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# Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn

**Leslie A. Cary, James F. Chang and Jun-Lin Guan***

Cancer Biology Laboratories, Department of Pathology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA

*Author for correspondence

## SUMMARY

**Cellular interactions with the extracellular matrix proteins play important roles in a variety of biological processes. Recent studies suggest that integrin-mediated cell-matrix interaction can transduce biochemical signals across the plasma membrane to regulate cellular functions such as proliferation, differentiation and migration. These studies have implicated a critical role of focal adhesion kinase (FAK) in integrin-mediated signal transduction pathways. We report here that overexpression of FAK in CHO cells increased their migration on fibronectin. A mutation of the major autophosphorylation site Y397 in FAK abolished its ability to stimulate cell migration, while phosphorylation of Y397 in a kinase-defective FAK by endogenous FAK led to increased migration. We also find that the wild-type and the kinase-defective FAK were associated with Src and Fyn in CHO cells whereas the F397 mutant was not. These results directly demonstrate a functional role for FAK in integrin signaling leading to cell migration. They also provide evidence for the functional significance of FAK/Src complex formation in vivo.**

Key words: FAK, Cell migration, Fibronectin, FAK/Src association

## INTRODUCTION

Like cell proliferation and differentiation, cell migration plays a critical role in many biological processes, such as embryonic development, wound healing and tumor metastasis (Hynes and Lander, 1992). In contrast to recent progress in our understanding of the intracellular signaling mechanisms involved in cell division and differentiation, less is known about potential signal transduction pathways leading to cell migration. Studies in many systems indicated that cell migration through the extracellular matrix (ECM) are mediated by cell surface integrin receptors (Hemler, 1990; Ruoslahti, 1991; Hynes, 1992; Brown and Yamada, 1995). Besides serving as a transmembrane linker between ECM and cytoskeleton, integrins are capable of transducing biochemical signals across the plasma membrane (Juliano and Haskill, 1993; Schaller and Parsons, 1993; Clark and Brugge, 1995). Although some of the components of the integrin signaling pathways have been identified, little is known about the molecular mechanisms by which integrin-initiated signal transduction mediates and/or regulates cell migration.

Focal adhesion kinase (FAK) (Schaller et al., 1992; Hanks et al., 1992) has been implicated in integrin signaling pathways (Schaller and Parsons, 1993). The activation of integrins by cell binding to ECM or by cross linking with antibodies leads to an increase in FAK tyrosine phosphorylation (Guan et al., 1991; Kornberg et al., 1991; Burridge et al., 1992) and its kinase activity (Guan and Shalloway, 1992; Lipfert et al., 1992). Furthermore, activation and subsequent autophosphorylation of FAK in response to cell adhesion lead to its association with several intracellular signaling molecules including Src (Cobb et al., 1994; Xing et al., 1994), Grb2 (Schlaepfer et al., 1994) and phosphatidylinositol 3-kinase (Chen and Guan, 1994; Guinebault et al., 1995). The major autophosphorylation site of FAK, Y397, has been determined to be a Src binding site through the SH2 domain of Src (Schaller et al., 1994). The binding site for Grb2 has been mapped to Y925 (Schlaepfer et al., 1994), while the binding site for phosphatidylinositol 3-kinase has not yet been identified. The downstream events and cellular consequences of FAK association with these intracellular signaling molecules are not clear at present, although MAP kinases are known to be activated in cell adhesion (Chen et al., 1994; Schlaepfer et al., 1994).

There are several indications that integrin signaling through FAK might be involved in cell migration. Increased FAK phosphorylation and activity have been observed in migrating endothelial cells, and inhibition of FAK activity by the tyrosine kinase inhibitor tyrphostin blocks their migration into wounded monolayers of cells (Romer et al., 1994). FAK phosphorylation and activity are also regulated by adhesion to the ECM in keratinocytes, and in repairing burn wounds FAK is localized to areas that coincide with rapidly migrating and proliferating keratinocytes (Gates et al., 1994). Furthermore, increased levels of FAK expression have been correlated with the invasive and metastatic potential of human tumors (Weiner et al., 1993; Owens et al., 1995). Finally, Ilic et al. (1995) reported that embryonic cells from FAK-deficient mice exhibited a decreased migration in culture, which was

suggested to be responsible for a defect in mesodermal migration resulting in an embryonic lethal phenotype of the FAK-deficient mice. Taken together, these data suggest strongly that increased expression and/or activation of FAK play a role in cell migration and invasiveness in biological processes such as embryonic development, wound healing and cancer.

To dissect the roles of FAK and its interaction with various cellular proteins in integrin-mediated cell migration, we have overexpressed FAK and its mutants in a Chinese hamster ovary (CHO) cell line. We have found that FAK overexpression caused a significant increase in cell migration on fibronectin (FN), and that autophosphorylation of FAK at Y397 and its subsequent association with Src and Fyn were crucial for this increased migration. These findings establish that at least one of the cellular consequences of FAK-mediated integrin signaling is enhanced cell migration through the ECM. They also provide evidence for a functional significance of FAK/Src complex formation in vivo.

