respectively. (**A**) Pyk2 IPs were labeled by the addition of [γ-^{32}P]ATP in an *in vitro* kinase (IVK) assay. ^{32}P-labeled proteins were transferred to membranes and visualized by autoradiography. (**B**) The same membrane shown in (A) was cut and analyzed by either anti-Pyk2 or anti-Src family PTK blotting. (**C**) FAK IPs were labeled by the addition of [γ-^{32}P]ATP in an IVK assay and the same membrane (**D**) was cut and analyzed by either anti-FAK or anti-Src family PTK blotting. (**E**) FAK$^-$ cells were serum-starved (lanes 1 and 2) and either stimulated by FN-replating for 20 min (lanes 3 and 4), KCl (75 mM, 5 min) treatment (lanes 5 and 6), sorbitol (300 mM, 5 min) treatment (lanes 7 and 8) or thapsigargin (5 μM, 5 min) treatment (lanes 9 and 10). As indicated, BAPTA (50 μM) was either added to suspended cells prior to replating or added 15 min prior to cell stimulation. Pyk2 IPs (500 μg WCL) were analyzed by anti-P.Tyr and anti-Pyk2 blotting. Separately, Pyk2 IPs (500 μg WCL) were analyzed for associated IVK activity through the phosphorylation of poly(Glu–Tyr)(4:1). Results are representative of three separate experiments.

confirming the calcium-mediated activation of Pyk2 (Yu *et al*., 1996). However, BAPTA treatment did not inhibit FN-stimulated ERK2 activation in the FAK⁻ cells (data not shown) nor did BAPTA inhibit the FN, KCl or sorbitol-stimulated increase in Pyk2-associated IVK activity (Figure 3E).

An ~5-fold increase in Pyk2-associated IVK activity was measured after FN stimulation of the FAK⁻ cells (Figure 3E, lane 3) under conditions where Src-family PTKs associate with Pyk2 (Figure 3A, lane 3). A 3- to 4-fold increase in Pyk2-associated IVK activity was measured after KCl and sorbitol stimulation of the FAK⁻ cells (Figure 3E, lanes 5–8) even though no association of Src-family PTKs with Pyk2 was detectable after these treatments (data not shown). It is possible that KCl and sorbitol stimulation of the FAK⁻ cells promotes increased Pyk2 tyrosine phosphorylation and increased IVK activity through the direct activation of Pyk2 (Lev *et al*., 1995; Tokiwa *et al*., 1996). Additionally, it is possible that FN stimulation of the FAK⁻ cells promotes increased Pyk2 tyrosine phosphorylation and associated IVK activity as a result of a Pyk2 association with Src-family PTKs. Therefore, these results show that there is not an exact correlation with the levels of Pyk2 tyrosine phosphorylation and associated IVK activity since KCl stimulation of the FAK⁻ cells promoted the highest level of Pyk2 tyrosine phosphorylation whereas the highest level of Pyk2-associated IVK activity were measured after FN stimulation of the FAK⁻ cells (Figure 3E).



Both Pyk2 and FAK transient overexpression can enhance FN-stimulated ERK2 activity in the FAK⁻ cells

To determine whether Pyk2 tyrosine phosphorylation events played a direct role in FN-stimulated ERK2 activation, transient co-transfection studies with epitope-tagged Pyk2 or FAK in combination with epitope-tagged ERK2 were performed in the FAK⁻ cells (Figure 4). As determined by immunoblotting, equivalent levels of either N-terminal Myc-tagged human Pyk2 (Figure 4A) C-terminal HA-tagged murine FAK (Figure 4B) and HA-tagged ERK2 (data not shown) were present in the FAK⁻ cell lysates prepared after cell suspension, FN- and PL-plating. In control transfected cells, FN-stimulation elevated HA-ERK2 activity 2-fold over that measured from suspended and PL-plated FAK⁻ cells (Figure 4C). This change in ERK2 activity after FN stimulation was slightly less than that measured for endogenous ERK2 in the FAK⁻ cells (Figure 1C).

In the FAK⁻ cells overexpressing either Pyk2 or FAK, FN-stimulated HA-ERK2 activity was elevated 6- and 5-fold compared with control cells, respectively (Figure 4C). Surprisingly, Myc-Pyk2 was highly tyrosine-phosphoryl-

Fig. 4. Pyk2 and FAK-enhanced signaling in the FAK⁻ cells. (A–D) Transient transfections were performed with either HA-tagged ERK2 alone (1 μg) or in combination with Myc-tagged Pyk2 (2.5 μg) or HA-tagged FAK (2.5 μg). Lysates of were prepared after incubation (45 min) in suspension (S) and following plating onto FN- or PL-coated dishes for 30 min. (**A**) Exogenous Pyk2 expression as visualized by anti-Myc blotting (9E10 mAb). (**B**) Exogenous FAK expression as visualized by anti-HA blotting (12CA5 mAb). (**C**) HA-ERK2 *in vitro* kinase activity as isolated in 12CA5 IPs was measured by phosphorylation of myelin basic protein (MBP). The amount of ^{32}P incorporated into MBP was determined by Cerenkov counting and the values (fold-activation from serum-starved FAK⁻ controls) represent the average of two separate experiments. (**D**) Regulation of either Myc-Pyk2 or HA-FAK tyrosine phosphorylation in the FAK⁻ cells by anti-P.Tyr blotting of either mAb 9E10 or mAb 12CA5 IPs. (**E**) FAK⁻ cells were transfected with HA-ERK2 (1 μg) along with (2.0 μg) pcDNA3 control vector (lane 1), or with 2.0 μg of either WT Pyk2 (lane 2), kinase-inactive Ala457 Pyk2 (lane 3), or Phe402 Pyk2 (lane 4). Lysates were prepared after FN stimulation (20 min) of the transfected cells. As indicated, Myc-Pyk2 expression was verified by 9E10 blotting and Pyk2 tyrosine phosphorylation levels determined by anti-P.Tyr blotting of 9E10 IPs. The level of endogenous Src-associated IVK activity (mAb 2-17 c-Src IPs) was determined by GST–FAK kinase assays and by anti-Src blotting of the c-Src IPs. Co-transfected HA-ERK2 activity (12CA5 IPs) was measured by MBP kinase assays and the amount of 12CA5-associated ERK2 was visualized by anti-ERK2 blotting. Results are representative of two separate transfection experiments.

ated in suspended, FN- and PL-plated cells, whereas HA-FAK exhibited enhanced tyrosine phosphorylation only after FN stimulation of the FAK⁻ cells (Figure 4D). The tyrosine phosphorylation of Myc-Pyk2 was clearly regulated differently compared with endogenous Pyk2 in the FAK⁻ cells (Figure 1B, lanes 2–4) and endogenous (Figure 1B, lanes 6–8) and exogenously expressed FAK. However, these results demonstrate that both Pyk2 and FAK are functional when overexpressed in the FAK⁻ cells and that both can enhance FN-stimulated signaling to ERK2.

