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FAK Regulates Tyrosine Phosphorylation of CAS, Paxillin, and PYK2 in Cells Expressing v-Src, But Is Not a Critical Determinant of v-Src Transformation

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Abstract FAK (focal adhesion kinase) is a nonreceptor protein-tyrosine kinase activated by tyrosine phosphorylation following integrin-mediated cell adhesion. Oncogenic Src promotes enhanced and deregulated FAK tyrosine phosphorylation which has been proposed to contribute to altered cell growth and/or morphological properties associated with transformation. In this study, an inducible FAK expression system was used to study the potential role of FAK in v-Src transformation. Our results portray FAK as a major v-Src substrate that also plays a role in recruiting v-Src to phosphorylate substrates CAS (Crk-associated substrate) and paxillin. The FAK Tyr-397 autophosphorylation site was necessary for this scaffolding function, but was not required for v-Src to stably interact with and phosphorylate FAK. FAK was also shown to negatively regulate v-Src mediated phosphorylation of the FAK-related kinase PYK2. Despite these effects, FAK does not play an essential role in targeting v-Src to major cellular substrates including CAS and paxillin. Nor is FAK strictly required to achieve the altered morphological and growth characteristics of v-Src transformed cells.
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Key words: focal adhesion kinase; Crk-associated substrate; integrin signaling; tetracycline-regulated expression

FAK (focal adhesion kinase) is a nonreceptor protein-tyrosine kinase activated to transduce intracellular signals following integrin-mediated adhesion to extracellular matrix ligands [reviewed by Hanks and Polte, 1997; Schlaepfer et al., 1999]. FAK activation results from it becoming phosphorylated at multiple tyrosine residues. FAK Tyr-397 is the only apparent site of autophosphorylation [Schaller et al., 1994]. Phosphorylated Tyr-397 is a binding site for SH2 domains of Src-family kinases (SFKs) [Schaller et al., 1994; Xing et al., 1994; Polte and Hanks, 1995]. This interaction serves both to recruit SFKs to focal adhesion sites and to activate their catalytic activity by releasing the autoinhibition due to SH2/SH3 intramolecular interactions [Schaller et al., 1999]. The FAK Tyr-397 site also mediates interactions with SH2 domains of other signaling effectors including phosphatidylinositol 3-kinase (PI3K) [Chen et al., 1996], Shc [Schlaepfer et al., 1998], Grb-7 [Han and Guan, 1999], and phospholipase C-γ1 [Zhang et al., 1999]. In addition to Tyr-397, FAK tyrosines 407, 576, 577, 861, and 925 have been identified as sites of phosphorylation by Src [Calalb et al., 1995, 1996; Schlaepfer and Hunter, 1996]. Phosphorylation of these residues in vivo does not occur when Tyr-397 is changed to phenylalanine [Ruest et al., 2000], indicating FAK is phosphorylated by SFKs bound to the autophosphorylation site. Phosphorylation of Tyr-925 promotes an interaction with the adaptor protein Grb2 which can contribute to adhesion-stimulated activation of the ERK2 mitogen-activated protein kinase (MAPK) [Schlaepfer et al., 1994, 1998]. Tyr-576 and Tyr-577 lie in the kinase domain activation loop and mutating these residues reduces FAK autophosphorylation activity

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[Calalb et al., 1995; Owen et al., 1999]. The potential for mutual activation by FAK and SFKs suggests a positive feedback loop leading to full activation of the tyrosine kinase complex. The functional significance of phosphorylation of FAK tyrosines 407 or 861 is currently unknown. In addition to phosphorylating FAK, SFKs recruited to Tyr-397 may be important for phosphorylation of FAK-associated substrates including CAS ($p130^{CAS}$, Crk-associated substrate) [Vuori et al., 1996; Owen et al., 1999] and paxillin [Thomas et al., 1999]. In this regard, FAK may function as a docking protein to recruit SFKs to phosphorylate focal adhesion substrates.

Much evidence indicates that signaling through FAK, requiring the Tyr-397 site, plays a role in diverse cellular responses including spreading and migration [Cary et al., 1996; Gilmore and Romer, 1996; Richardson et al., 1997; Owen et al., 1999; Sieg et al., 1999], anchorage-dependent survival [Frisch et al., 1996; Ilic et al., 1998; Sonoda et al., 2000], and proliferation [Zhao et al., 1998; Oktay et al., 1999]. An important question concerns the role of FAK in the alteration of these properties observed upon cell transformation. FAK phosphotyrosine is elevated in cells transformed by oncogenic Src and is retained when Src-transformed cells are held in suspension [Guan and Shalloway, 1992]. Thus it is conceivable that enhanced and deregulated FAK activity contributes to the altered cell growth and/or morphological properties induced by oncogenic SFKs. In addition to its potential role in oncogenic transformation, FAK has been implicated in cancer pathogenesis based on its overexpression in a variety of human tumors and tumor cell lines [Tremblay et al., 1996; Kornberg, 1998; Licato and Brenner, 1998; Judson et al., 1999].

In this study, we used mouse embryo fibroblasts that stably express v-Src and inducibly express FAK variants to examine the potential role for FAK in regulating the ability of v-Src to target major cellular substrates and to achieve transformed cell properties. Our results confirm FAK as a major v-Src substrate and demonstrate both positive and negative roles for FAK in regulating v-Src phosphorylation of other cellular substrates. Despite these effects, our analysis of v-Src transformed cell properties in the presence or absence of FAK expression do not support an essential role for FAK in promoting v-Src transformation.