## MATERIALS AND METHODS

### Antibodies

The monoclonal antibody KT3, which recognizes the COOH-terminal epitope tag derived from the SV40 large T antigen (KPPTPPPEPET), has been described previously (MacArthur and Walter, 1984), and was a generous gift from Gernot Walter (UCSD, La Jolla, CA). The rabbit polyclonal anti-FAK serum has also been described previously (Chen and Guan, 1994). The monoclonal anti-phosphotyrosine antibody PY20 was purchased from Transduction Laboratories (Lexington, KY). The anti-Src monoclonal antibody 2-17 was a generous gift from David Shalloway (Cornell University, Ithaca, NY). The rabbit polyclonal anti-Fyn antibody FYN3 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

### Construction of expression plasmids

The cDNAs encoding FAK and its kinase-defective mutant with the epitope tag derived from the final 11 COOH-terminal residues of SV40 large T antigen (KPPTPPPEPET) were kindly provided by J. T. Parsons (University of Virginia, Charlottesville, VA) and were described by Hildebrand et al. (1993). The point mutant F397 was introduced to FAK using site-directed mutagenesis by overlap extension using the polymerase chain reaction, as described previously (Ho et al., 1989). The desired mutation was confirmed by dideoxy DNA sequencing (USB Biochemicals, Cleveland, OH). A second epitope tag derived from the influenza virus hemagglutinin sequence (YPYDVPDYA) was then ligated to the 5′ end of the FAK coding sequence, as described previously (Chen et al., 1995). Epitope-tagged FAK or its mutants were cloned into expression vector pCDM8 (Invitrogen, San Diego, CA) to generate the expression plasmids pCDM8-FAK, pCDM8-KD and pCDM8-F397, respectively.

### Cells and transfections

Chinese hamster ovary (CHO) cells were maintained in F-12 medium (Gibco BRL, Gaithersburg, MD) with 10% FBS (Gibco BRL, Gaithersburg, MD). Cells were transfected with 20 μg of the expression plasmids or pCDM8 and 2 μg of pSV2neo (Southern and Berg, 1982), using LipofectACE (Gibco BRL, Gaithersburg, MD). Two days after transfection, 0.5 mg/ml of G418 (Gibco BRL, Gaithersburg, MD) was added to the growth medium. Neomycin-resistant cells were selected after approximately 14 days as either colonies or pools, from which colonies were isolated later. The clones were then analyzed for expression of exogenous FAK by immunostaining and western blotting using KT3. Multiple positive clones were obtained for further analysis for each expression plasmid in two independent transfections.

### Immunoprecipitations, western blotting and kinase assays

Lysates were prepared from cells growing as monolayers or in suspension. Cells were washed with cold phosphate-buffered saline (PBS) and lysed in NP-40 lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, and 1 mM $Na_3VO_4$) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.1 trypsin inhibitory units/ml aprotinin and 20 μg/ml leupeptin). The lysates were centrifuged for 10 minutes at 4°C to remove debris, and the protein concentrations were determined using the Bio-Rad Protein Assay (Hercules, CA). For immunoprecipitations, lysates were incubated with primary antibody for 1 hour at 4°C (for KT3) or for 5 hours at 4°C (for anti-Src or anti-Fyn). For monoclonal antibodies, Protein A-Sepharose beads coupled to rabbit anti-mouse IgG were added for 1 hour at 4°C. For polyclonal antibodies, Protein A-Sepharose beads were added for 1 hour at 4°C. The beads were then washed 4 times with lysis buffer, and the precipitates were eluted by boiling for 5 minutes in SDS sample buffer. The proteins were resolved by SDS-PAGE and transferred to nitrocellulose (Schleicher and Schuell, Inc., Keene, NH). Western blots were carried out as described previously (Guan et al., 1991), using horseradish peroxidase-conjugated IgG (donkey anti-rabbit or sheep anti-mouse, Amersham, Arlington Heights, IL) as a secondary antibody and the Amersham Enhanced Chemiluminescence system (Arlington Heights, IL) for detection. Assays to determine kinase activity were carried out as described previously (Guan and Shalloway, 1992).

### Migration assays

Human plasma FN, mouse Type IV collagen and mouse vitronectin were purchased from Gibco BRL (Gaithersburg, MD). Mouse laminin was from Sigma (St Louis, MO). Migration experiments were carried out in a Neuro Probe 48-well chemotaxis chamber (Cabin John, MD). Various extracellular matrix proteins in F-12 medium were added to the lower chamber; F-12 medium only was added to some wells as a control. Cells were harvested by trypsinization, washed once with F-12 medium containing 0.5 mg/ml soybean trypsin inhibitor (Sigma, St Louis, MO), and then washed twice with F-12 medium. Cells were resuspended in F-12 medium and added to the upper chamber at $1.25\times10^4$ cells per well. The lower and upper chambers were separated by a polycarbonate membrane (8 μm pore size, Poretics, Livermore, CA). Cells were allowed to migrate for 3, 6 or 9 hours at 37°C in a humidified atmosphere containing 5% $CO_2$. The membrane was fixed in methanol for 8 minutes and stained with modified Giemsa stain (Sigma, St Louis, MO) for 1 hour. Cells on the upper side of the membrane were then removed mechanically. Cells on the lower side of the membrane were enumerated using a light microscope at ×200 magnification. One data point comprised the average number of cells in two random fields (each counted twice). In some experiments, FN was coated onto both sides of the membrane overnight at 4°C prior to use.