Since previous studies have shown that both Pyk2 kinase activity and phosphorylation at Tyr402 are important for Pyk2-enhanced G-protein-stimulated signaling events (Lev *et al*., 1995; Dikic *et al*., 1996), comparisons were made between WT Myc-tagged Pyk2, kinase-inactive (Ala457) Pyk2, and Phe402 Pyk2 in transiently transfected and FN-stimulated FAK⁻ cells (Figure 4E). Compared with the P.Tyr levels of WT Pyk2, Ala457 Pyk2 was weakly tyrosine-phosphorylated after FN stimulation of the FAK⁻ cells (Figure 4E, lane 3) whereas Phe402 Pyk2 was not detectably tyrosine phosphorylated (Figure 4E, lane 4). Interestingly, both WT and Ala457 Pyk2 but not Phe402 Pyk2 associated with (data not shown) and enhanced the level of FN-stimulated endogenous c-Src IVK activity in the FAK⁻ cells (Figure 4E, lanes 2 and 3). Although the binding interactions promoting the association of Ala457 Pyk2 with Src-family PTKs were not determined, these Pyk2 overexpression results suggest that an SH2-mediated interaction of Src-family PTKs with Pyk2 at Tyr402 may lead to the Pyk2 kinase-independent enhancement of Src-family PTK activity in the FAK⁻ cells.

Significantly, whereas both WT and Ala457 Pyk2 overexpression enhanced endogenous FAK⁻ c-Src IVK activity, only WT Pyk2 overexpression enhanced the level of FN-stimulated HA-ERK2 activity compared with control cells (Figure 4E, lanes 1 and 2). Ala457 Pyk2 expression did not affect the level of FN-stimulated HA-ERK2 activity (Figure 4E, lane 3) and Phe402 Pyk2 overexpression slightly inhibited HA-ERK2 activity compared with control transfected cells (Figure 4E, lane 4). These results suggest that enhanced activation of Src-family PTKs in the FAK⁻ cells may not be singularly sufficient to deliver a FN-stimulated signal to ERK2. In addition, these results support the conclusion that Pyk2 kinase activity and the phosphorylation of the Src SH2 binding site at Tyr402 are important components of the FN-stimulated signaling cascade to ERK2 in the FAK⁻ cells.

Elevated levels of Src-associated PTK activity in FAK⁻ compared with FAK⁺ cells

Since Pyk2 expression is elevated in the FAK⁻ cells and transient Pyk2 overexpression enhanced the level of FN-stimulated c-Src IVK activity, analyses were performed to measure and compare c-Src-associated IVK activity in lysates of non-transfected FAK⁺ and FAK⁻ cells (Figure 5). Immunoblotting analyses of whole cell lysates showed that the expression levels of both c-Src and Fyn were slightly elevated in the FAK⁻ compared with the FAK⁺ cells (data not shown). After normalization for equal amounts of immunoprecipitated c-Src protein, IVK assays were performed using a fusion protein of the FAK C-terminal domain as a substrate [glutathione-*S* transferase–FAK (GST–FAK)]. This domain of FAK is a good substrate for Src PTK activity and a poor substrate for FAK (Schlaepfer and Hunter, 1996) and Pyk2 (data not shown) transphosphorylation events. Whereas similar levels of basal c-Src PTK activity were found in either serum-starved FAK⁺ or FAK⁻ lysates (data not shown), an ~5-fold elevated level of c-Src PTK activity was found in lysates of suspended FAK⁻ compared with FAK⁺ cells (Figure 5A, lanes 1 and 4).

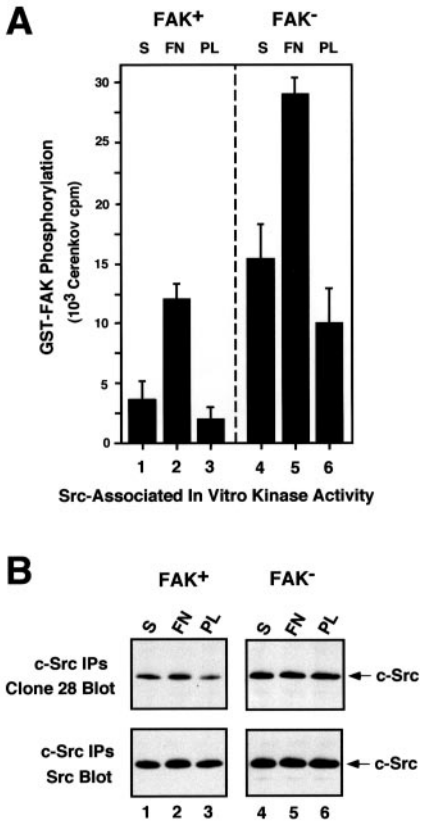


Fig. 5. Measurement of c-Src-associated IVK activity in the FAK⁺ and FAK⁻ cells. (**A**) Monoclonal antibody c-Src IPs were made from lysates (~500 μg) of FAK⁺ (lanes 1–3) or FAK⁻ (lanes 4–6) cells held (45 min) in suspension (S) and then either plated onto FN- or PL-coated plates for 30 min. Associated c-Src IVK activity was determined by the phosphorylation of a GST–FAK C-terminal fusion protein (2.5 μg) in the presence of [γ-^{32}P]ATP. This fusion protein was not significantly phosphorylated *in vitro* by Pyk2 isolated from serum-starved FAK⁻ cells (data not shown). The values represent the average of three separate experiments and error bars represent standard deviation. (**B**) Monoclonal antibody c-Src IPs were made from lysates (~500 μg) of FAK⁺ (lanes 1–3) or FAK⁻ (lanes 4–6) cells held (45 min) in suspension (S) and then either plated onto FN- or PL-coated plates for 20 min. Src IPs were analyzed by Clone 28 (mAb to dephosphorylated C-terminal of Src) blotting followed by anti-Src polyclonal blotting of the same membrane.

This elevated level of Src-associated PTK activity in suspended FAK⁻ is consistent with the observed weak interaction with Pyk2 (Figure 3A and B, lane 2) and was reduced after pre-clearing suspended FAK⁻ cell lysates with antibodies to Pyk2 (data not shown). Mechanistically, the increased level of c-Src IVK activity in the suspended FAK⁻ lysates may be a result of interactions with Pyk2. Alternatively, increased c-Src PTK activity may be due in part to the enhanced dephosphorylation of the regulatory Tyr529 site of p50^{csk} phosphorylation in c-Src (Figure 5B). Immunoblotting of c-Src IPs with a monoclonal (Clone

28) antibody which selectively recognizes the dephosphorylated motif surrounding Tyr529 in the C-terminal tail region of c-Src (Kawakatsu *et al*., 1996), showed the enhanced reactivity with c-Src from suspended FAK$^-$ compared with FAK$^+$ lysates (Figure 5B, lanes 1 and 4). After FN stimulation of the FAK$^+$ cells, a 3- to 4-fold increase in c-Src PTK activity was detected compared with low-level IVK activity measured from suspended and PL-plated FAK$^+$ cells (Figure 5A, lanes 1–3). Increased c-Src PTK activity after FN stimulation of the FAK$^+$ cells was associated with the enhanced dephosphorylation of c-Src Tyr529 (Figure 5B, lane 2) and these changes in c-Src PTK activity after FN stimulation are consistent with previous studies (Kaplan *et al*., 1995; Schlaepfer and Hunter, 1997; Schlaepfer *et al*., 1998).

In the FAK$^-$ cells, the level of c-Src PTK activity after FN-stimulation was increased ~2-fold above the high level found in suspended cells and was only slightly decreased in PL-plated FAK$^-$ cells (Figure 5A, lanes 4–6). In the FN-stimulated FAK$^-$ cells, the Clone 28 signal is higher than that observed in the FN-stimulated FAK$^+$ cells (Figure 5B, lanes 2 and 5). However, the increased level of c-Src-associated IVK activity upon FN stimulation of the FAK$^-$ cells was not accompanied by further dephosphorylation of c-Src at Tyr529 in the FAK$^-$ cells (Figure 5B, lanes 4 and 5). Although the total level of c-Src activity was higher in the FAK$^-$ cells, a similar 2- to 3-fold increase in FN-stimulated c-Src activity was measured between the suspended and FN-stimulated points in the FAK$^+$ and FAK$^-$ cells, respectively (Figure 5A). These results suggest that increased levels of c-Src-associated IVK activity may also play a role in a compensatory signaling pathway in the FAK$^-$ cells.

