v-Src Induces Tyrosine Phosphorylation of Focal Adhesion Kinase Independently of Tyrosine 397 and Formation of a Complex with Src*

Received for publication, November 18, 1999, and in revised form, May 15, 2000
Published, JBC Papers in Press, May 16, 2000, DOI 10.1074/jbc.M909322199

Gordon W. McLean‡, Valerie J. Fincham, and Margaret C. Frame
From the Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Bearsden, Glasgow, G61 1BD, United Kingdom

The non-receptor tyrosine kinase FAK plays a key role at sites of cellular adhesion. It is subject to regulatory tyrosine phosphorylation in response to a variety of stimuli, including integrin engagement after attachment to extracellular matrix, oncogene activation, and growth factor stimulation. Here we use an antibody that specifically recognizes the phosphorylated form of the putative FAK autophosphorylation site, Tyr\(^{397}\). We demonstrate that FAK phosphorylation induced by integrins during focal adhesion assembly differs from that induced by activation of a temperature-sensitive v-Src, which is associated with focal adhesion turnover and transformation. Specifically, although v-Src induces tyrosine phosphorylation of FAK, there is no detectable phosphorylation of Tyr\(^{397}\). Moreover, activation of v-Src results in a net decrease in fibronectin-stimulated phosphorylation of Tyr\(^{397}\), suggesting possible antagonism between v-Src and integrin-induced phosphorylation. Our mutational analysis further indicates that the binding of v-Src to Tyr\(^{397}\) of FAK in its phosphorylated form, which is normally mediated, at least in part, by the SH2 domain of Src, is not essential for v-Src-induced cell transformation. We conclude that different stimuli can induce phosphorylation of FAK on distinct tyrosine residues, linking specific phosphorylation events to ensuing biological responses.

Focal adhesion kinase (FAK)\(^1\) is a non-receptor protein-tyrosine kinase that has been implicated in a variety of signaling pathways and cellular processes. It is widely expressed in most tissues, and at particularly high levels in neuronal tissue (1, 2). Phosphorylation of FAK occurs in response to a wide variety of stimuli, including integrin engagement, oncogenic transformation, and growth factors or mitogenic neuropeptides (3–5). FAK was cloned simultaneously from mouse and chicken (6, 7), and was cloned from human keratinocytes in vivo (8), and an association between FAK and v-Src was cloned simultaneously from mouse and chicken (6, 7), and was cloned from human keratinocytes in vivo (8), and an association between FAK and v-Src has been demonstrated in human keratinocytes in vivo (9). The carboxyl terminus contains two proline-rich sequences that can bind to SH3 domain-containing proteins, such as p130\(^{cas}\) or Graf (10, 11), as well as the sequences responsible for focal adhesion targeting of FAK (FAK domain; residues 919–1042) (12). In addition to these functional regulatory sequences, alternative splicing provides another mode of FAK regulation, with autonomous expression of the carboxyl-terminal portion of FAK (13), termed FRNK. However, while enforced overexpression of FRNK inhibits FAK-mediated cell spreading (14), its role as a physiological regulator of FAK function in vivo remains to be established.

To date, six tyrosine phosphoacceptor sites in FAK have been identified, namely tyrosines 397, 407, 576, 577, 861, and 925 (15–18). Artificial integrin clustering induced by antibodies, or cell adhesion to fibronectin (FN), induces FAK phosphorylation (19, 20), with Tyr\(^{397}\) believed to be a site of autophosphorylation (21). FAK is also heavily tyrosine phosphorylated in Src-transformed cells (19), with sites other than Tyr\(^{397}\) also being phosphorylated (15–17). A model has been proposed whereby integrin-ligand binding triggers autophosphorylation of Tyr\(^{397}\), creating a high affinity binding site for the Src family kinases via their SH2 domains (7, 15, 19, 21). The process of Src binding itself, which requires displacement of the intramolecular Src SH2 domain-Tyr\(^{397}\) interaction might contribute to Src activation (22), enabling further phosphorylation of FAK on the carboxyl-terminal tyrosine residues, i.e. Tyr\(^{407}\), Tyr\(^{576}\), Tyr\(^{577}\), Tyr\(^{581}\), and Tyr\(^{585}\), and activation of downstream signaling initiated by association of SH2 domain containing proteins, such as Grb2 (17, 23) and the p85 regulatory subunit of phosphoinositide 3-kinase (24, 25).