### Adhesion assays

Adhesion assays were carried out in 96-well Immulon-4 microtiter plates (Dynatech Laboratories, Chantily, VA). The plates were coated overnight at 4°C with human plasma FN (Gibco BRL, Gaithersburg, MD) at various concentrations in PBS, washed twice with PBS, and blocked with 2 mg/ml heat-inactivated BSA (Sigma, St Louis, MO) for 1 to 2 hours at 37°C. Cells were harvested by trypsinization, washed once with F-12 medium containing 0.5 mg/ml soybean trypsin inhibitor (Sigma, St Louis, MO), and then washed twice with F-12 medium. Cells were resuspended in F-12 medium and added to the coated plate at $5\times10^4$ to $1\times10^5$ cells per well. After 30 minutes at 37°C in a humidified atmosphere containing 5% $CO_2$, nonadherent cells

were washed twice with PBS, and adherent cells were fixed with 70% ethanol for 20 minutes. Cells were stained with 5 mg/ml crystal violet in 20% methanol for 10 minutes and washed 4 times with PBS. Bound stain was extracted with 0.1 M sodium citrate, pH 4.2, for 30 minutes, and the optical density (OD) was read at 562 nm. The background OD value from a well without cells was subtracted from the OD value for each well, and for each cell type the resulting values were normalized to 100% adhesion at 8 μg/ml. The percent adhesion was then determined for each cell type at each FN concentration.

## RESULTS

### Overexpression of FAK increases CHO cell migration on FN

In order to determine the cellular consequences of integrin signaling through FAK, CHO cells which stably express exogenous FAK were generated. CHO cells were co-transfected with a plasmid encoding chicken *FAK* cDNA and a plasmid encoding the neomycin-resistance gene. The exogenous FAK was 'tagged' with an epitope which is recognized by the monoclonal antibody KT3 in order to distinguish it from the endogenous hamster FAK. After selection in G418-containing medium, clonal cell lines were screened for FAK expression by immunostaining, and the expression was subsequently verified by SDS-PAGE and western blotting of cell lysates.

An analysis of three representative FAK-expressing clones, designated 'WT', is shown in Fig. 1. A 125 kDa protein which is recognized by the KT3 antibody was detected in all three WT clones (lanes 2-4) but not in a control clone transfected with vector alone (lane 1). An increase in overall FAK expression was also observed in these three WT clones (lanes 6-8) compared with the control clone (lane 5) by western blotting with a polyclonal anti-FAK serum. Densitometric analysis indicated an approximate fourfold level of overexpression of FAK in these clones. A protein of apparent molecular mass 90 kDa, which is recognized by the anti-FAK serum, was observed in all CHO cell clones (lanes 5-8). To confirm that the tagged proteins of 125 kDa shown in lanes 2-4 are recognized by anti-FAK, lysates were immunoprecipitated with KT3 and western blotted with anti-FAK (lanes 9-12).

To examine if integrin signaling through FAK is involved in cell migration, CHO cell clones overexpressing FAK were evaluated in their migration on various matrix proteins using modified Boyden chamber assays, as described in Materials and Methods. As shown in Fig. 2A, all three independent WT clones (b, c and d) exhibited increased migration to FN in comparison with the control cells (a). About 2- to 3-fold increases were observed for the WT clones in response to 3 μg/ml, 6 μg/ml or 12 μg/ml FN, while no apparent cell migration was observed for any of the cells without FN (data not shown and see Fig. 5). Similar levels of increased migration (2- to 3-fold) were observed for the three WT clones in assays where both side of the membranes were coated with FN (data not shown), suggesting that overexpression of FAK increased random migration of CHO cells on FN. The three WT clones studied were obtained from two independent transfections, thus it is unlikely that the increased migration was due to clonal variation. Furthermore, no significant differences were observed between WT clones and the control clone in their migration in response to type IV collagen (Fig. 2B) or to vitronectin (Fig. 2C) at several different concentrations. No apparent cell migration was detected for any of the CHO cells in response to laminin (2.5-20 μg/ml) under the same conditions (data not shown). Therefore the increased migration of CHO cells overexpressing FAK appears to be specific to FN. Fig. 3 shows that the increased migration of WT clones to FN can be observed at 3 hours in the modified Boyden chamber assays, but was more significant after longer incubations (6 and 9 hours).

To determine if the increased migration of WT clones was due to altered cell adhesion to FN, adhesion of the WT clones to FN was also evaluated in standard cell adhesion assays as described in the Materials and Methods. Fig. 4 shows that all three WT clones exhibited adhesion similar to the control clone to FN over a range of concentrations. In addition, no apparent differences in cell spreading between WT clones and control cells were observed (data not shown). Therefore, FAK may not be involved directly in cell adhesion or spreading to FN, and in addition, increased migration of WT cells was not caused by altered cell adhesion to FN.