Pathways of FN-stimulated signaling to ERK2 in the FAK$^+$ and FAK$^-$ cells

FN-stimulated FAK autophosphorylation promotes Src-family PTK binding and the Src-mediated phosphorylation of FAK at sites such as Tyr925. Grb2 binding to FAK at Tyr925 is one of several Grb2-mediated FN-stimulated signaling pathways to ERK2 (Schlaepfer *et al*., 1998). To visualize the tyrosine-phosphorylated proteins specifically associated with Grb2 in the FAK$^+$ cells, anti-P.Tyr blotting was used to analyze Grb2 IPs made from lysates of suspended, FN, and PL-plated cells (Figure 6A). FN stimulation of FAK$^+$ cells promoted the association of ~116, 52 and 46 kDa tyrosine-phosphorylated proteins with Grb2 (Figure 6A, lanes 1–3). The 116 kDa protein was positively identified as FAK (data not shown). Similar replating experiments with FAK$^-$ cells showed that only a pair of 52 and 46 kDa tyrosine-phosphorylated co-immunoprecipitated with antibodies to Grb2 after FN stimulation (Figure 6A, lanes 4–6). These proteins were positively identified as p52 and p46 Shc (Figure 6A, lanes 7–9). Although Pyk2 contains a consensus Grb2 binding site at Tyr881 and the GST–Grb2 SH2 domain weakly binds to Pyk2 in lysates of FN-stimulated FAK$^-$ cells (data not shown), Pyk2 could not be detected in association with Grb2 IPs made from FAK$^-$ cell lysates.

Previous studies have shown that Grb2 binding to Shc is important for the transmission of integrin-stimulated signals to ERK2 (Wary *et al*., 1996; Mainiero *et al*., 1997; Schlaepfer *et al*., 1998). FN stimulation promotes the

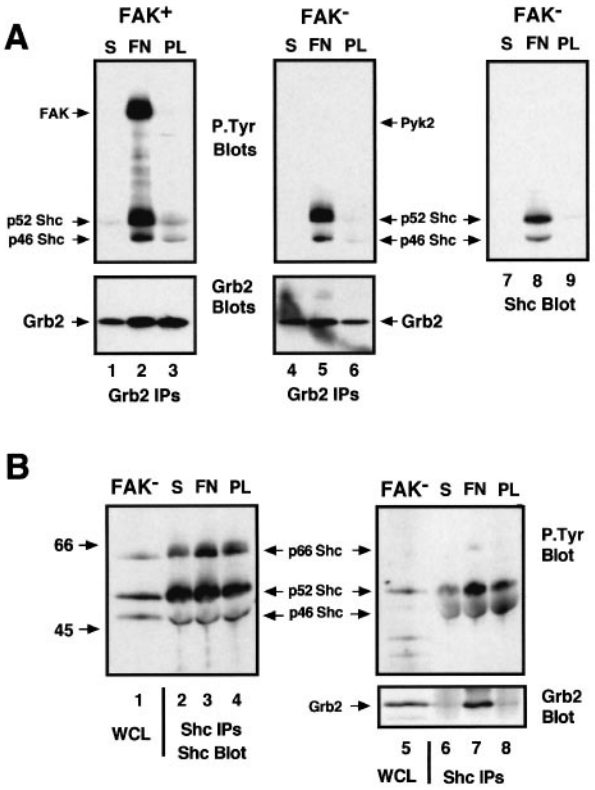


Fig. 6. Grb2-associated proteins in the FAK$^+$ and FAK$^-$ cells. (**A**) Lysates of FAK$^+$ (lanes 1–3) or FAK$^-$ (lanes 4–9) cells were prepared after 1 h in suspension (S) and following plating onto FN- or PL-coated dishes for 30 min. Cell lysates were equalized for protein content (~1 mg total cell protein) and polyclonal Grb2 IPs were resolved by SDS–PAGE and Grb2 was visualized by anti-Grb2 blotting. The proteins associated with Grb2 were visualized by anti-P.Tyr blotting (lanes 1–6) and reprobed by anti-Shc blotting (lanes 7–9). (**B**) Lysate (~100 μg total cell protein) from serum-starved FAK$^-$ cells (lane 1) or Shc IPs from suspended, and FN- or PL-replated FAK$^-$ cells were resolved by SDS–PAGE and Shc was visualized by anti-Shc blotting (lanes 1–4) followed by anti-P.Tyr blotting (lanes 5–8). Lower panel: duplicate Shc IPs were resolved by 15% SDS–PAGE and Grb2 associated with Shc was visualized by anti-Grb2 blotting (lanes 5–8).

association of p52 and p46 Shc with Grb2 in both FAK$^+$ and FAK$^-$ cells (Figure 6A). In whole-cell lysates or in Shc IPs, three Shc isoforms of p46, p52 and p66 were expressed in the FAK$^-$ cells (Figure 6B, lanes 1–4). Anti-P.Tyr immunoblotting showed that p52 Shc exhibited the highest P.Tyr level after FN stimulation of the FAK$^-$ cells (Figure 6B, lane 7) and no other P.Tyr-containing proteins were detectably associated with Shc in the FAK$^-$ cells (data not shown). Increased p52 Shc tyrosine phosphorylation after FN stimulation of the FAK$^-$ cells paralleled the time-course of increased Pyk2 tyrosine phosphorylation and ERK2 activation (data not shown; Figure 1D). By anti-Grb2 immunoblotting of the Shc IPs, Grb2 was detected in association with Shc only after FN-stimulation of the FAK$^-$ cells (Figure 6B, lane 7). Thus, Grb2 binding to Shc may be a major signaling pathway promoting FN-stimulated ERK2 activation in the FAK$^-$ cells.

Src-family PTKs regulate enhanced Pyk2 tyrosine phosphorylation, Grb2 binding to Shc and ERK2 activation after FN-stimulation of FAK$^-$ cells

Previous studies have shown that FN-stimulated FAK and c-Src PTK activity can promote the phosphorylation of