Despite the model proposed above, the exact nature of the phosphoacceptor sites targeted by individual stimuli, the temporal sequence of events and how these are linked to cellular responses, is still unclear. To address this with respect to Tyr\(^{397}\) of FAK, we generated an antibody that specifically recognized the phosphorylated form of FAK-Tyr\(^{397}\). This was used to detect phosphorylation of Tyr\(^{397}\) after plating cells on fibronectin. In contrast, the Tyr\(^{397}\)-phospho-specific antibody failed to react with FAK after activation of a ts v-Src mutant, although v-Src did induce phosphorylation of FAK on other tyrosine residues. In addition, a Tyr\(^{397}\) to Phe\(^{397}\) mutant of FAK (397F) was still tyrosine phosphorylated after v-Src activation, although direct association of the two proteins was ablated. Furthermore, v-Src induced phosphorylation after v-Src activation, although direct association of the two proteins was ablated. Furthermore, v-Src-induced phosphorylation on v-Src does not affect the targeting of either v-Src or FAK to focal

* This work was supported by the Cancer Research Campaign. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 0141-330-3954; Fax: 0141-942-6521; E-mail: g.mclean@beatson.gla.ac.uk.

The abbreviations used are: FAK, focal adhesion kinase; SH, Src homology; FN, fibronectin; PI-3K, phosphoinositide 3-kinase; CEF, chicken embryo fibroblast; ts, temperature sensitive; PBS, phosphate-buffered saline; ECM, extracellular matrix; TRITC, tetramethyl rhodamine isothiocyanate; FRNK, FAK-related non-kinase.

dimensional: 605.0x786.0
[50x737]© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
[50x48]mine isothiocyanate; FRNK, FAK-related non-kinase.
[50x57]buffered saline; ECM, extracellular matrix; TRITC, tetramethyl rhoda-
[50x744]Complex with Src*
[50x695]Kinase Independently of Tyrosine 397 and Formation of a
[50x744]THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 275, No. 30, Issue of July 28, pp. 23333–23339, 2000
[50x125]of page charges. This article must therefore be hereby marked "
Tyrosine 397-independent Phosphorylation of FAK by v-Src

adhesions, v-Src-induced tyrosine phosphorylation of FAK on sites other than Tyr397, or cell transformation.

EXPERIMENTAL PROCEDURES

Cells and Viruses—Primary chicken embryo fibroblasts (CEF) cultures were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% newborn calf serum, 1% chick serum, and 10% tryptose phosphate broth, and were buffered with 5% CO2. For FN plating experiments, dishes were coated by preincubating overnight at 4°C in a solution of 10 μg/ml FN (Stratagene) in PBS before being used for cell plating. The neomycin-selectable avian retrovirus, SFCV, constructs encoding temperature sensitive (ts) LA-29 v-src (26), or a kinase-defective variant of ts LA-29 that contains a Lys-Arg mutation at position 295, have been described previously (27). Cells expressing ts v-Src mutants were grown either at restrictive temperature (41°C), or were shifted to permissive temperature (35°C) for the indicated times. Myc-tagged FAK was generated by ligating a double stranded oligonucleotide (sense strand, 5'-gatcggagacaaacctatcagagaggatgataaa-3'; antisense strand, 5'-gattctagctctctttctgagatgagtttttgttcgct-3') into the BclI site at position 3186. Y397F-FAK was produced by conventional polymerase chain reaction and cloning methods, and verified by sequencing. Both Myc-tagged variants were expressed in the replication competent avian retrovirus RCAS.