### Y397 of FAK is necessary for the increased CHO cell migration on FN

To investigate the mechanisms of stimulation of cell migration in CHO cells overexpressing FAK, we examined cellular effects of overexpression of two different FAK mutants, F397 and KD, in CHO cells. F397 is a point mutation of Y397 to F, abolishing the major autophosphorylation site of FAK (Schaller et al., 1994). KD is a kinase-defective FAK variant with a K454 to R mutation in the ATP binding site of the catalytic domain (Hildebrand et al., 1993). The mutants were transfected into CHO cells as described in Materials and Methods. Clonal cell lines were screened for FAK expression

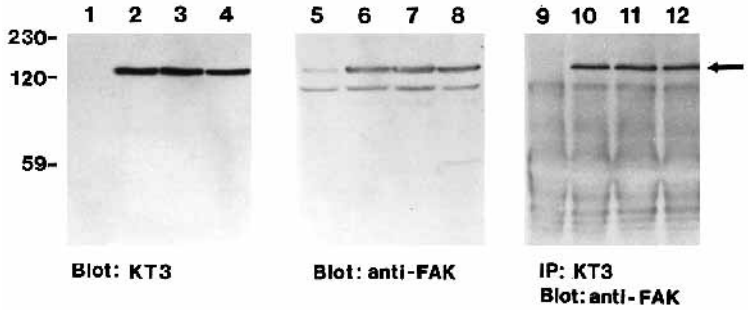


**Fig. 1.** Overexpression of FAK in CHO cells. Lysates from three CHO cell WT clones (lanes 2-4, 6-8 and 10-12) and one control clone (lanes 1, 5 and 9) were analyzed for FAK expression. Lysates were analyzed by SDS-PAGE followed by western blotting with KT3 (lanes 1-4) or anti-FAK (lanes 5-8). Lysates were immunoprecipitated with KT3 and the immune complexes were analyzed by SDS-PAGE followed by western blotting with anti-FAK (lanes 9-12). The positions of molecular mass markers (kDa) are shown on the left. The position of FAK is indicated on the right by an arrow.

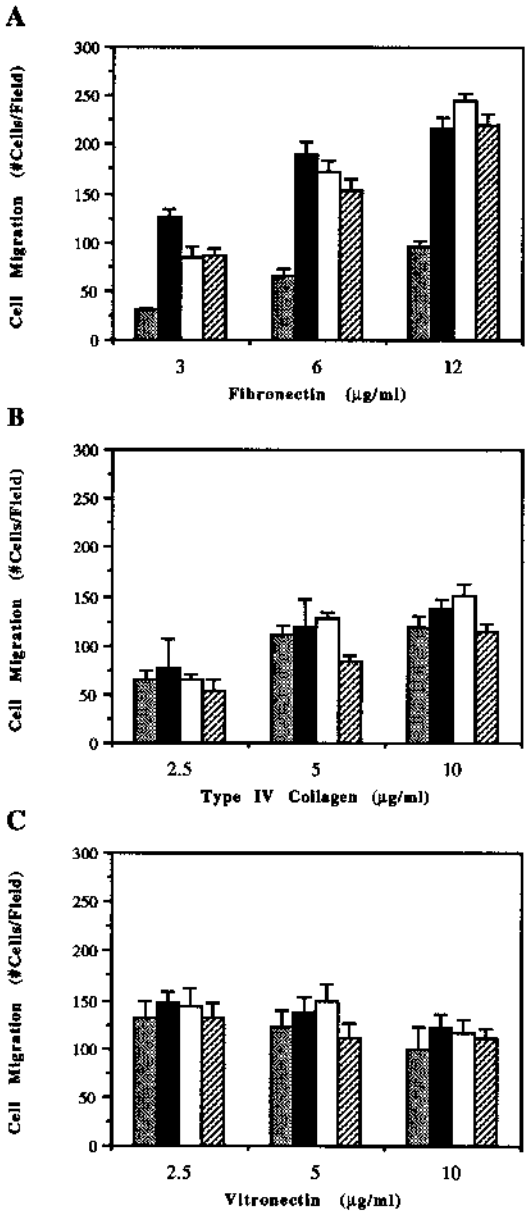


**Fig. 2.** Migration of CHO cells which overexpress FAK. Cell migration of three WT clones (b, filled bars; c, open bars; d, striped bars) and one control clone (a, stippled bar) were analyzed using a modified Boyden chamber. Cells were added to the upper chamber and allowed to migrate for 6 hours through an 8 μm porous membrane towards the lower chamber to which FN (A), type IV collagen (B) or vitronectin (C) had been added at different concentrations, as indicated. Cells which migrated to the lower chamber were fixed, stained and enumerated using a light microscope. Mean data from at least seven data points from four independent experiments are shown. Error bars represent standard errors. The WT clones are the same as those in Fig. 1.

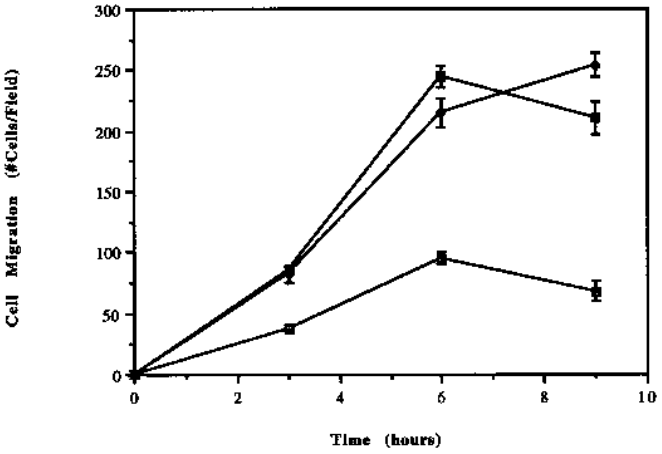


**Fig. 3.** Time course of migration on FN of CHO cells which overexpress FAK. Cell migration assays were performed for one control clone (a, □) and two WT clones (b, ◆; c, ■) using 12 μg/ml FN, as described in Fig. 2. After various times as indicated, cells which migrated to the lower chamber were fixed, stained and enumerated using a light microscope. Mean data from at least seven data points from four independent experiments are shown. Error bars represent standard errors.