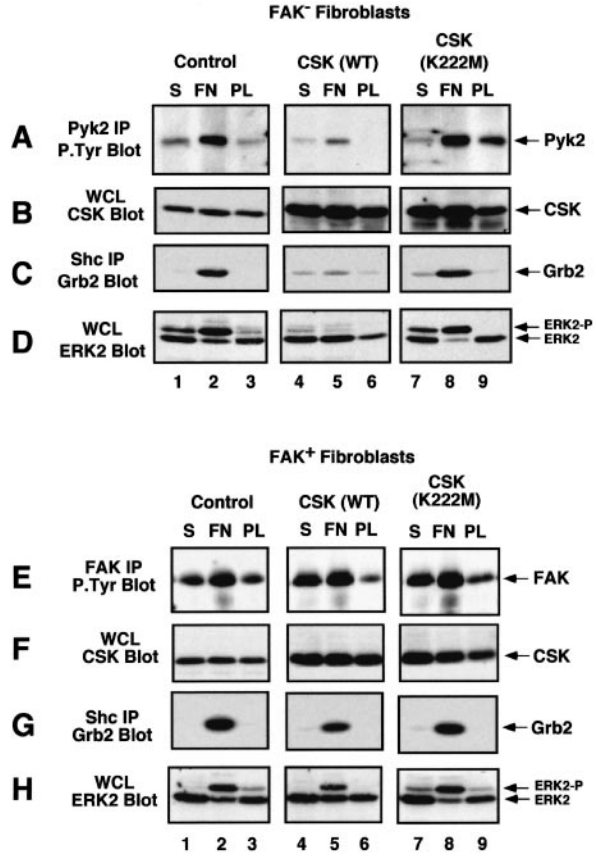


Fig. 7. Regulation of FN-stimulated signaling in the FAK⁻ and FAK⁺ fibroblasts. Lysates of FAK⁻ (A–D) or FAK⁺ (E–H) cells were prepared after incubation (45 min) in suspension (S) and following plating onto FN- or PL-coated dishes for 30 min (lanes 1–3). Replating assays were also performed after retroviral infection with either wild-type (WT) $p50^{csk}$ (lanes 4–6) or kinase-inactive (K222M) $p50^{csk}$ (lanes 7–9). Pyk2 IPs from FAK⁻ cells (**A**) or FAK IPs from FAK⁺ cells (**E**) were analyzed by anti-P.Tyr blotting. Whole cell lysates (~100 μg) were analyzed by either anti-CSK blotting (**B** and **F**) or by anti-ERK2 blotting (**D** and **H**). Shc IPs (from ~1 mg WCL) were analyzed by anti-Grb2 blotting (**C** and **G**). Data presented are representative of two separate infection and replating experiments for both the FAK⁻ and FAK⁺ cells.

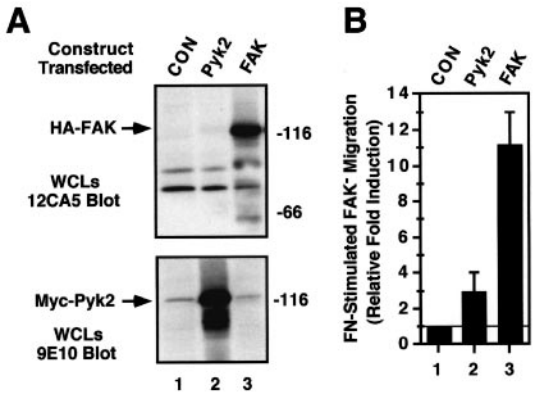


Fig. 8. Stimulation of FAK⁻ cell migration toward FN. (**A**) FAK⁻ cells were transiently transfected with either pcDNA3.1 control vector (lane 1) Myc-tagged Pyk2 (lane 2), or HA-tagged FAK (lane 3). Expression of the transfected proteins was visualized by either anti-HA blotting (12CA5 mAb) or by anti-Myc blotting (9E10 mAb) of ~100 μg WCLs. (**B**) Transiently transfected cells were placed into modified Boyden chambers and the number of migrating cells to immobilized FN (10 μg/ml) after 3 h were determined as described in Materials and methods. Trypan blue exclusion assays demonstrated that all transfected cells utilized were >95% viable. Background cell migration to 0.5% BSA was found to be <0.1%. Relative fold induction was calculated by normalization of the transfection-induced number of migrating cells to that of the number of migrating controls cells in each separate experiment. Each bar represents the mean ± SD for triplicates from five separate experiments.

multiple Grb2 binding sites on Shc (Schlaepfer *et al*., 1998). Since transient WT Pyk2 overexpression in the FAK⁻ cells enhanced the level of FN-stimulated endogenous Shc tyrosine phosphorylation (data not shown), replating assays were performed after transient retroviral-mediated $p50^{csk}$ overexpression to constrain the level of Src-family PTK activation and to test whether Src-family PTK activity is important for FN-stimulated signaling events in the FAK⁻ cells (Figure 7A–D). Phosphorylation of Src-family PTKs by $p50^{csk}$ negatively regulates Src kinase activity (for a review see Thomas and Brugge, 1997). Anti-C-terminal Src kinase (anti-CSK) blotting of WCLs showed that $p50^{csk}$ expression in the FAK⁻ cells was elevated ~3- to 4-fold after infection with either WT or kinase-inactive K222M $p50^{csk}$ retroviral vectors (Figure 7B). Anti-Src C-terminal-directed Clone 28 blotting of FAK⁻ WCLs showed decreased c-Src/Fyn reactivity after WT $p50^{csk}$ compared with either control or K222M $p50^{csk}$-infected FAK⁻ cells confirming the activity of transiently expressed $p50^{csk}$ in these assays (data not shown).

WT-, but not K222M $p50^{csk}$-overexpression dramatically inhibited FN-stimulated FAK⁻ cell spreading after attachment to FN (data not shown). Compared with controls (Figure 7A–D, lane 2), WT $p50^{csk}$ overexpression also reduced FN-stimulated Pyk2 tyrosine phosphorylation, Grb2 binding to Shc and ERK2 activation (Figure 7A–D, lane 5) whereas K222M $p50^{csk}$ expression did not affect these FN-stimulated events (Figure 7A–D, lane 8). As measured by *in vitro* GST–FAK C-terminal phosphorylation, overexpression of WT, but not K222M $p50^{csk}$, reduced the level of FN-stimulated c-Src IVK activity ~4- to 5-fold compared with controls (data not shown). Treatment of suspended FAK⁻ cells with the Src-specific PP1 (Hanke *et al*., 1996) PTK (10 μM) inhibitor had identical effects on FN-stimulated Pyk2 tyrosine phosphorylation, Grb2 binding to Shc and ERK2 activation as $p50^{csk}$ overexpression in the FAK⁻ cells (data not shown). Thus, although Pyk2 kinase activity is important for enhanced FN-stimulated signaling to ERK2 in the FAK⁻ cells, these results support the conclusion that Src-family PTKs may regulate the levels of Pyk2 tyrosine phosphorylation and signaling to ERK2 in the absence of FAK.

To determine whether Src-family PTK activation is also essential for FN-stimulated ERK2 activation in the FAK⁺ cells, replating assays also were performed under similar conditions of retroviral-mediated $p50^{csk}$ overexpression (Figure 7E–H). WT $p50^{csk}$ overexpression did not detectably affect FAK⁺ cell spreading after attachment to FN (data not shown) and the level of FAK tyrosine phosphorylation after FN stimulation of the $p50^{csk}$-infected FAK⁺ cells was similar to that observed in control FAK⁺ cells (Figure 7E, lanes 2 and 5). This result contrasts with the dramatic reduction in Pyk2 P.Tyr levels after $p50^{csk}$ infection and FN stimulation of FAK⁻ cells (Figure 7A,

lane 5). In the FAK$^+$ cells, WT p50^{csk} overexpression reduced the amount of Grb2 binding to Shc and FN-stimulated ERK2 activation ~2- to 3-fold compared with controls (Figure 7E–H, lanes 2 and 5) whereas K222M p50^{csk} expression did not affect these FN-stimulated events (Figure 7E–H, lane 8).