Generation of a Phosphospecific Antibody Against Tyr397 of FAK—For the generation of antibodies specific for the putative FAK autophosphorylation site, Tyr397, the following 17-aminoc acid peptide was synthesized SVSETDDYYEIDEE(D/C). This represents amino acids 390–405 of avian and human FAK (1, 6) with tyrosine 397 being chemically phosphorylated. The carboxyl-terminal cysteine residue was added to facilitate coupling to a carrier protein, keyhole limpet hemocyanin. Peptides were estimated to be greater than 90% pure by high performance liquid chromatography, and composition was confirmed by amino acid analysis. The peptide representing amino acids 390–405 of the avian and human FAK sequence that was used to immunize rabbits. The tyrosine residue at position 397 was chemically phosphorylated. Also shown are the position of the focal adhesion targeting sequence (FAT) and the COOH-terminal proline-rich region (PR) that mediate binding to SH3 domain containing proteins. The putative Src-specific phosphorylation sites Tyr397, Tyr576, Tyr611, and Tyr725 are indicated. B, FAK was detected by immunoblotting lysates of CEF that had been either retained in suspension (lanes 1, 3, and 5) or plated onto FN-coated dishes (lanes 2, 4, and 6). Membranes were probed with a FAK-specific antibody (anti-397-P), either preincubated with excess immunizing peptide in the unphosphorylated form (lanes 1 and 2) or in the phosphorylated form (lanes 3 and 4), or probed with a FAK specific mAb to detect total cellular FAK (lanes 5 and 6).

antibody or anti-Myc conjugated to horseradish peroxidase (Invitrogen) and visualization was by enhanced chemiluminescence (ECL, Amer sham Pharmacia Biotech) according to the manufacturer’s guidelines.

Immunofluorescence—Cells were grown on glass coverslips, fixed at 4°C for 15 min with 3.7% formaldehyde, permeabilized with 0.5% Triton X-100, and incubated with 1:500 anti-Src EC10 (UBI), 2 μg/ml anti-Myc mAb (Jackson) or 2.5 μg/ml anti-paxillin mAb (Transduction Laboratories). Antibody detection was via fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma) and/or Texas Red-conjugated goat anti-rabbit IgG (Jackson), for 45 min at room temperature. Actin stress fibers were visualized by staining with 10 μg/ml phalloidin-TRITC (Sigma) for 40 min at room temperature. Fluorescence was visualized using a Bio-Rad MRC 600 confocal microscope and images were printed on a dye sublimation printer (Kodak).

RESULTS

Generation of a Phospho-specific Antibody Recognizing FAK-Tyrosine 397—In order to study stimulus-induced phosphorylation of the putative autophosphorylation site in FAK, i.e., Tyr397, we generated an antiserum which specifically recognized Tyr397 in its phosphorylated state. A 17-aminoc acid peptide encompassing residues 390–405 of FAK, with a phospho-specific antibody Tyr397, was coupled to keyhole limpet hemocyanin via a carboxyl-terminal cysteine residue and used to immunize rabbits (as described under “Experimental Procedures”). Antibodies present in the crude sera that recognized nonphosphorylated epitopes were removed by affinity absorption. Purified phospho-specific IgG was then used to identify FAK autophosphorylation by immunoprecipitation and Western blotting. Unoccupied binding sites were blocked with 5% dried milk powder in PBS + 0.1% Tween 20 (PBST) followed by probing with either anti-Tyr397 (anti-397-P) purified IgG (2 μg/ml in 3% bovine serum albumin in PBST or general anti-phosphotyrosine (PY20; Transduction Laboratories) at 0.25 μg/ml or FAK monoclonal antibody at 0.5 μg/ml (clone 77, Transduction Laboratories). Detection was by incubation with either anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies or anti-Myc conjugated to horseradish peroxidase (Invitrogen) and visualization was by enhanced chemiluminescence (ECL, Amer sham Pharmacia Biotech) according to the manufacturer’s guidelines.