MATERIALS AND METHODS

Antibodies

Anti-FAK polyclonal antibody (C-20) was obtained from Santa Cruz Biotechnology. Monocolonal antibodies against avian Src (clone EC10) and phosphotyrosine (clone 4G10) were from Upstate Biotechnology. Monocolonal antibodies against phosphotyrosine (clone PY20), paxillin (clone 349), PYK2 (PYK2/CAKβ, clone 11), and CAS (p130Cas, clone 21) were from BD Transduction Laboratories. HRP-conjugated rabbit anti-mouse and HRP-conjugated goat anti-rabbit antibodies were from BD Transduction Laboratories. Rabbit anti-mouse IgG polyclonal antibody was from Cappel.

Cells and Culture

Parental TetFAK cells, TFW-46 (Tet-FAK(WT), clone 46) and TFF-21 (Tet-FAK(F397), clone 21) are mouse embryo fibroblasts previously derived from FAK-null embryos and engineered to inducibly express WT-FAK or F397-FAK, respectively, under the control of the tetracycline repression system [Owen et al., 1999]. Tet-FAK cells were routinely grown in monolayer culture in DMEM containing 4,500 mg/L D-glucose and 54 mg/L L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml Amphotericin B, 1 mM nonessential amino acids (all from Life Technologies) and 10% fetal bovine serum (FBS) (Atlanta Biologicals). Tetracycline (1 μg/ml) (Calbiochem) was included in the culture media to maintain Tet-FAK cells in the *noninduced* state. FAK expression was induced by replacing the growth media with fresh media lacking tetracycline. Two days after tetracycline withdrawal, the *induced* cells were harvested for biochemical analysis.

To isolate Src-TetFAK(WT) (STFW) clones, noninduced TFW-46 cells were transfected using Lipofectamine (Life Technologies) with plasmid pMv*src* (5 μg plasmid/60-mm dish) that expresses v-Src under control of MoMLV LTR [Johnson et al., 1985]. Two days later, the cells were transfered to a 100-mm dish and given fresh media containing 1 μg/ml tetracycline every two days for a period of 4 weeks, during which transformed foci appeared. In parallel, transfected cell cultures were maintained in media lacking tetracycline to induce FAK expression. The numbers of foci appearing in these induced cultures did not vary significantly

from the noninduced cultures. Identical transfections with plasmid pM5Hc-Src that expresses chicken c-Src [Johnson et al., 1985] did not produce foci above background levels seen with mock transfected cells. About two dozen foci were picked and expanded, and screened for potential v-Src expression by anti-phosphotyrosine immunoblotting to reveal elevated cellular phosphotyrosine content. From these procedures, four STFW clones (7, 21, 22, and 25) that exhibited highest phosphotyrosine levels were selected for further study. STFF (Src-TetFAK(F397)) clones were isolated from pMv*src*-transfected STF-21 cells by co-transfection with pBABE-hygromycin (0.5 μg). Colonies resistent to 200 μg/ml hygromycin were isolated 2 weeks after transfection and screened as above for elevated phosphotyrosine levels. One clone, STFF-18, was selected for further study.

Immunoprecipitation and Immunoblotting

Subconfluent cell cultures, induced or noninduced, were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 1% aprotinin and 0.1 mM Na_3VO_4) and protein concentrations were determined using the BCA assay (Pierce). Lysates equivalent to 20–30 μg protein were diluted in 2× SDS–PAGE sample buffer for immunoblot analysis of whole cell extracts. Immunoprecipitations were carried out from 1 ml RIPA lysates (~500 μg protein). The lysates were incubated 4–12 h on ice using 1 μg primary antibody (followed by 10 μg rabbit anti-mouse IgG polyclonal antibody for monoclonal primaries), and immune complexes were collected on protein-A-Sepharose (25 μl 50% slurry, Zymed). The immunoprecipitates were washed three times with 1 ml ice-cold RIPA buffer, resuspended in 2× SDS–PAGE sample buffer, and processed for immunoblotting.

For immunoblotting, samples were separated on either 7% or 10% SDS–polyacrylamide gels, and transferred to Immobilon-P membrane (Millipore). Nonspecific reactivity was blocked by incubating the membrane for 1 h at room temperature in Tris-buffered saline containing 0.2% Tween 20 (TBST) and either 5% nonfat dry milk or, for phosphotyrosine antibodies, 3% bovine serum albumin, and 1% ovalbumin. Membranes were then incubated overnight with either 1 μg/ml monoclonal antibody or 0.4 μg/ml polyclonal antibody, washed extensively in TBST, and then incubated for 1 h either in 0.1 μg/ml HRP-conjugated rabbit anti-mouse antibody to detect bound monoclonal antibodies or in 0.05 μg/ml HRP-conjugated goat anti-rabbit antibody to detect polyclonal antibodies. After washing in TBST, the blots were developed by enhanced chemiluminescence (Amersham Pharmacia) and exposed to autoradiographic film.

Analysis of Cell Growth in Monolayer and Suspension

The indicated cell lines were maintained either in the presence of tetracycline (1 μg/ml, +tet) or grown for two days in the absence of tetracycline (−tet) to induce WT-FAK expression. Cells were then replated into multiple 100-mm diameter dishes at 1.5×10^5 cells/dish in medium containing either 10% or 0.5% FBS. At 2–4 day intervals following replating, cells were harvested by trypsinization and counted using a Coulter counter. During the course of the experiment, cells were re-fed every second day with fresh media containing the indicated concentration of serum. The re-feeding medium of the +tet cultures also contained fresh tetracycline to maintain the cells in the noninduced state.

For assessing colony formation in suspension culture, cells (1×10^4) were suspended in 1 ml of DMEM containing 10% serum and 0.4% sea plaque agarose (FMC Bioproducts), with or without tetracycline, and poured over a base layer of 0.8% agarose. For noninduced cultures, both agarose layers contained 1 μg/ml tetracycline. The top layer was allowed to set and the suspension cultures were incubated at 37°C. Cultures were fed every four days with media containing 10% serum, with or without tetracycline. Colonies were photographed after four weeks.