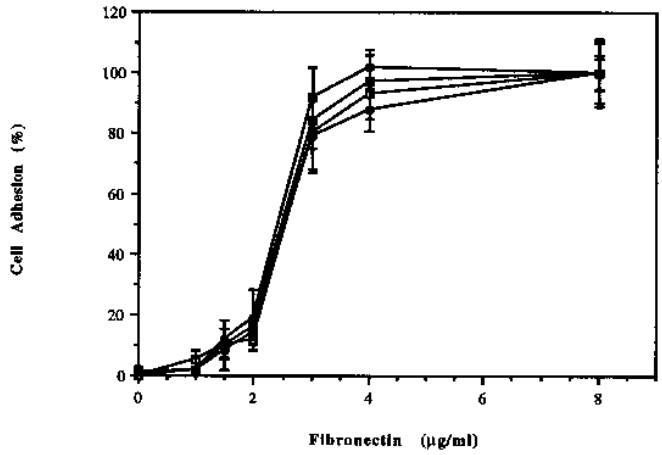


**Fig. 4.** Adhesion of CHO cells which overexpress FAK. Microtiter plates were coated with fibronectin at the indicated concentrations. A control clone (a, ○) and three WT clones (b, □; c, ●; d, ■) were suspended in serum-free F-12 medium and added to the coated plate for 30 minutes. Nonadherent cells were removed by washing. Adherent cells were fixed and stained, and the adhesion was quantified by reading the optical density at 562 nm. Mean data from at least three independent exeriments are shown. Error bars represent standard deviations.

by immunostaining, which was then verified by SDS-PAGE and western blotting of cell lysates. Multiple clones expressing the two mutants were isolated and expanded for analysis from two independent transfections. Analyses of the expression levels by western blotting of representative KD clones and F397 clones indicated a comparable level of exogenous FAK expression as compared with WT clones (data not shown). Immunofluorescent staining with mAb KT3 of cells plated on FN-coated coverslips showed that the wild-type, kinase-defective and F397 FAK were all localized in the focal contacts (data not shown).

To determine the effects of the two FAK mutations on integrin signaling leading to increased cell migration, F397 and KD clones were evaluated by modified Boyden chamber assays using FN as attractants. Fig. 5 shows results from representative clones that overexpress the wild-type, kinase-defective or

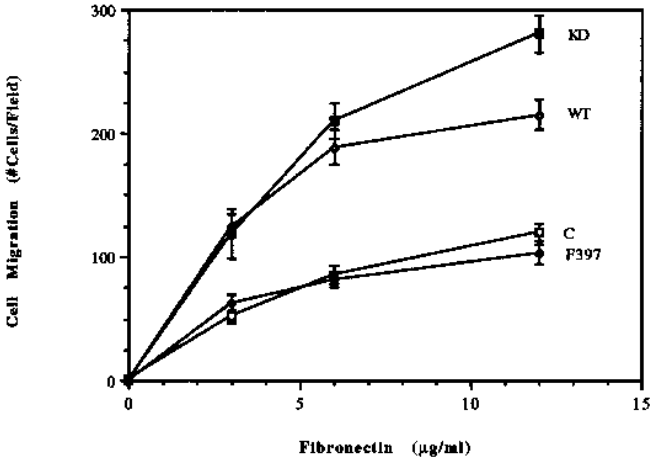


**Fig. 5.** Migration of CHO cells which overexpress wild-type or mutant FAK. Cell migrations of a WT clone, a KD clone, an F397 clone and a control clone (C) were analyzed using a modified Boyden chamber. Cells were added to the upper chamber and allowed to migrate for 6 hours through an 8 μm porous membrane towards the lower chamber to which soluble FN had been added at the indicated concentrations. Cells which migrated to the lower chamber were fixed, stained and enumerated using a light microscope. Mean data from at least ten data points from four independent experiments are shown. Error bars represent standard errors.