Results similar to WT p50^{csk} overexpression were also obtained when FAK$^+$ replating was performed in the presence of 10 μM PP1 (data not shown). Although the partial reduction in FN-stimulated ERK2 activation in the FAK$^+$ cells overexpressing p50^{csk} is consistent with that measured upon inhibition of c-Src PTK activity in NIH 3T3 fibroblasts (Schlaepfer *et al*., 1998), FN-stimulated signaling events in FAK$^+$ cells overexpressing p50^{csk} are higher than measured in Src-deficient fibroblasts (Schlaepfer *et al*., 1997). The incomplete WT p50^{csk} inhibition of FN-stimulated signaling events in the FAK$^+$ cells is consistent with studies performed in NIH 3T3 fibroblasts where direct FN-stimulated FAK-mediated phosphorylation of Shc can promote Grb2 binding and signaling to ERK2 under conditions of inhibited Src PTK activity (Schlaepfer *et al*., 1998). In addition, the results from these p50^{csk} overexpression studies show that the FN-stimulated linkage leading to increased FAK and Pyk2 tyrosine phosphorylation differ between the FAK$^+$ and FAK$^-$ cells.

Pyk2 overexpression is unable to rescue the FAK$^-$ cell-migration defect

Compared with FAK$^+$ cells, the FAK$^-$ fibroblasts exhibit enhanced focal contact formation and decreased rates of cell migration *in vitro* (Ilić *et al*., 1995). ERK2 activation has been shown to be important for both collagen (Klemke *et al*., 1997) and FN-stimulated cell migration events (Anand-Apte *et al*., 1997). Since both Pyk2 and FAK expression can enhance FN-stimulated ERK2 activity in the FAK$^-$ cells, FAK$^-$ cell migration toward FN was measured after the transient overexpression of either Pyk2 or FAK (Figure 8). Immunoblot analyses to the epitope tags verified equivalent levels of either Myc-Pyk2 (Figure 8A, lane 2) and HA-FAK (Figure 8A, lane 3) were being expressed in the FAK$^-$ cells used in the migration assays.

As analyzed by a Transwell chamber haptotaxis migration assay to immobilized FN, overexpression of Pyk2 resulted in a 2- to 3-fold increase in the number of migrating cells compared with FAK$^-$ controls (Figure 8B, lane 2). However, transient FAK expression dramatically enhanced FAK$^-$ cell migration ~10- to 12-fold greater than control FAK$^-$ cells and ~3- to 4-fold greater than FAK$^-$ cells overexpressing Pyk2 (Figure 8B, lane 3). The number of migrating FAK-transfected FAK$^-$ cells was ~75% of the number of migrating control β-galactosidase transfected FAK$^+$ cells (data not shown). In addition, the FAK-enhanced level of FAK$^-$ cell migration was partially dependent upon FAK kinase activity and completely dependent upon FAK tyrosine phosphorylation at Tyr397 (data not shown). Thus, while Pyk2 has the ability to enhance FN-stimulated signaling to ERK2 in the absence of FAK (Figure 6), Pyk2 can only partially compensate for the defect in migration that is observed in FAK$^-$ cells. In addition, these results suggest that the Pyk2-elevated levels of FN-stimulated ERK2 activity are not sufficient to overcome the migration defect of the FAK$^-$ cells.

Discussion

Integrin signaling in FAK-deficient fibroblasts

The FAK PTK links transmembrane integrin receptors to an intracellular signaling network which can promote the activation of downstream targets such as the ERK2/MAP kinase. Previous studies have shown that this signaling network downstream of integrins in fibroblasts consists of FAK, Src-family PTKs and SH2/SH3 domain-containing adaptor proteins such as Shc, Grb2, p130^{Cas}, Nck and Crk (Schlaepfer and Hunter, 1998). One major finding of this present study is that FN stimulation of FAK$^-$ fibroblasts can activate ERK2, indicating that FAK is not essential for FN-receptor integrin-stimulated signaling to ERK2. This result is consistent with previous studies showing that either antibody-mediated clustering of integrins (Wary *et al*., 1996) or plating of NIH 3T3 fibroblasts on integrin binding fragments of FN (Lin *et al*., 1997) could activate ERK2 in the absence of detectable FAK tyrosine phosphorylation. However, in the absence of FAK, the expression of the FAK-related PTK, Pyk2, was elevated in the FAK$^-$ cells. Although our results do not provide evidence that Pyk2 is essential for ERK2 activation in the FAK$^-$ cells, we show that Pyk2 and Src-family PTK activities combine to promote FN receptor-stimulated signaling to ERK2 in the absence of FAK.

Pyk2 localization and tyrosine phosphorylation in the FAK$^-$ cells

Pyk2 is expressed in both the FAK$^+$ and FAK$^-$ primary mouse embryo fibroblasts. As compared by SDS–PAGE, the ~110 kDa Pyk2 band detected in the FAK$^-$ cells migrated slightly slower compared with Pyk2 expressed in mouse macrophages (D.Schlaepfer, unpublished results). Therefore, it is likely that the FAK$^-$ fibroblast-expressed Pyk2 protein represents full-length rather than the alternative splice variant of Pyk2 expressed in hematopoietic cells (Dikic and Schlessinger, 1998; Li *et al*., 1998). Pyk2 expression is greatly elevated in the FAK$^-$ compared with the FAK$^+$ fibroblasts. Although previous studies have linked Pyk2 overexpression with increased levels of apoptosis of cultured cells (Xiong and Parsons, 1997), our studies do not agree with these findings since the FAK$^-$ cells expressing high levels of Pyk2 proliferate readily in cell culture.

Interestingly, whereas FAK primarily co-localizes with integrins at regions of cell–substratum contact upon FN stimulation and in unstimulated cells, Pyk2 exhibited a punctate perinuclear distribution in both FN-stimulated FAK$^+$ and FAK$^-$ cells. This lack of a strong Pyk2 co-localization with integrins is surprising since Pyk2 can bind to paxillin (Salgia *et al*., 1996; Li and Earp, 1997) and paxillin binding to FAK has been hypothesized to be important for FAK localization to cellular focal contacts (Tachibana *et al*., 1995). However, our Pyk2 localization results are in agreement with the perinuclear distribution of Pyk2 in rat fibroblasts (Sasaki *et al*., 1995; Matsuya *et al*., 1998) and in rat smooth muscle cells (Zheng *et al*., 1998).

Since the distribution of Pyk2 is primarily perinuclear

in both the FAK^+ and FAK^- cells, then a compensatory mechanism of Pyk2 action in the FAK^- cells does not involve a significant change in Pyk2 localization. What may be important is the enhancement of Pyk2 tyrosine phosphorylation by FN receptor stimulation which occurred in the FAK^- but not in the FAK^+ cells. In the FAK^- cells, the regulation of Pyk2 tyrosine phosphorylation in serum-starved, suspended and FN-stimulated cells was very similar to the adhesion-mediated regulation of FAK tyrosine phosphorylation in the FAK^+ cells. However, analyses of Pyk2 *in vitro* autophosphorylation activity revealed that Pyk2 exhibited a moderate level of IVK activity which was not potently activated by FN stimulation of the FAK^- cells. In contrast, FAK autophosphorylation activity was greatly enhanced upon FN stimulation of the FAK^+ cells. Since FAK localized to sites of integrin receptor clustering in the FAK^+ cells and Pyk2 exhibits a perinuclear distribution in the FAK^- cells, the differences in FN-stimulated FAK and Pyk2 autophosphorylation activities may represent the direct activation of FAK by FN receptor clustering and the indirect enhancement of Pyk2 tyrosine phosphorylation through its association with another PTK.