RESULTS

Effects of FAK Expression on Phosphorylation of v-Src Substrates

Our studies utilized immortalized TetFAK mouse fibroblasts that were initially derived from FAK −/− embryos [Ilic et al., 1995], and subsequently engineered to allow inducible expression of either wild type (WT) FAK or a FAK variant in which Tyr-397 was changed to phenylalanine (F397-FAK) upon tetracycline withdrawal from the culture medium [Owen

et al., 1999]. TetFAK derivatives stably expressing v-Src were isolated (see Materials and Methods). Those expressing v-Src and WT-FAK were designated STFW (Src-TetFAK(WT)) and those expressing v-Src and mutant F397-FAK were designated STFF (Src-TetFAK(F397)). The parental clones not expressing v-Src are designated TFW-46 and TFF-21, respectively. Representative results are presented for one STFW clone (STFW-25) and one STFF clone (STFF-18). Immunoblotting with an avian-specific Src antibody documented stable v-Src expression in these cells (Fig. 1A, bottom, lanes 3, 4, 7, and 8). The cells retain the characteristic of inducible FAK expression upon tetracycline withdrawal (Fig. 1B, bottom, compare +Tet and −Tet lanes).

v-Src expression in STFW-25 and STFF-18 cells, even in the absence of FAK, was accompanied by a large increase in phosphotyrosine content of numerous cellular proteins as evident from comparison of anti-phosphotyrosine immunoblot profiles with those of parental cells (Fig. 1A, top, compare +Tet lanes of TFW-46 vs. STFW-25 and TFF-21 vs. STFF-18). WT-FAK expression in STFW-25 cells resulted in a visible increase in tyrosine phosphorylation of protein(s) migrating in the 120–130 kDa range, but otherwise did not dramatically alter the overall phosphotyrosine profile. The

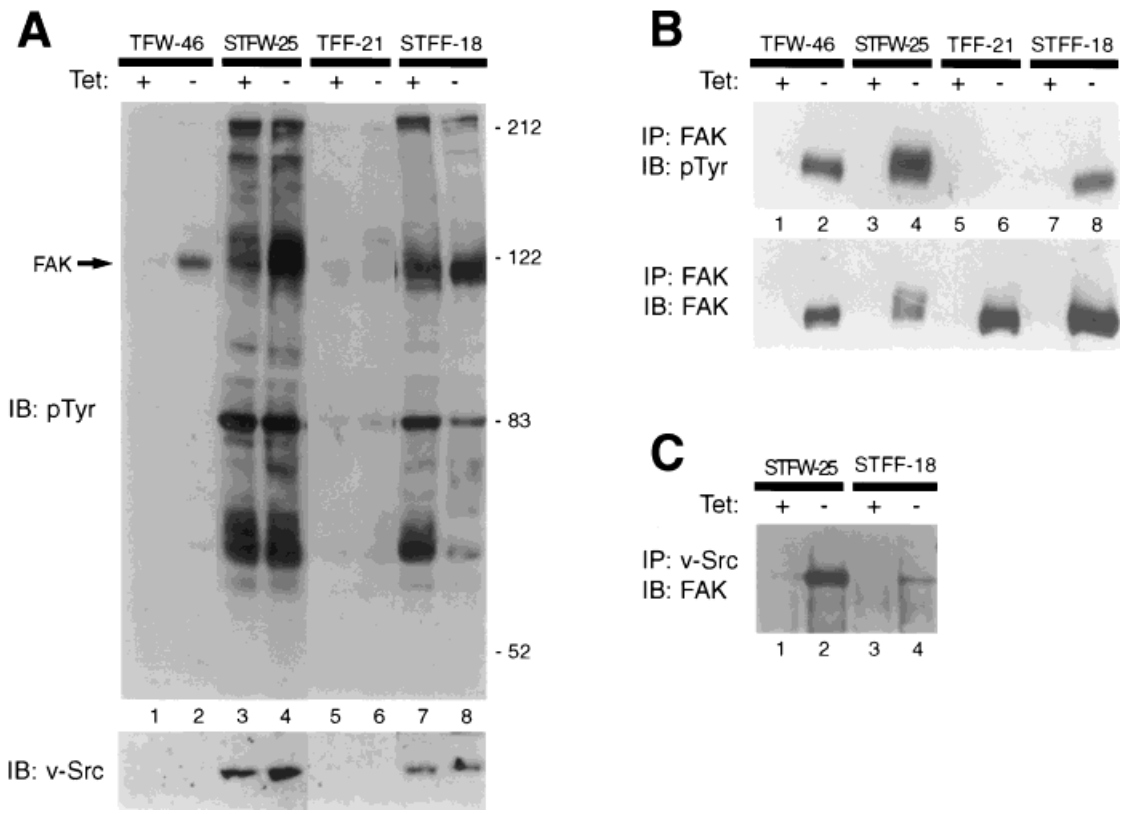

Fig. 1. Effects of v-Src and FAK (WT or F397) expression on cellular protein phosphotyrosine profiles. Exponentially-growing cultures of TFW-46, TFF-21, STFW-25, and STFF-18 cells were either maintained in the presence of tetracycline (Tet+) or were induced by tetracycline withdrawal (Tet−) for 2 days prior to lysis in RIPA buffer. **A**: Immunoblot (IB) detection of tyrosine phosphorylated proteins and v-Src from whole cell lysates. Samples from the lysates (30 μg total protein) were subjected to 8% polyacrylamide SDS–PAGE, transfered to nitrocellulose filters, and analyzed by immunoblotting with monoclonal antibodies against either antiphosphotyrosine (4G10, top panel) or avian Src (EC10, bottom panel). **B**: FAK expression and tyrosine phosphorylation. FAK immunoprecipitates (IP) were prepared from RIPA buffer lysates containing 500 μg total protein using C-20 FAK antibody, then proteins in the immunoprecipitates were separated by 8% polyacrylamide SDS–PAGE, transferred to filters and immunoblotted (IB) with either an antibody against phosphotyrosine (4G10, top panel) or the FAK C-20 antibody (top panel). **C**: Co-precipitation of WT- and F397-FAK with v-Src. v-Src immunoprecipitates (IP) were prepared from RIPA buffer lysates containing 500 μg total protein using EC10 antibody, immunoblots (IB) were prepared as above, and probed with the FAK C-20 antibody. In all panels, immunoblots were developed by enhanced chemiluminescence.