Immunofluorescence—Cells were grown on glass coverslips, fixed at 4°C for 15 min with 3.7% formaldehyde, permeabilized with 0.5% Triton X-100, and incubated with 1:500 anti-Src EC10 (UBI), 2 μg/ml anti-Myc mAb (Jackson) or 2.5 μg/ml anti-paxillin mAb (Transduction Laboratories). Antibody detection was via fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma) and/or Texas Red-conjugated goat anti-rabbit IgG (Jackson), for 45 min at room temperature. Actin stress fibers were visualized by staining with 10 μg/ml phalloidin-TRITC (Sigma) for 40 min at room temperature. Fluorescence was visualized using a Bio-Rad MRC 600 confocal microscope and images were printed on a dye sublimation printer (Kodak).

RESULTS

Generation of a Phospho-specific Antibody Recognizing FAK-Tyrosine 397—In order to study stimulus-induced phosphorylation of the putative autophosphorylation site in FAK, i.e., Tyr397, we generated an antiserum which specifically recognized Tyr397 in its phosphorylated state. A 17-aminoc acid peptide encompassing residues 390–405 of FAK, with a phospho-specific antibody Tyr397, was coupled to keyhole limpet hemocyanin via a carboxyl-terminal cysteine residue and used to immunize rabbits (as described under “Experimental Procedures”). Antibodies present in the crude sera that recognized nonphosphorylated epitopes were removed by affinity absorption. Purified phospho-specific IgG was then used to identify FAK autophosphorylation by immunoprecipitation and Western blotting. Unoccupied binding sites were blocked with 5% dried milk powder in PBS + 0.1% Tween 20 (PBST) followed by probing with either anti-Tyr397 (anti-397-P) purified IgG (2 μg/ml in 3% bovine serum albumin in PBST or general anti-phosphotyrosine (PY20; Transduction Laboratories) at 0.25 μg/ml or FAK monoclonal antibody at 0.5 μg/ml (clone 77, Transduction Laboratories). Detection was by incubation with either anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies or anti-Myc conjugated to horseradish peroxidase (Invitrogen) and visualization was by enhanced chemiluminescence (ECL, Amer sham Pharmacia Biotech) according to the manufacturer’s guidelines.
induce rapid de-phosphorylation of FAK (15). Confirmation of the sequence and phosphorylation-specific reactivity of this antibody was obtained by blotting cell lysates with anti-397-P IgG which had been preincubated with excess immunizing peptide, either in the unphosphorylated (Fig. 1B, lanes 1 and 2) or phosphorylated form (Fig. 1B, lanes 3 and 4). Preincubation with excess Tyr\(^{397}\)-containing phosphopeptide inhibited FAK reactivity after plating cells on FN (Fig. 1B, lane 4), while excess unphosphorylated peptide had no effect, indicating that purified anti-397-P IgG reacted specifically with the Tyr\(^{397}\)-phosphorylated form of FAK.

**Phosphorylation of FAK Induced by FN Differs from That Induced by the v-Src Oncoprotein**—Although both FN attachment and v-Src activity stimulate tyrosine phosphorylation of FAK, the biological outcome of these two stimuli is quite different. Specifically, integrin engagement mediated by FN attachment induces focal adhesion formation and cell spreading, while v-Src induces focal adhesion turnover and cell transformation. We therefore compared FAK-Tyr\(^{397}\) phosphorylation induced by either plating cells on FN or by activating a v-Src mutant (ts-LA29 v-Src) in the absence of FN, which induces phosphorylation, and subsequent proteolysis, of FAK, events that are visibly linked to focal adhesion disruption during transformation (31). As expected, plating of cells on FN triggered a substantial increase in FAK tyrosine phosphorylation (Fig. 2, middle panel, lanes 2–4), which coincided with an increase in the phosphorylation of Tyr\(^{397}\) (Fig. 2, upper panel, lanes 2–4). However, although FAK was also tyrosine phosphorylated by activation of ts v-Src after switch to the permissive temperature (35 °C), as detected by a general phosphotyrosine antibody (Fig. 2, middle panel, lanes 5–7), FAK-Tyr\(^{397}\) failed to react with anti-397-P (Fig. 2, upper panel, lanes 5–7). This indicates that phosphorylation of FAK induced by v-Src can occur at phosphoacceptor sites that are distinct from Tyr\(^{397}\), although the latter is clearly phosphorylated after plating on FN.