In the FN-stimulated FAK^- cells, Pyk2 was associated with Src-family PTKs, and, under these conditions, maximal Pyk2-associated IVK activity was measured. Interestingly, other treatments such as FAK^- cell depolarization with KCl lead to the highest levels of Pyk2 tyrosine phosphorylation but yet lower levels of measurable Pyk2-associated IVK activity and no detectable co-association with Src family PTKs. The fact that cellular stimuli can increase Pyk2 tyrosine phosphorylation levels either through the direct activation of Pyk2 IVK activity or through its association with other PTKs makes the analysis of Pyk2 activation events difficult to interpret. However, since FN-stimulated increases in Pyk2 tyrosine phosphorylation were inhibited in FAK^- cells overexpressing $p50^{csk}$ or in the presence of Src-family PTK inhibitors and since FN-stimulated increases in Pyk2-associated IVK activity were not inhibited by BAPTA treatment, we hypothesize that increased FN-stimulated Pyk2 tyrosine phosphorylation in the FAK^- cells results from an association with active Src-family PTKs. This is consistent with increased Pyk2 tyrosine phosphorylation after T-cell receptor stimulation being dependent upon Fyn PTK activity (Qian *et al*., 1997) and increased Pyk2 tyrosine phosphorylation after FcεRI receptor stimulation being dependent upon Syk PTK activity (Okazaki *et al*., 1997). Although Pyk2 can be activated by stimuli that increase intracellular calcium levels in the FAK^- cells, our BAPTA results suggest that these pathways are not essential for both FN-stimulated increases in Pyk2 tyrosine phosphorylation and ERK2 activation in the FAK^- cells.

FN receptor-stimulated signaling pathways in the FAK^+ and FAK^- cells

Previous studies have shown that Ras GTP binding (Clark and Hynes, 1996; Wary *et al*., 1996; Schlaepfer and Hunter, 1997) and multiple Grb2/SOS-mediated signaling pathways downstream of both FAK and Src-family PTKs are important for maximal FN receptor-stimulated ERK2-activation events (Schlaepfer *et al*., 1998). FN stimulation of the FAK^- cells promoted the *in vitro* binding of the SH2 domain of Grb2 bound to Pyk2, but this level of stimulation was not enough to promote the detectable co-immunoprecipitation of Pyk2 with antibodies to Grb2. In contrast, FN stimulation promoted detectable *in vivo* FAK association with Grb2 in the FAK^+ cells. Significantly, a common Grb2-binding target in both the FN-stimulated FAK^+ and FAK^- cells was the tyrosine-phosphorylated p52 Shc adaptor protein. Since the average level of FN-stimulated ERK2 activity was higher in the FAK^+ compared with the FAK^- cells, it is possible that the summation of multiple Grb2-mediated signaling pathways in the FAK^+ cells may promote a higher level of FN-stimulated ERK2 activity.

FAK autophosphorylation is directly stimulated by FN receptor stimulation in the FAK^+ cells. Overexpression of $p50^{csk}$ did not inhibit FN-stimulated FAK tyrosine phosphorylation, Grb2 binding to Shc or ERK2 activation in the FAK^+ cells. These results support the existence of Src-independent signaling pathways to ERK2. In the FAK^- cells, overexpression of $p50^{csk}$ inhibited Pyk2 tyrosine phosphorylation, Grb2 binding to Shc, and ERK2 activation after FN stimulation. These results point to the importance of Src-family PTKs for FN receptor-stimulated signaling events in the absence of FAK. Therefore, from the same FN stimulus, cellular context may dictate which PTK is the primary mediator of FN receptor-stimulated signals to ERK2.

In the FAK^- cells, transient overexpression of either FAK or Pyk2 augmented the level of FN-stimulated signaling to ERK2. For FAK, enhanced FN-stimulated ERK2 activation could result from the direct phosphorylation of Shc to promote Grb2 binding (Schlaepfer *et al*., 1998) or through the elevation of FN-stimulated Src-family PTK activity (Schlaepfer and Hunter, 1997). For Pyk2, both kinase activity and the Tyr402 autophosphorylation/Src SH2 domain binding site were required for Pyk2-enhanced FN-stimulated signaling to ERK2. In PC-12 cells, where Pyk2 overexpression has been shown to enhance G-protein-linked Shc tyrosine phosphorylation and ERK2 activation (Lev *et al*., 1995), Pyk2-enhanced signaling is attenuated by co-expression of $p50^{csk}$ (Dikic *et al*., 1996). Since expression of WT but not Phe402 Pyk2 led to elevated c-Src PTK activity in the FN-stimulated FAK^- cells, we speculate that elevated Pyk2 expression, phosphorylation at Tyr402 and association with Src-family PTKs in the FAK^- cells acts to maintain or extend c-Src PTK activity upon FN stimulation. This Pyk2 enhancement of c-Src IVK activity is independent of Pyk2 kinase activity and is consistent with Src-family PTK activation occurring upon SH2 domain binding to the motif surrounding Pyk2 Tyr402 and displacement of regulatory intramolecular c-Src domain interactions (Xu *et al*., 1997)

Although previous studies have shown that both FAK and c-Src can be independently activated by FN receptor stimulation of cells (Schlaepfer *et al*., 1998), the mechanisms of FN-stimulated Src-family PTK activation in the absence of FAK are not clearly defined. In the FAK^- cells, c-Src IVK activity is elevated in suspended, FN- and PL-replated cells compared with the level of c-Src activity in the FAK^+ cells. One component leading to higher c-Src activity in the FAK^- cells involves the enhanced dephosphorylation of the negative/regulatory $p50^{csk}$

phosphorylation site (Tyr529) in the C-terminal tail region of murine c-Src. Src dephosphorylatiоn at Tyr529 has been previously suggested as a mechanism of FN-stimulated c-Src PTK activation (Kaplan *et al*., 1995). Interestingly, FN stimulation of the FAK$^-$ cells did not cause the further dephosphorylation of c-Src Tyr529 whereas p50^{csk} overexpression in FAK$^-$ cells led to a reduction of FN-stimulated c-Src PTK activation. Therefore, we conclude that dephosphorylation of c-Src Tyr529 plays a permissive role in FAK$^-$ cell c-Src PTK activation events. We speculate that other events such as c-Src phosphorylation in the activation loop within the kinase domain or conformational changes upon SH2 domain binding to target proteins such as Pyk2 also play important roles in the regulation of FN-stimulated c-Src activity.

FN-stimulated FAK$^-$ migration

It has been proposed that ERK2 activation is important for FN-stimulated cell migration (Anand-Apte *et al*., 1997) and targets of elevated ERK2 kinase activity include myosin light chain kinase (MLCK) (Klemke *et al*., 1997) or transcription factors in the nucleus that can promote matrix metalloprotease expression (Simon *et al*., 1996). The FAK$^-$ cells exhibit decreased rates of cell migration potentially due to the fact that they form an increased number of cell–substratum contacts (Ilić *et al*., 1995). FN stimulation of the FAK$^-$ cells promotes ERK2 activation to a level that is slightly less than that measured in the FAK$^+$ cells. Although it is possible that this level of ERK2 activity is below some threshold for cell migration, both Pyk2 and FAK overexpression enhance FN-stimulated ERK2 activity in the FAK$^-$ cells whereas only FAK, but not Pyk2, effectively promotes FAK$^-$ cell migration. Even though the role of ERK2 activation in FAK-mediated migration events is controversial (Klemke *et al*., 1997; Cary *et al*., 1998), we speculate that the differences in the ability of FAK and Pyk2 to stimulate FAK$^-$ cell migration may involve the differential localization of signaling complexes involving FAK to regions of cell–substratum contact.