increased anti-phosphotyrosine immunoreactivity at ~120–130 kDa is due in part to antibody recognition of FAK itself, which exhibits increased tyrosine phosphorylation and mobility retardation when examined from the STFW-25 cells (Fig. 1B, compare lanes 2 and 4). WT-FAK expressed in the parental TFW-46 cells migrates as a narrower band of Mr ~120 kDa and appears as the major anti-phosphotyrosine immunoreactive protein in these cells (Fig. 1A, top, lane 2). Although F397-FAK is not significantly tyrosine-phosphorylated in the parental TFF-21 cells, F397-FAK apparently serves as a v-Src substrate and becomes tyrosine phosphorylated when expressed in the STFF-18 cells (Fig. 1B, compare lanes 6 and 8). Again, this could account for the increased anti-phosphotyrosine antibody immunoreactivity observed in the ~120 kDa range when STFF-18 cells are induced (Fig. 1A, compare lanes 7 and 8). Thus it appears that v-Src can be recruited to phosphorylate F397-FAK through a mechanism other than the SH2-mediated interaction with the phosphorylated Tyr-397 site. Further supporting this, F397-FAK is readily detected in v-Src immunoprecipitates (Fig. 1C).

To more specifically address the role of FAK as a docking protein important for recruitment of v-Src to focal adhesion substrates, we examined the phosphotyrosine state in adherent cells of two v-Src substrates, CAS and paxillin, known to directly interact with FAK in focal adhesions. Induced WT-FAK expression in the absence of v-Src expression (parental TFW-46 cells) significantly increased levels of CAS and paxillin phosphotyrosine in adherent subconfluent cells (Fig. 2A,B; compare lanes 1 and 2). CAS and paxillin phosphotyrosine levels were also elevated as a result of v-Src expression in the STFW-25 cells in the absence of FAK, but were more dramatically elevated when WT-FAK was also expressed in these cells (Fig. 2A,B; compare lanes 1–4). For CAS, elevated phosphotyrosine content in FAK-expressing TFW-46 and STFW-25 cells was also apparent when CAS is first immunoprecipitated and then immunoblotted with antiphosphotyrosine antibody (data not shown). In contrast, expression of F397-FAK in the TFF-21 cells did not enhance the phosphotyrosine content of either CAS or paxillin, but instead resulted in slightly reduced tyrosine phosphorylation of these substrates (Fig. 2A,B; compare lanes 5 and 6). F397-FAK

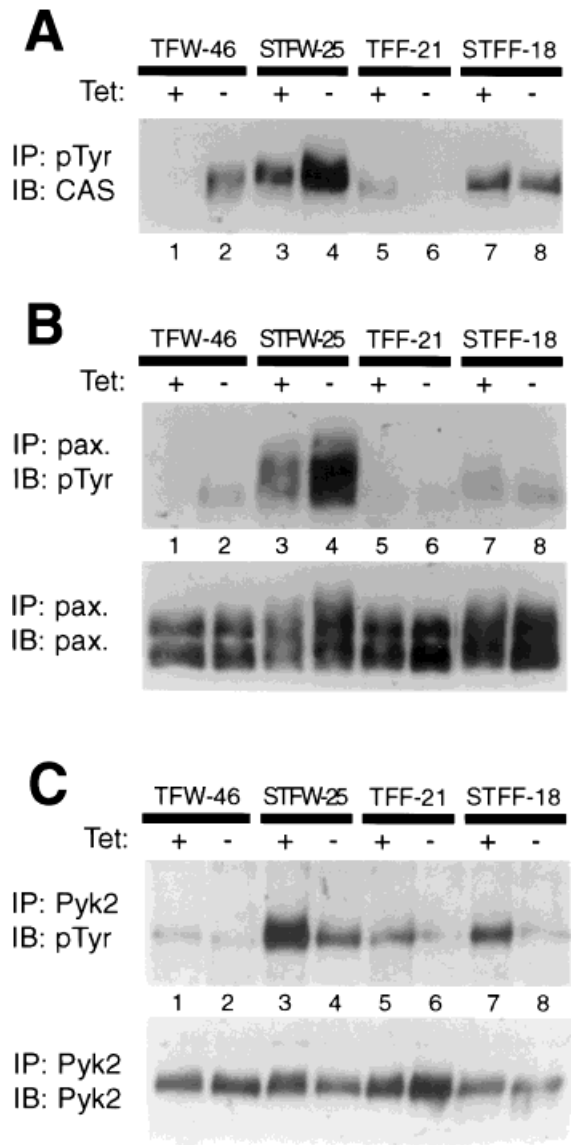

Fig. 2. Analysis of CAS, paxillin, and PYK2, phosphotyrosine levels. RIPA lysates were prepared from exponentially-growing cultures of induced or noninduced TFW-46, TFF-21, STFW-25, or STFF-18 cells and the indicated proteins were immunoprecipitated for analysis of their phosphotyrosine contents. **A**: CAS Immunoprecipitates (IP) were prepared using PY20 antibody against phosphotyrosine, and the relative amounts of tyrosine-phosphorylated CAS was determined by probing immunoblots (IB) prepared from the PY20 immunoprecipitates with an antibody against CAS. Paxillin (**B**) and PYK2 (**C**); Immunoprecipitates were prepared using primary antibodies against paxillin (pax.) or PYK2. For immunoblot analysis, duplicate blots were prepared for the immunoprecipitated proteins and then probed with either an antibody against phosphotyrosine (4G10, top panels) or the same antibody used to form the immunoprecipitate (bottom panels). In all panels, immunoblots were developed by enhanced chemiluminescence.

also did not further elevate the v-Src-enhanced tyrosine phosphorylation of CAS and paxillin in the STFF-18 cells (Fig. 2A,B; compare lanes 7 and 8). These data support a docking function for FAK in promoting CAS and paxillin tyrosine phosphorylation by v-Src, but also indicate that phosphorylation of these substrates by v-Src can occur even in the absence of FAK expression.