**Activation of ts v-Src Results in a Net Decrease in FN-stimulated FAK-Tyr\(^{397}\) Phosphorylation**—Since our results indicated that different phosphorylation sites in FAK might act as recipients for signals from FN or v-Src, we next examined phosphorylation after attachment to FN when v-Src was active. CEF expressing ts v-Src, or its kinase-defective variant (ts LA29-KD), were either retained in suspension after trypsinization (S), or plated onto FN for 60 min, either at the restrictive (41 °C) or permissive (35 °C) temperatures. After plating on FN, specific phosphorylation of Tyr\(^{397}\) occurred both at the permissive and the non-permissive temperatures and in cells expressing both kinase-active and kinase-defective v-Src (Fig. 3A, upper panel). However, we noted that ts v-Src activity at the permissive temperature was consistently associated with reduced phosphorylation of FAK-Tyr\(^{397}\), when compared with cells plated on FN at restrictive temperature (Fig. 3A, compare lanes 4 and 5). To investigate this further, adhesion to FN and activation of v-Src were stimulated sequentially, and phosphorylation of FAK-Tyr\(^{397}\) examined. Following adhesion to FN for 45 min, cells expressing ts v-Src were either switched to 35 °C for various times (Fig. 3B, lanes 4–6), or were maintained at 41 °C (Fig. 3B, lanes 7–9). As expected, plating cells on FN following suspension caused a robust increase in phosphotyrosine content, which correlated with specific phosphorylation of Tyr\(^{397}\) (Fig. 3B, upper and middle panels). However, following switch to the permissive temperature, there was a time-dependent decrease in the specific phosphorylation of FAK-Tyr\(^{397}\) mediated by ts v-Src. This was contrasted with the unaffected phosphorylation of cells expressing kinase-defective v-Src (ts LA29-KD) when plated on FN at the permissive temperature (Fig. 3B, lane 8). Thus, v-Src activity antagonizes integrin-induced phosphorylation of FAK-Tyr\(^{397}\).
Tyrosine 397-independent Phosphorylation of FAK by v-Src

FAK-Tyr\textsuperscript{397} (Fig. 3B, upper panel, lanes 4–6). Furthermore, although there was also some decrease in phosphorylation of FAK-Tyr\textsuperscript{397} after 120 min at 41 °C (Fig. 3B, lanes 9), indicating that phosphorylation of FAK-Tyr\textsuperscript{397} after plating on FN was transient, the net decrease was exacerbated and was evident by 60 and 90 min when v-Src was active at 35 °C (Fig. 3B, upper panel, lanes 4–6). Thus, v-Src-induced FAK phosphorylation is associated with reduced specific FAK-Tyr\textsuperscript{397} phosphorylation that is normally stimulated by attachment to FN, raising the possibility that v-Src-induced tyrosine phosphorylation of FAK may antagonize integrin-induced phosphorylation.