Since Pyk2 binds to the same signaling molecules such as p130^{Cas} proposed to be important in FAK-mediated cell migration events (Cary *et al*., 1998; Klemke *et al*., 1998) and since studies have shown that only a small fraction of overexpressed Pyk2 localizes to focal contact sites in chicken embryo fibroblasts (Schaller and Sasaki, 1997), we hypothesize that the inability of Pyk2 to stimulate efficient FAK$^-$ cell migration may be related to its observed perinuclear distribution in the FAK$^-$ cells. Thus, the ability of FAK but not Pyk2 to effectively stimulate FAK$^-$ cell migration to FN may involve the localization of FAK-associated proteins to sites of FN receptor clustering leading to the turnover or remodeling of focal contact sites. In this way, the FAK PTK would be at the crossroads of both FN-stimulated signaling and cell migration events.

Materials and methods

Cells

Primary mouse fibroblasts were isolated from embryonic day 8 *fak*$^{+/+}$ or *fak*$^{-/-}$ embryos as described (Ilić *et al*., 1995). Both FAK$^+$ and FAK$^-$ cells carry mutations in the *p53* gene introduced by crossing mice heterozygous for the *FAK* and *p53* alleles as described (Furuta *et al*., 1995). Experiments were performed either on either pools of early passage (5–20) cells or on individual cell clones isolated by dilution plating. Cells were maintained on gelatin-coated [0.1% in phosphate-buffered saline (PBS)] cell culture dishes in Dulbecco's modified Eagle's medium (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).

Reagents, antibodies and fusion proteins

The Src-specific inhibitor PP1 and BAPTA/AM were purchased from Calbiochem (San Diego, CA). All other reagents or chemicals were purchased from Sigma Chemical Co. (St Louis, MO). Polyclonal rabbit anti-FAK antiserum (#5904) was produced against a peptide corresponding to mouse FAK residues 8–27, PNLNHTPSSSTKTHLG-TGME-(C), coupled to keyhole limpet hemocyanin (KLH) through the C-terminal cysteine residue as described (Schlaepfer and Hunter, 1996). No sequence similarity is shared in this N-terminal region of FAK and Pyk2. Rabbit polyclonal anti-Pyk2 antiserum (#5906) was produced to a peptide corresponding to human Pyk2 residues 4–24, VSEPLSRVKLGTLRRPEGPAE-(C) coupled to KLH. The sequence encoding Pyk2 residues Ser-2 through His-106 was amplified by PCR using the primers (5′-AAAGGATCCTCTGGGGTGTCCGAGCC-3′ and 5′-AAAGAATTCTCAGTGCAGCCAGTGGATCTC-3′) with the human Pyk2 cDNA as a template. The ~312 bp PCR product was cloned into the *Bam*HI and *Eco*RI sites of pGEXKT and the resulting GST fusion protein (38 kDa) was expressed and purified from BL-21 *Escherichia coli*. The GST–Pyk2 fusion protein was covalently coupled to glutathione-agarose and used to affinity-purify the anti-Pyk2 antiserum as described (Schlaepfer and Hunter, 1996).

Affinity-purified polyclonal antibodies to ERK2 (C-14), Src-family PTKs (Src-2) and p130^{Cas} (C-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antiserum to Grb2 was produced and affinity-purified as described (Schlaepfer and Hunter, 1996). Rabbit antiserum to human Csk (#5363) was affinity-purified as described (Neet and Hunter, 1995). Rabbit antiserum to Shc and to Fyn were kindly provided by P.van der Geer (UC San Diego) and B.Sefton (The Salk Institute), respectively. Affinity-purified rabbit antibody to Shc was purchased from Upstate Biotechnology (UBI, Lake Placid, NY). Monoclonal antibodies to c-Src (mAb 2–17 and Clone 28) and to ERK2 (mAb B3B9) were kindly provided by S.Simon (The Salk Institute), H.Kawakatsu (University of California at San Francisco) and by M.Weber (University of Virginia), respectively. Monoclonal antibodies to Pyk2 and phosphotyrosine (4G10) were purchased from Transduction Laboratories (Lexington, KY) and UBI, respectively.

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

Cells were serum-starved in DMEM containing 0.5% FBS for 24 h and harvested by limited trypsin/EDTA treatment (0.05% trypsin, 2 mM EDTA, in PBS). The trypsin was inactivated by soybean trypsin inhibitor (0.5 mg/ml) with 0.25% BSA (Fraction V, ICN Biomedicals, Aurora, OH) in DMEM, cells were collected by centrifugation, resuspended in DMEM containing 0.1% BSA and held in suspension for 1 h at 37°C (2×10^5 cells/ml). Cell culture dishes (10 cm) were pre-coated with FN purified from bovine plasma (10 μg/ml) or poly-L-lysine (100 μg/ml) in PBS overnight at 4°C, rinsed with PBS and warmed to 37°C for 1 h prior to replating. Suspended cells were distributed onto ligand-coated dishes (1×10^6 cells per dish), incubated at 37°C, and at various times following plating, the attached cells were rinsed in PBS (4°C) and protein extracts were made in modified RIPA lysis buffer containing 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS as described (Schlaepfer and Hunter, 1996).

Cell lysis, immunoprecipitation and immunoblotting

Antibodies were added to the cell lysates, incubated for 2 h at 4°C and 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 or *in vitro* ^{32}P-labeling.

For immunoblotting, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). Blots were incubated with either 1 μg/ml anti-phosphotyrosine (4G10) or 1 μg/ml monoclonal anti-Pyk2, 1:5000 dilution of monoclonal anti-ERK2, or a 1:1000 dilution of polyclonal anti-FAK, anti-Pyk2, anti-Src, anti-Shc,

anti-Csk, or anti-Grb2 polyclonal antibodies for 2 h at RT. Bound primary antibody was visualized by enhanced chemiluminescent detection and subsequent reprobing of membranes was performed as described (Schlaepfer *et al*., 1998).

Immune complex ^{32}P kinase reactions

Pyk2, FAK, c-Src or Fyn immunoprecipitates (IPs) were washed in Triton-only lysis buffer, followed by HNTG buffer, and then in kinase buffer (20 mM HEPES pH 7.4, 10% glycerol, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 150 mM NaCl). To initiate kinase reactions, excess buffer was removed from the IPs, 2.5 μl of [γ-^{32}P]ATP (3000 Ci/mmol, 10 μCi/μl) was added and the IPs were incubated for 15 min at 32°C (~30 μl total volume). Reactions were stopped with the addition of 2× SDS–PAGE sample buffer and boiled for 2 min. To measure Pyk2-associated IVK activity, 50 μg of poly(Glu–Tyr)(4:1) was added to Pyk2 IPs and kinase reactions were initiated by ATP addition (5 μl, final concentration 20 μM ATP, 10 μCi/nmol [γ-^{32}P]ATP). Reactions were incubated at 32°C for 15 min, resolved by SDS–PAGE, transferred to PVDF membrane, stained with Coomassie Blue, visualized by autoradiography, and the radioactivity associated with poly(Glu:Tyr) determined by Cerenkov counting of the entire gel lane.

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 and then in ERK2 kinase buffer (25 mM HEPES pH 7.4, 10 mM $MgCl_2$). Myelin basic protein (MBP) (2.5 μg) was added to each IP as a substrate. Kinase reactions (~35 μl total volume) were initiated by ATP addition (5 μl, final concentration 20 μM ATP, 10 μCi/nmol [γ-^{32}P]ATP), incubated at 32°C for 10 min and stopped by the addition of 2× SDS–PAGE sample buffer. The phosphorylated MBP was resolved on a 17.5% acrylamide gel, visualized by Coomassie Blue staining followed by autoradiography, and the ^{32}P incorporated into MBP was determined by Cerenkov counting.