The FAK-related kinase PYK2 is expressed in TetFAK cells [Owen et al., 1999] and could possibly compensate for some FAK functions in the absence of FAK expression. It was, therefore, of interest to examine the effect of v-Src on PYK2 tyrosine phosphorylation, both in the presence and absence of FAK expression. In noninduced STFW-25 and STFF-18 cells, PYK2 tyrosine phosphorylation was greatly elevated relative to the respective TFW-46 and TFF-21 parental cells (Fig. 2C, compare lanes 1 to 3 and 5 to 7). In contrast to the results obtained for CAS and paxillin, expression of WT-FAK in the STFW-25 cells resulted in a substantial reduction of PYK2 phosphotyrosine (Fig. 2C, compare lanes 3 and 4). A similar reduction of PYK2 phosphotyrosine content occurred upon expression of F397-FAK in the STFF-18 cells. As reported previously [Owen et al., 1999], induced expression of either WT- or F397-FAK in the respective TFW-46 and STFF-21 parental cells was also effective in reducing the relatively low PYK2 phosphotyrosine levels seen in these cells (Fig. 2C). These data identify PYK2 as a likely v-Src substrate, and indicate that FAK acts to inhibit PYK2 phosphorylation by v-Src as well as by endogenous SFKs.

Morphological and Growth Properties of v-Src-Expressing Cells in the Presence or Absence of FAK

To examine the potential role for FAK in v-Src morphological transformation, we compared the morphologies of STFW-25 cells in the presence or absence of FAK expression. A spindle-shaped morphology characteristic of v-Src-transformed fibroblasts was observed for these cells when FAK was not expressed (Fig. 3, upper right; compare to parental cells in lower right). Withdrawal of tetracycline from the STFW-25 cultures to induce expression of WT-FAK did not further enhance cell rounding but, rather, resulted in a more flattened appearance of the cells (Fig. 3, upper left). Thus FAK is not required for morphological transformation by v-Src and may act to oppose this cellular response.

To evaluate the potential role for FAK in achieving mitogenic transformation by v-Src, we measured growth properties of STFW and parental TFW-46 monolayer cultures in the presence or absence of WT-FAK expression. In the absence of WT-FAK, STFW-25 cells exhibited enhanced growth properties relative to TFW-46 cells, indicative of v-Src-transformation. This included growth to a higher saturation density in high (10%) FBS (Fig. 4A, + Tet) and ability to proliferate slowly in low (0.5%) FBS (Fig. 4B, + Tet). WT-FAK expression failed to enhance either the high-serum saturation density or the low-serum growth rate of either STFW-25 or parental TFW-46 cells (Fig. 4A,B, compare-Tet to +Tet curves). Finally, we examined the capacity of STFW cells to undergo anchorage-independent growth in the presence or absence of WT-FAK expression. Both noninduced and induced STFW-25 cells formed colonies in soft agar suspension, while the parental TFW-46 cells did not (Fig. 5). Induction of the STFW-25 cells to express WT-FAK did not enhance soft agar colony formation, but rather resulted in a reduction in colony size and number. We have obtained monolayer growth and soft agar colony formation results similar to those presented in Figures 4 and 5 for three other WT-FAK expressing STFW clones examined as well as for the STFF-18 clone expressing F397-FAK (data not shown).

DISCUSSION

In this study, we made use of an inducible FAK expression system to address the role of FAK in v-Src-mediated substrate phosphorylation and cell transformation. We found that FAK is expressed as the major tyrosine phosphorylated protein in adherent, randomly growing fibroblasts and, consistent with past reports [Kanner et al., 1990; Guan and Shalloway, 1992], undergoes further tyrosine phosphorylation as a result of v-Src expression (Figs. 1 and 2A). The prevailing view that FAK phosphorylation by SFKs occurs as a result of SFK SH2 domains binding to the phosphorylated FAK Tyr-397 site is supported by the observation that F397-FAK fails to exhibit adhesion-regulated tyrosine-phosphorylation in nontransformed TFF-21 cells (Fig. 1) [Ruest et al., 2000]. But we found that the F397-FAK