Phosphorylation of FAK by Src Does Not Require Binding of Src to Tyr\textsuperscript{397}—Extracellular matrix (ECM)-induced autophosphorylation of FAK on Tyr\textsuperscript{397} creates a high affinity binding site for the SH2 domain of c-Src, and mutation (Tyr to Phe) of FAK-Tyr\textsuperscript{397} in its phosphorylated form, was essential for v-Src to induce tyrosine phosphorylation of FAK on other sites. To address this, we used CEF expressing ts v-Src which also overexpressed Myc-tagged FAK proteins, either wt FAK or FAK in which the Tyr at position 397 had been changed by mutation to Phe (Y397F-FAK). We tested whether the Y397F mutation inhibited the association of v-Src with FAK, by carrying out anti-Src immunoprecipitations from wt- and Y397F-FAK-expressing cells and probing immunoblots with anti-Myc. As expected, although expressed wt FAK co-precipitated with v-Src (Fig. 4A, top panel, lane 1), an interaction that persisted after activation of v-Src by shift to the permissive temperature (Fig. 4A, top panel, lanes 2 and 3), the Y397F-FAK mutant failed to co-precipitate with v-Src (Fig. 4A, top panel, lanes 4–6). Thus, consistent with the findings of others on c-Src (21, 32), v-Src also requires FAK-Tyr\textsuperscript{397} for binding. In addition, re-probing of the immunoblots with an anti-FAK mAb failed to detect any substantial binding of endogenously expressed FAK to v-Src (Fig. 4A, bottom panel, lanes 4–6).

To determine whether v-Src-induced phosphorylation of FAK occurred in the absence of Tyr\textsuperscript{397} and formation of the v-Src-FAK complex, cells expressing the Myc-tagged FAK proteins were either maintained at 41 °C or shifted to 35 °C for 2 h. We detected an increase in tyrosine phosphorylation of wt-FAK and to a lesser extent Y397F-FAK after activation of v-Src (Fig. 4B, top panel, lanes 2 and 4, respectively), indicating that binding of v-Src to FAK-Tyr\textsuperscript{397} was not essential for phosphorylation of other tyrosines residues within FAK. Additional confirmation of the specificity of the anti-397-P IgG was obtained by its failure to recognize Myc-tagged Y397F-FAK immunoprecipitated from these cells with anti-Myc (Fig. 4B, middle panel, lanes 1 and 2).

To test whether integrin-dependent tyrosine phosphorylation of FAK on sites other than Tyr\textsuperscript{397}, we used CEF expressing ts v-Src, which also overexpressed Myc-tagged wt-FAK (lanes 1–3) or Myc-tagged Y397F-FAK (lanes 4–6), that had been either retained at 41 °C (0 h) or shifted to 35 °C (1 h, 2 h). Samples were transferred to nitrocellulose and probed with an anti-Myc mAb to detect co-precipitated FAK (upper panel), an anti-Src mAb EC10 to confirm immunoprecipitation of v-Src (middle panel), or a FAK mAb to detect any endogenously expressed FAK binding (lower panel). B, FAK was immunoprecipitated with an anti-Myc mAb from CEF expressing ts LA29 v-Src and either Myc-tagged Y397F-FAK (lanes 1 and 2) or Myc-tagged wt-FAK (lanes 3 and 4), that had been either retained at 41 °C or shifted to 35 °C for 2 h. Proteins were blotted and probed with PY20 anti-phosphotyrosine (upper panel), anti-397-P (middle panel), or anti-FAK (bottom panel). C, FAK was immunoprecipitated with an anti-Myc mAb from CEF expressing either Myc-tagged Y397F-FAK (lanes 1–4) or Myc-tagged wt-FAK (lanes 5 and 6). Cells were plated onto FN-coated dishes for the indicated times, either in the absence (lanes 1–2 and 5–6) or presence of 2 μM PD162531, a selective Src family kinase inhibitor (lanes 3 and 4). Proteins were blotted and probed with either PY20 anti-phosphotyrosine (upper panel) or anti-FAK (bottom panel).