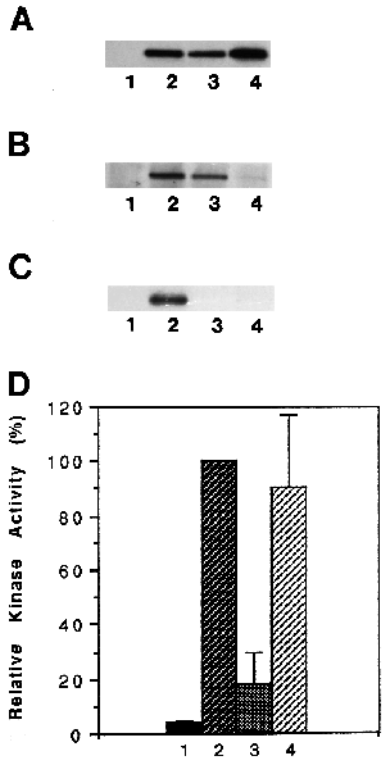


**Fig. 6.** Phosphorylation and kinase activity of exogenous FAK in CHO cells. Cell lysates from a control clone (lane 1), a WT clone (lane 2), a KD clone (lane 3) and an F397 clone (lane 4) were immunoprecipitated with KT3, and part of the immune complexes were analyzed by SDS-PAGE followed by western blotting with KT3 (A) or PY20 (B). Part of the immune complexes were incubated in kinase buffer with [γ-$^{32}$P]ATP, subjected to SDS-PAGE and visualized by autoradiography to determine the in vitro kinase activity (C). The remainder of the immune compexes were incubated in kinase buffer with [γ-$^{32}$P]ATP and poly(Glu,Tyr), subjected to SDS-PAGE and analyzed by autoradiography to determine the in vitro kinase activity to an exogenous substrate. The region containing the labeled substrate was excised and quantified by scintillation counting. Relative kinase activities were normalized to the WT clone. The mean and standard deviations of relative kinase activities from two independent experiments are shown (D).

F397 FAK. As shown above, the WT clone exhibited a significantly increased level of migration as compared with the control clone. The F397 clone demonstrated a level of migration comparable to the basal level of migration of a control clone, indicating that Y397 is necessary for increased cell migration upon integrin signaling through FAK. Unexpectedly, the KD clone also showed an increased level of cell migration over the control clone, which is similar to that of the WT clone at 3 μg/ml or 6 μg/ml FN and slightly higher at 12 μg/ml FN. Therefore, kinase activity of FAK appeared to be dispensable for stimulation of cell migration on FN under our experimental conditions. Similar results were obtained with several independent clones from two transfections of each expression plasmid, excluding the possibility of clonal variation in our results (data not shown). Again, no differences were observed in cell adhesion to FN for CHO cells expressing the wild-type, kinase-defective and F397 FAK (data not shown).

### Y397 phosphorylation may be responsible for the stimulation of cell migration by kinase-defective FAK

Autophosphorylation of FAK upon integrin-mediated cell adhesion may be accomplished by either intramolecular or intermolecular mechanisms. Previous reports have shown transphosphorylation of a kinase-defective FAK by co-transfection of a kinase-active FAK in COS cells (Chan et al. 1994; Eide et al., 1995). Therefore, it is possible that transphosphorylation of the kinase-defective FAK by endogenous hamster FAK is responsible for increased cell migration in KD clones.

To test this possibility, experiments were carried out to determine the in vivo phosphorylation state of the exogenous FAK in CHO cells. Cell lysates were prepared from the representative WT, KD, F397 and control clones. Lysates were immunoprecipitated with KT3, and the immune complexes were western blotted with KT3 to verify expression levels (Fig. 6A), or with an anti-phosphotyrosine antibody, PY20, to determine the in vivo phosphotyrosine levels of the exogenous FAK (Fig. 6B). As expected, the wild-type FAK was highly phosphorylated on tyrosine residues in vivo (Fig. 6B, lane 2) while the majority of phosphorylation of the F397 mutant FAK was abolished (Fig. 6B, lane 4). Interestingly, the kinase-defective FAK was found to be highly phosphorylated in vivo (Fig. 6B, lane 3). Under these conditions (growing monolayer cells), the endogenous hamster FAK was phosphorylated in vivo and exhibited high kinase activity in vitro (data not shown). Therefore, these results suggest a transphosphorylation mechanism for the kinase-defective FAK, as discussed above.

To confirm that the kinase-defective FAK indeed lacks kinase activity, in vitro kinase assays of the KT3 immune complexes were performed either with or without poly(Glu,Tyr) as exogenous substrates. As shown in Fig. 6C, the wild-type FAK was able to autophosphorylate to a high level in vitro (lane 2), while the kinase-defective FAK and F397 mutant FAK showed no autophosphorylation in vitro (lanes 3 and 4, respectively). A longer exposure demonstrated a weak level of autophosphorylation of the F397 mutant FAK (data not shown). Fig. 6D shows that the wild-type (lane 2) and F397 (lane 4) FAK had comparable levels of in vitro kinase activity to the exogenous substrate, while the kinase-defective

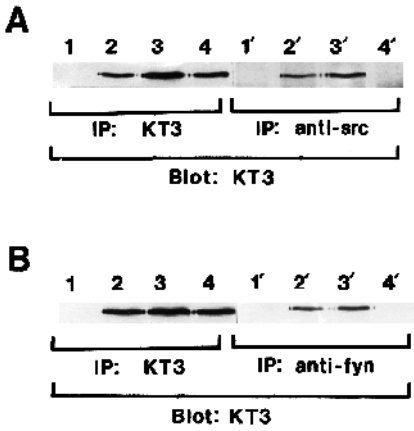


**Fig. 7.** In vivo association of exogenous FAK with Src or Fyn in CHO cells. Lysates from a control clone (lanes 1, 1′), a WT clone (lanes 2, 2′), a KD clone (lanes 3, 3′) and an F397 clone (lanes 4, 4′) were analyzed for exogenous FAK association with Src or Fyn. (A) Lysates were immunoprecipitated with KT3 (lanes 1-4) or the anti-Src antibody 2-17 (lanes 1′-4′), subjected to SDS-PAGE and western blotted with KT3. (B) Lysates were immunoprecipitated with KT3 (lanes 1-4) or the anti-Fyn antibody FYN3 (lanes 1′-4′), subjected to SDS-PAGE and western blotted with KT3.