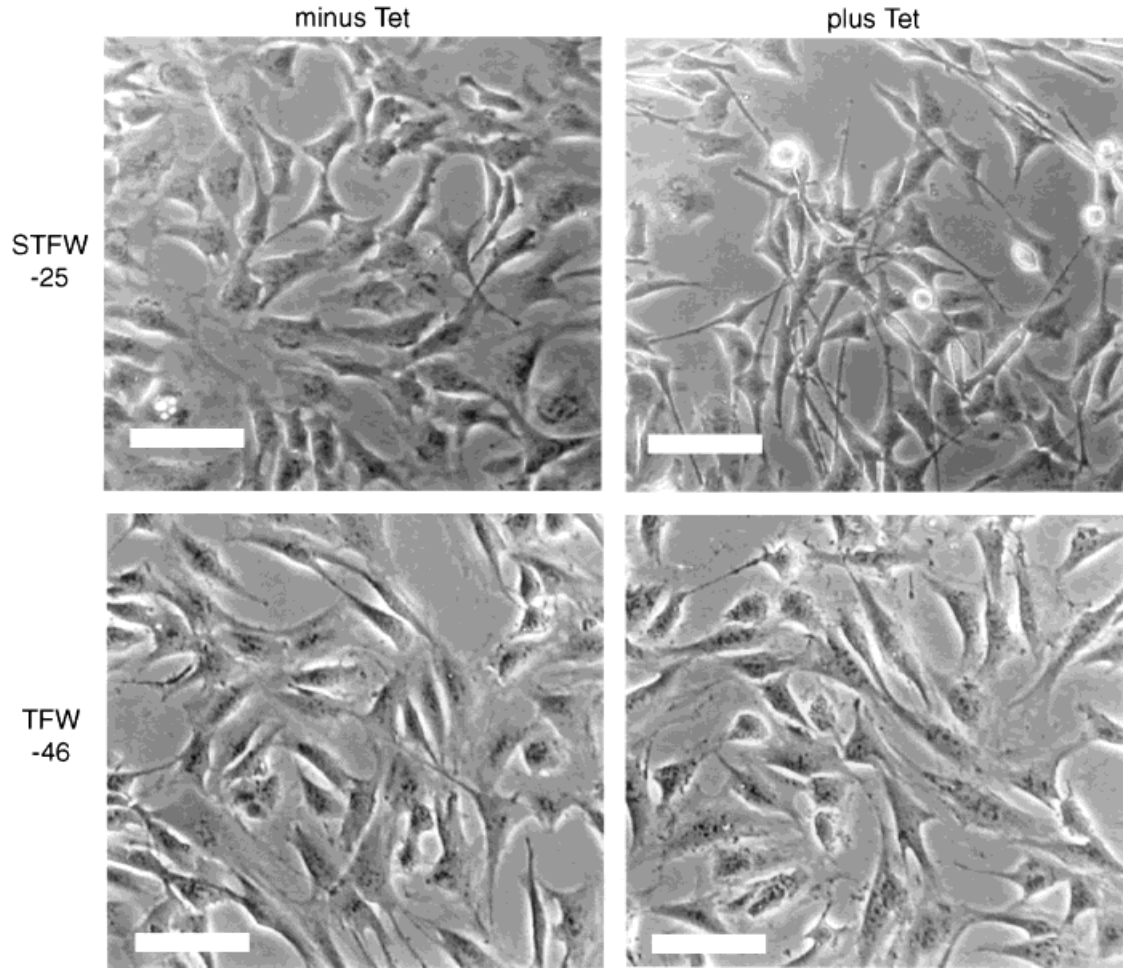

Fig. 3. Effects of WT-FAK and v-Src expression on cell morphology. Subconfluent cultures of STFW-25 and TFW-46 cells growing either in the presence of tetracycline or 48 h after tetracycline withdrawal were examined by phase-contrast microscopy. The fusiform transformed cell morphology is evident in the STFW-25 cells growing in the noninduced (+Tet) conditions. Bars = 100 μm.

mutant still undergoes substantial tyrosine phosphorylation in cells expressing v-Src and also co-immunoprecipitates with v-Src (Fig. 1). These findings are in agreement with other recent reports [McLean et al., 2000; Hauck et al., 2001].

By analyzing the tyrosine phosphorylation state of CAS and paxillin, we addressed the potential role for FAK as a docking protein in recruiting v-Src to phosphorylate these two major focal adhesion substrates. Our results indicated that FAK expression is not absolutely required for v-Src to target these substrates. Even when FAK was not expressed, v-Src expression significantly elevated phosphotyrosine content for both CAS and paxillin. Nonetheless, when WT-FAK was expressed along with v-Src the phosphotyrosine levels of both CAS and paxillin rose substantially (Fig. 2C,D). F397-FAK was unable to enhance CAS and paxillin phosphotyrosine, indicating that FAK-dependent phosphorylation of these substrates likely occurs as a result of SH2-mediated recruitment of v-Src to phosphorylate FAK-associated CAS and paxillin. In TetFAK cells not expressing v-Src, expression of WT-FAK (but not F397-FAK) was also able to promote tyrosine phosphorylation of CAS and paxillin, suggesting that FAK's docking function also normally regulates phosphorylation of these substrates by endogenous nononcogenic SFKs. The requirement for Tyr-397 could indicate that FAK itself exhibits little or no direct kinase activity toward CAS and paxillin. However, it is also possible that FAK's own kinase activity toward these substrates is regulated through