To measure c-Src kinase activity, IPs (mAb 2-17 covalently coupled to protein G-agarose) from ~500 μg total cell protein in RIPA lysis buffer were washed in Triton-lysis buffer, followed by HNTG buffer and then in kinase buffer (20 mM PIPES pH 7.0, 10 mM $MnCl_2$, 1 mM DTT). As a substrate, 2.5 μg of purified GST–FAK C-terminal fusion protein was added to each IP. Previous studies have shown that c-Src can phosphorylate FAK tyrosine residues Tyr861 and Tyr925 in the FAK C-terminal domain (Calalb *et al*., 1996; Schlaepfer and Hunter, 1996). Kinase reactions were performed as described for ERK2, were resolved on a 10% acrylamide gel and transferred to a PVDF membrane. GST–FAK was visualized by Coomassie Blue staining followed by autoradiography and the amount of ^{32}P incorporated was determined by Cerenkov counting. Equal recovery of c-Src in the IPs was verified by immunoblotting.

Immunofluorescent staining

Cells were replated on FN-coated (10 μg/ml) glass coverslips and fixed after 2 h in either ice cold acetone for 10 min or with 3.7% paraformaldehyde in PBS. Paraformaldehyde-fixed samples were permeabilized with 0.2% Triton X-100 in PBS for 2 min. Coverslips were washed in PBS and blocked as recommended (Vector Laboratories, Burlingame, CA). Rabbit polyclonal affinity-purified #5906 to Pyk2, A-17 to FAK (Santa Cruz Biotechnology), or mouse anti-vinculin VIN-11-5 (Sigma) primary antibodies at 100 ng/ml were incubated at 4°C overnight in PBS containing either ChromPure goat or donkey IgGs at 100 ng/ml (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). After three washes in PBS, coverslips were incubated for 30 min at 37°C with appropriate secondary antibodies (biotin-conjugated donkey anti-rabbit and rhodamine-conjugated goat anti-mouse) (Jackson Labs Inc.) followed by FITC-streptavidin (Molecular Probes) for 15 min at 37°C. Hoechst 33342 (Molecular Probes) at 10 μg/ml was added to visualize nuclei. Control stainings were performed without either primary or secondary antibodies and the specificity of Pyk2 staining was performed in the presence of excess GST–Pyk2 fusion protein. Slide mounting was done in Vectashield (Vector Labs) and a Zeiss Axiophot equipped with appropriate filters was used for epifluorescence microscopy. Images were photographed with Kodak TMAX 400 film, scanned, and reconstructed using Adobe PhotoShop software.

CSK retroviral production and FAK$^-$ cell infection

293T cells were transiently transfected by standard calcium phosphate methods with 15 μg human CSK or 15 μg kinase-inactive CSK (K222M) cloned into the pSLX-CMV retroviral expression vector along with 10 μg pCL-Eco packaging vector in growth media containing chloroquin (25 μM). The cells were incubated at 37°C (5% CO_2) for 8 h, the precipitate was removed by washing with PBS, and the cells were incubated with DMEM containing 10% calf serum for 72 h after which time the retroviral supernatant was collected and debris removed by 1000 *g* centrifugation. Polybrene (6 μg/ml) was added to the retroviral supernatants and 5 ml was used to infect FAK$^-$ or FAK$^+$ cells in log-phase growth on 10 cm dishes. At 16 h post-infection, the cells were placed into growth media and after 48 h, the cells were placed into starvation media (DMEM with 0.5% FBS) for 18 h prior to further experimental manipulations. Percentage infectivity was measured by the addition of 5 μg pCL-lacZ to the 293T transfections and subsequent staining of the FAK$^-$ cells for β-galactosidase (β-gal) activity.

Myc-tagged Pyk2 and HA-tagged FAK constructs

The human Pyk2 cDNA (generously provided by J.Schlessinger) was subcloned into the *Eco*RI site of pBluescript SK$^+$ (containing mutations in the *Cla*I and *Spe*I sites within the polylinker region). QuickChange oligonucleotide-directed mutagenesis was used to destroy a *Kpn*I site within Pyk2 and add an *Xba*I site following the TGA stop codon. The oligonucleotides (5′-AAAGGTACCGGATCCATGTCTGGGGTGTCC-GAGCCCCTG-3′ and 5′-TTTACTAGTCAGCTGGCGTATCCCTTT-AGG-3′) in combination with PCR were used to add *Kpn*I and *Bam*HI sites to the 5′-end of the Pyk2 cDNA and to destroy an internal *Bam*HI site preceding the *Spe*I site within the Pyk2 cDNA. This ~885 bp PCR Pyk2 fragment was digested with *Kpn*I and *Spe*I and subcloned into the same sites of pBluescript Pyk2. All mutations and manipulations were confirmed by DNA sequencing.

To add human c-Myc epitope tags to the N-terminal of Pyk2, the reading frame following the *Bgl*II site of the pCS3-MT vector (from Jon Cooper, Seattle, WA) was altered by the QuickChange mutagenesis protocol. The ~3 kb *Bam*HI–*Xba*I digested Pyk2 was cloned into *Bgl*II–*Xba*I digested pCS3-MT which adds six Myc epitope tags to the N-terminus of Pyk2. For Myc-tagged Pyk2 protein production, the 3.3 kb *Bam*HI–*Xba*I Pyk2 fragment was cloned into the same sites in the pcDNA3.1 (Invitrogen, La Jolla, CA) mammalian expression vector. Mutagenesis to produce kinase-inactive Ala457 Pyk2 and Phe402 Pyk2 was performed as described (Lev *et al*., 1995; Dikic *et al*., 1996). Hemagglutinin (HA)-epitope tagged murine WT FAK was used as described (Schlaepfer and Hunter, 1996). Transient transfections of either FAK$^+$ or FAK$^-$ cells were performed using Lipofectamine Plus (Gibco-BRL, Gaithersberg, MD) and control pcDNA3-LacZ transfections followed by staining for β-gal activity showed that ~20% of the cell populations could be transfected.

Cell migration assays

Transwell chamber (Costar, Cambridge, MA) migration assays were performed as described previously (Klemke *et al*., 1998). FAK$^-$ cells were transiently transfected with either 2.5 μg WT Pyk2 or 2.5 μg WT FAK along with 2.5 μg pcDNA3-LacZ. Control FAK$^+$ or FAK$^-$ cells were transfected with 2.5 μg pcDNA3 with 2.5 μg pcDNA3-LacZ. Briefly, haptotaxis assays were performed by pre-coating the under surface of the polycarbonate membrane with FN (10 μg/ml in PBS) for 2 h at 37°C. The membrane was washed to remove excess ligand, the lower chamber filled with 0.5 ml DMEM containing 0.5% BSA, and serum-starved (24 h in DMEM with 0.5% FBS) trypsin/EDTA suspended FAK$^-$ cells (1×10^5 cells in 0.1 ml DMEM with 0.5% BSA) were added to the upper chamber. After 3 h at 37°C, 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 and analyzed for β-gal activity using X-gal as a substrate. The number of β-gal-expressing migratory cells were counted using an inverted microscope (cells/field using a 40× objective). Each determination represents the average of three individual wells and error bars represent standard deviation (SD). Background levels of cell migration in the presence of 0.5% BSA were less than 0.1% of FAK$^-$ control cell migration. Data presented are representative of five separate experiments.

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