FAK exhibited significantly reduced activity (lane 3), as expected. The residual activity observed in the immune complex (compare lane 3 with lane 1) may be contributed by the associated Src and/or Fyn (see below). Therefore, the lack of activity of the kinase-deficient FAK was confirmed, indicating that it was not able to autophosphorylate in vivo.

The major autophosphorylation site on FAK, Y397, has been shown to direct its binding to Src in vivo, which may be responsible for triggering downstream events of integrin signaling through FAK (Schaller et al., 1994; Schlaepfer et al., 1994). Thus it is possible that transphosphorylation of kinase-defective FAK by endogenous FAK also occured on Y397, allowing its binding to Src (or src family members) and triggering the signaling cascade responsible for increased CHO cell migration on FN. To test this possibility, we examined if the FAK variants expressed in CHO cells were associated with either Src or Fyn in vivo. To detect FAK association with Src, lysates prepared from the representative WT, KD, F397 and control clones were immunoprecipitated with KT3 or the anti-Src antibody 2-17, and the immune complexes were western blotted with KT3 (Fig. 7A). As expected, the wild-type FAK was associated with Src while the F397 mutant FAK was not (lanes 2′ and 4′). The kinase-defective FAK was associated with Src to a similar extent as the wild-type FAK (compare lanes 3′ and 2′). Similarly, the four lysates were immunoprecipitated with a polyclonal anti-Fyn antibody and western blotted with KT3 to detect FAK associations with Fyn. Fig. 7B shows that both the kinase-defective and the wild-type FAK were associated with Fyn (lanes 3′ and 2′), but the F397 FAK was not (lane 4′). Taken together, these results indicated that the kinase-defective FAK in CHO cells was transphosphorylated on Y397 and formed functional complexes with Src and Fyn, which may be responsible for triggering downstream events leading to increased cell migration on FN.

## DISCUSSION

Recent studies have implicated a critical role of FAK in integrin-mediated signal transduction pathways that regulate various cellular functions (Juliano and Haskill, 1993; Schaller and Parsons, 1993; Clark and Brugge, 1995). In particular, a recent report by Ilic et al. (1995) indicates that FAK plays a role in cell motility. It was shown that embryonic cells from FAK-deficient mice exhibited a decreased migration on FN, which was suggested to be responsible for a defect in mesodermal migration resulting in an embryonic lethal phenotype of the FAK-deficient mice. Consistent with these results, in the present study we have found that overexpression of FAK in CHO cells increased their migration on FN. Furthermore, our system allowed us to dissect the molecular mechanisms involved in FAK-dependent cell motility by analyzing various FAK mutants in promoting cell migration. In this report, we have shown that a mutation of the major autophosphorylation site Y397 in FAK abolished its ability to stimulate cell migration, while phosphorylation of Y397 in a kinase-defective FAK by endogenous FAK led to increased migration. These results suggest that FAK phosphorylation at Y397 is critical in triggering the downstream events in the integrin signaling pathway leading to cell migration. Y397 has previously been determined to be a binding site for the SH2 domain of Src family kinases (Schaller et al., 1994). Therefore, these results suggest that FAK association with Src and/or Fyn is important for integrin signaling through FAK leading to cell migration, even though we cannot exclude completely that other cellular proteins also coupling with FAK at Y397 are responsible for the failure of F397 FAK to promote cell migration.

One surprising finding here is that overexpression of the kinase-defective FAK in CHO cells also stimulated their migration on FN (Fig. 5). Although the mechanism of FAK activation by integrins is still unclear, it is likely to involve oligomerization of FAK with integrins and other cytoskeletal proteins at the focal contacts (Burridge et al., 1988). Treatment of cells with cytochalasin D, which selectively disrupts F-actin filaments, can block FAK activation and phosphorylation induced by integrins binding to ligands or other stimuli (Burridge et al., 1992; Sinnett-Smith et al., 1993). The oligomerization of FAK in response to integrin activation could be mediated by its interactions with cytoskeletal proteins talin (Chen et al., 1995) and/or tensin (Miyamoto et al., 1995), or direct binding to the cytoplasmic domain of integrins (Schaller et al., 1995). It has been shown that the kinase activity of FAK is not necessary for either its localization to focal contacts (Hildebrand et al., 1993; data not shown) or binding to talin (Chen et al., 1995). Therefore, the kinase-defective FAK may form oligomer aggregates together with the endogenous FAK, allowing transphosphorylation of the kinase-defective FAK by the endogenous FAK, which has been shown previously in other cells (Chan et al., 1994; Eide et al., 1995). Complete phosphorylation of the kinase-defective FAK at Y397 will allow it to bind Src and trigger downstream events such as cell migration, as observed (Fig. 5). Indeed, we have found that the kinase-defective FAK was phosphorylated and bound to Src and Fyn as efficiently as the transfected wild-type FAK in vivo (Figs 6 and 7).

The mechanisms by which overexpression of FAK and its association with Src and/or Fyn led to increased cell migration on FN are not understood at present. However, it has been proposed that binding of Src or other src family members (e.g.