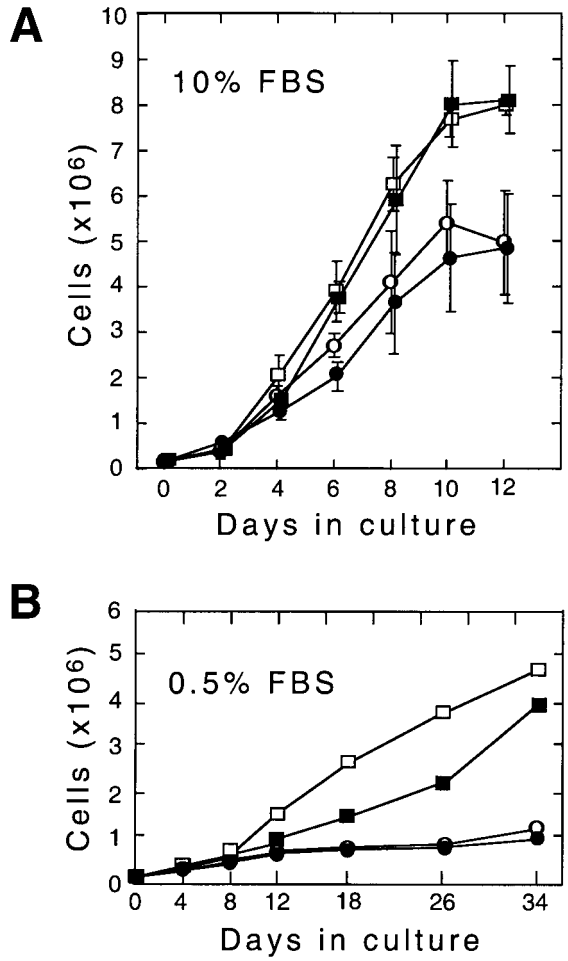

Fig. 4. Effects of WT-FAK and v-Src expression on monolayer growth. Induced or noninduced TFW-46 and STFW-25 cultures were sparsely plated then maintained in medium containing either 10% (**A**) or 0.5% (**B**) FBS and at the indicated days following replating, cells were harvested by trypsinization and counted. Data shown in (A) represent the mean number of cells (± SEM) of three independent experiments. Data in (B) is from a single experiment. Open squares, noninduced (+ Tet) STFW-25 cells; closed squares, induced (− Tet) STFW-25 cells; open circles, noninduced (+ Tet) TFW-46 cells; closed circles, induced (− Tet) TFW-46 cells.

phosphorylation of FAK activation loop tyrosines 576 and 577 by SFKs bound to the Tyr-397 site. FAK-dependent and -independent mechanisms for paxillin phosphorylation were also indicated by findings that paxillin mutations abolishing FAK binding reduce but do not eliminate paxillin tyrosine phosphorylation in cells expressing oncogenic Src [Thomas et al., 1999]. In the absence of FAK expression, phosphorylation of paxillin and CAS could be achieved through direct binding of v-Src and endogenous SFKs to these substrates via SH3 and/or SH2 interactions [Weng et al., 1993; Nakamoto et al., 1996]. PYK2, which has conserved domains for interaction with SFKs, CAS, and paxillin [Lev et al., 1995], may also function to either directly phosphorylate CAS and paxillin or to recruit SFKs to phosphorylate these substrates in the absence of FAK. However, the paucity of CAS and paxillin tyrosine phosphorylation in the absence of FAK or v-Src expression argues that PYK2 does not contribute substantially to the phosphorylation of these substrates in the TetFAK cells.

PYK2 phosphotyrosine content was also observed to be elevated in cells expressing v-Src (Fig. 2C), identifying PYK2 as a likely v-Src substrate. This is not surprising given the homology between PYK2 and FAK. Of interest was the observation that FAK expression, either WT or F397, suppressed tyrosine phosphorylation of PYK2 by v-Src. Although the suppression did not require Tyr-397, a mechanism involving v-Src sequestration from PYK2 through stable interaction with FAK cannot be ruled out, since v-Src can stably associate with F397-FAK. An alternative mechanism would involve FAK displacing PYK2 from focal adhesion sites due to competition with focal adhesion targeting proteins, and thereby blocking the ability of PYK2 to associate with v-Src and/or endogenous SFKs at focal adhesion sites. This is supported by previous studies indicating that the FAK C-terminal domain is more efficient in focal adhesion targeting than the homologous domain of PYK2 [Schaller and Sasaki, 1997; Zheng et al., 1998] and can inhibit PYK2 phosphorylation when overexpressed [Li et al., 1999].

Our tyrosine phosphorylation findings are consistent with the speculation that FAK may play a key regulatory and/or signaling role in promoting v-Src morphological transformation such that either v-Src phosphorylation of FAK or the docking function provided by FAK to target v-Src to other cellular substrates could be an essential step in disrupting focal adhesion contacts to promote the fusiform or rounded cell morphology. Yet, from our observation that the cells expressing v-Src exhibit the fusiform appearance in the absence of FAK expression (Fig. 3), it can be concluded that FAK is neither critical for morphological transformation by v-Src nor does its expression lead to a further rounding of the STFW-25 cells. Rather, FAK expression was associated with a

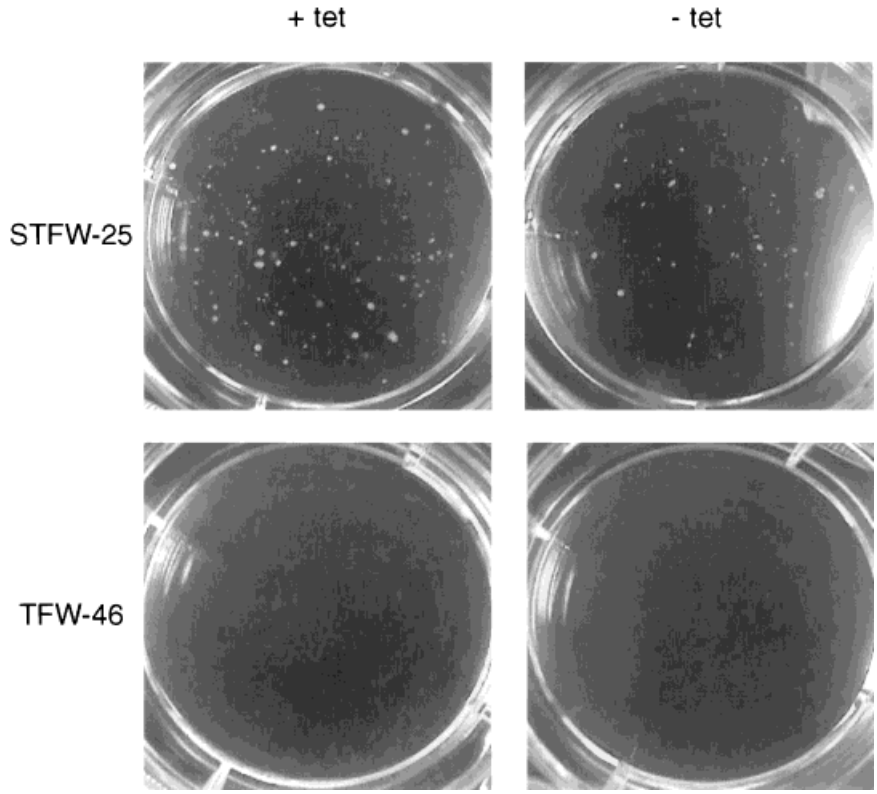

Fig. 5. Effects of WT-FAK and v-Src expression on anchorage-independent growth. TFW-46 or STFW-25 cells were suspended in soft agar and maintained in growth media containing 10% FBS under either induced (− Tet) or noninduced (+ Tet) conditions. The dishes were photographed 4 weeks following the initial plating. Colony formation is evident in the STFW-25 cultures under both noninduced and induced conditions, but note the reduced size and number of colonies appearing under induced conditions.

flattening of the v-Src-transformed cells. This suggests a role for FAK, antagonistic to v-Src, in promoting the spread cell phenotype. Other studies have shown that FAK can enhance the rate of cell spreading on fibronectin [Richardson et al., 1997; Owen et al., 1999]. A role for FAK in opposing v-Src morphological transformation is consistent with findings of Fincham et al. [1995], who observed that increased FAK tyrosine phosphorylation following activation of a temperature-sensitive v-Src correlated with FAK degradation and speculated that this could promote focal adhesion disassembly leading to cell rounding. However, in the cells used in our study, v-Src expression does not dramatically alter the half-life of FAK (data not shown). Other recent studies have questioned the role of FAK signaling in v-Src morphological transformation by dissociating high levels of FAK and/or CAS tyrosine phosphorylation from the transformed cell phenotype [Burnham et al., 1999; Hakak et al., 2000]. Morphological transformation by v-Src has been linked to tyrosine phosphorylation of p190RhoGAP which, in turn, inactivates Rho by stimulating GTPase activity [Fincham et al., 1999; Mayer et al., 1999]. In this context, it is intriguing that PYK2 has been shown to bind to p190RhoGAP and promote its tyrosine phosphorylation by v-Src [Zrihan-Licht et al., 2000]. Our observation that PYK2 tyrosine phosphorylation is suppressed by FAK (Fig. 2C) suggests that the ability of PYK2 to bind to v-Src and promote p190RhoGAP phosphorylation is also impaired by FAK. This may elevate levels of GTP-bound Rho and thereby promote cell flattening.

Our study also did not indicate a critical role for FAK in promoting the elevated growth and survival properties of v-Src-transformed cells. Monolayer growth in reduced serum and to increased saturation density, and anchorage-independent growth in soft agar were all observed in STFW and STFF cells in the absence of FAK expression. Nor were any of these

properties enhanced by FAK expression, as might be expected if FAK plays a nonessential but substantial contributory role in v-Src signaling events that lead to these altered growth properties. Current evidence indicates that enhanced growth and survival characteristics of Src-transformed fibroblasts result from activation of multiple parallel signaling pathways including Ras/MAPK, PI3K/Akt, and STAT3 pathways [Bromberg et al., 1998; Turkson et al., 1998, 1999; Penuel and Martin, 1999; Odajima et al., 2000]. v-Src mitogenic transformation in the absence of FAK expression indicates that FAK is not a required element in activating these or other relevant signaling events. A potential role for FAK in anchorage-independent growth of v-Src transformed cells is suggested by the findings of Renshaw et al. [1999], who showed that expression of a dominant-negative FAK reduces v-Src stimulated ERK2 activity by 60–70%. However, these authors also reported that dominant-negative FAK expression does not decrease growth of v-Src-transformed cells in soft agar, further indicating that FAK is not essential for v-Src transformation. The reduced growth in low serum and in soft agar we observed associated with FAK induction suggest that FAK may suppress a relevant mitogenic pathway, perhaps by binding to v-Src and sequestering the oncoprotein away from other substrates more relevant to proliferation. However, we cannot rule out the possibility that the tetracycline-controlled transactivator (tTA) protein that is inducibly expressed along with FAK could account for negative proliferation effects [Gallia and Khalili, 1998].

Although our findings do not support a role for FAK in promoting v-Src transformation of TetFAK fibroblasts, we do not exclude the possibility that in other cell lines, FAK plays a more crucial role in promoting the transformed characteristics resulting from v-Src expression. Such a role could have been concealed by unique characteristics of the TetFAK cells employed in our study. PYK2 could compensate for the lack of FAK by providing signaling and/or docking functions that contribute to v-Src transformation. This idea is supported by the finding that PYK2 promotes adhesion-regulated ERK2 activation in FAK-null cells [Sieg et al., 1998]. However, other recent findings indicate that PYK2 has opposing effects to FAK in both cell survival and serum-stimulated proliferation responses [Xiong and Parsons, 1997; Zhao et al., 2000]. Another complicating factor is the origin of the TetFAK cells from FAK-null cells that were also deficient in p53 [Ilic et al., 1995]. Since FAK can transduce an adhesion survival signal in primary fibroblasts through suppression of p53-regulated apoptosis [Ilic et al., 1998], it is possible that the p53 deficiency could diminish a FAK requirement for anchorage-independent survival triggered by v-Src. Nevertheless, the p53 deficiency alone is not sufficient for transformation and our results clearly demonstrate that FAK expression is not a required element in the v-Src signaling events that confer the ability of TetFAK cells to grow in reduced serum, to higher saturation density, or in suspension.

In summary, an inducible FAK expression system was used to document FAK as a major v-Src substrate that functions to enhance v-Src-mediated tyrosine phosphorylation of CAS and paxillin. Yet, FAK expression was found not to be required for v-Src to promote either morphological transformation or altered growth in suspension and in reduced serum. It remains possible that signaling through FAK plays a more critical role in the enhanced migratory/invasive properties of v-Src transformed cells, and this will be assessed in future studies.

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