Fyn) to FAK at Y397 leads to their activation (Schaller et al., 1994). Therefore it is possible that activation of the kinase complexes stimulated cell migration by acting on the cytoskeleton and/or focal contacts. Increased phosphorylation of cytoskeletal proteins may regulate the ability of these proteins to physically interact with other proteins, thereby modulating the structure of cytoskeleton and/or focal contacts. Remodeling of the cytoskeleton and assembly/disassembly of focal contacts are critical processes of cell migration (Burridge et al., 1988; Stossel, 1993). Two cytoskeletal proteins, paxillin and tensin, are coordinately phosphorylated with FAK during cell adhesion to FN (Burridge et al., 1992; Bockholt and Burridge, 1993). They also colocalize with FAK in focal contacts. They are therefore potential substrates of FAK. Furthermore, paxillin exhibited an increased tyrosine phosphorylation in chicken embryo fibroblasts overexpressing FAK under certain conditions (in the presence of vanadate, a protein tyrosine phosphatase inhibitor) (Schaller and Parsons, 1995). In vanadate-treated CHO cells overexpressing FAK variants, the phosphotyrosine levels of paxillin were also increased in a FAK-dependent manner in WT, KD and F397 clones, although to a lesser extent in F397 cells (data not shown). In contrast, no apparent differences were observed in the phosphotyrosine levels of tensin among these clones (data not shown). These results suggest that paxillin phosphorylation may be partially responsible for the increased migration of CHO cells overexpressing FAK.

It has been suggested that association of FAK at Y397 with Src allows phosphorylation of additional tyrosine residues on FAK, which mediate binding of FAK to other proteins via phosphotyrosine-SH2 interactions (Schlaepfer et al., 1994). The adaptor protein Grb2 has been shown to bind to phosphorylated Y925 of FAK, which is a Src phosphorylation site. Association of Grb2 with FAK at the plasma membrane could then lead to the formation of multiprotein signaling complexes, which promote activation of the Ras signal transduction pathway. Indeed, integrin-mediated cell adhesion has been shown to cause activation of MAP kinases (Chen et al., 1994; Schlaepfer et al., 1994) and altered gene expression (Dike and Farmer, 1988; Tremble et al., 1995). Therefore it is also possible that association of the wild-type and kinase-defective FAK with Src and/or Fyn in CHO cells leads to altered gene expression, such as increased expression of the ECM-degrading metalloproteinases collagenase, stromelysin-1 and 92-kD gelatinase, which may be responsible for increased cell migration (Werb et al., 1989; Huhtala et al., 1995).

It is interesting to note that we did not observe any changes in adhesion or spreading on FN of CHO cells overexpressing FAK or its mutants (Fig. 4 and data not shown). Consistent with this, Hildebrand et al. (1993) have previously reported that overexpression of FAK and its variants in chicken embryo fibroblasts did not cause any change in cellular morphology or cell spreading, although potential differences in cell migration were not examined. Therefore, FAK may not be involved directly in cell adhesion or spreading on FN, even though previous studies have correlated cell spreading with FAK phosphorylation (Guan et al., 1991; Burridge et al., 1992). However, we cannot exclude the possibility that subtle differences in cell adhesion were not detected in this or the previous study because of sensitivity limitations of the assays used. In any case, the cell migration assays described here may be a more sensitive method to dissect the role of FAK and its interactions with other signaling molecules in signal transduction pathways initiated by integrins.

Because FAK has been proposed to be a point of convergence of signaling by integrins, growth factors and other extracellular stimuli (Zachary and Rozengurt, 1992), our results here raised the possibility that FAK may be involved in growth factor-stimulated cell migrations. Furthermore, recent results by Matsumoto et al. (1994) and Abedi et al. (1995) suggested that FAK may play a role in cell migration in response to hepatocyte growth factor/scatter factor and platelet-derived growth factor, respectively. However, epidermal growth factor induced similar migration of CHO cells overexpressing FAK or its mutants in preliminary experiments using the modified Boyden chamber assays (data not shown). It is clear that future experiments are necessary to determine if, in addition to a role in cell migration on FN, intracellular signaling pathways through FAK are also involved in directional migration in response to growth factors or cytokines.

In conclusion, results from this study provided evidence for a functional role of FAK and its association with Src and/or Fyn in an integrin-triggered signaling pathway leading to cell migration. As discussed above, Ilic et al. (1995) have shown that embryonic cells from FAK-deficient mice exhibited a decreased migration in culture, which was suggested to be responsible for a defect in mesodermal migration resulting in an embryonic lethal phenotype of the FAK-deficient mice. Therefore, cell migration triggered by integrin signaling through FAK is likely to play a critical role in normal development. Furthermore, our results also raised the possibility that FAK plays a role in tumor invasion and metastasis. Tyrosine phosphorylation of proteins associated with focal contacts has been reported to mediate cellular invasion by transformed cells (Mueller et al., 1992). The invasive ability of tumor cells has been correlated with their migrations through the ECM (Partin et al., 1989; Aznavoorian et al., 1990). More recently, it was reported that an increased level of FAK expression was highly correlated with the invasive potential of human tumors (Weiner et al., 1993; Owens et al., 1995). Understanding the molecular and cellular mechanisms by which increased FAK expression contributes to embryonic development and tumor progression will clearly be an important area for future investigation.

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