We found that Y397F-FAK was tyrosine phosphorylated after plating of cells on FN (Fig. 4C, upper panel, lane 2), although phosphorylation was considerably delayed when compared with wt-FAK (Fig. 4C, upper panel, lanes 5 and 6). Furthermore, this delayed integrin-induced tyrosine phosphorylation of Y397F-FAK was likely due to Src family kinase activity, since phosphorylation was impaired in the presence of a Src inhibitor, PD162531 (Fig. 4C, upper panel, lanes 3 and 4), that we have previously shown to inhibit the activity of the Src family kinases in vitro and in vivo (34). Thus, as is the case...
Tyrosine 397-independent Phosphorylation of FAK by v-Src

with v-Src, formation of a complex between c-Src SH2 and FAK, or prior phosphorylation of Tyr397, is not absolutely required for Src-dependent phosphorylation of FAK on sites other than Tyr397, but may be necessary for optimal, rapid phosphorylation after integrin engagement.

The Association of Src with FAK, and Phosphorylation of Tyr397, Is Not Required for Cell Transformation—As the proposed model for FAK regulation suggested a critical role for phosphorylation of FAK-Tyr397, and the consequent association with Src, during ECM-induced intracellular signaling, we examined whether vast overexpression of Y397F-FAK that blocks association with v-Src (Fig. 4A), and also endogenous c-Src (not shown), influenced v-Src’s biological activity. Specifically, v-Src activation in CEF is accompanied, at early times, by the assembly of new focal adhesions containing the oncoprotein (27, 35), and subsequently, by tyrosine kinase-induced focal adhesion disruption as cells round up during transformation (27). Thus, we tested whether mutation of Tyr397 of FAK to Phe397 influenced the intracellular targeting of ts v-Src to focal adhesions after switch to the permissive temperature, or subsequent focal adhesion disruption and transformation. The extent of overexpression of Myc-tagged wt- and Y397F-FAK, compared with endogenous FAK in vector-transfected controls, is shown (Fig. 5A, lanes 5–8). Double immunofluorescence with anti-Src and anti-Myc demonstrated that both exogenous FAK proteins were localized in focal adhesion structures, irrespective of v-Src activity (Fig. 5B). In addition, v-Src was translocated from the perinuclear region of the cell to focal adhesions at the periphery after shift to the permissive temperature, even in the presence of overexpressed Y397F-FAK that prevented formation of a Src-FAK complex (Fig. 5B, upper panels). These data indicate that both FAK and v-Src are targeted to focal adhesions under conditions where they are not associated. Moreover, overexpression of Y397F-FAK had no obvious consequences for focal adhesions formed at early times after activation of v-Src, as judged by visualization of focal adhesions containing the oncoprotein (Fig. 5B), and cells with focal adhesions containing Y397F-FAK remained spread. In addition, v-Src-induced morphological transformation that is associated with focal adhesion disruption (31) was not impaired by vast overexpression of Y397F-FAK (Fig. 5C). Staining for actin stress fibers and the focal adhesion component paxillin, demonstrated that both cells expressing wt FAK and Y397F-FAK exhibited characteristic signs of the transformed phenotype when switched to 35 °C, with loss of actin stress fibers and disruption of paxillin containing focal adhesions associated with cell rounding (Fig. 5C).

**DISCUSSION**

Engagement of cell surface integrins with ECM, and transformation of cells with v-Src, results in increased protein tyrosine phosphorylation content, particularly in a subset of pro-

---

**FIG. 5.** Y397F-FAK overexpression does not inhibit new focal adhesion assembly in adherent cells, or transformation. **A,** cellular FAK was detected by immunoblotting lysates of CEF (lanes 1 and 2) or CEF-expressing ts LA29 v-Src alone (lanes 3 and 4), or with either Myc-tagged Y397F-FAK (lanes 5 and 6) or Myc-tagged wt-FAK (lanes 7 and 8). CEF were either maintained at 41 °C or shifted to 35 °C for 2 h as indicated. **B,** CEF cells expressing ts LA29 v-Src and either Y397F-FAK or wt-FAK were fixed and stained with rabbit anti-Src serum and mouse anti-Myc mAb to visualize exogenous Myc-tagged FAK. Subsequent detection was via appropriate secondary antibodies coupled to either fluorescein isothiocyanate (Src) or Texas Red (Myc). **C,** CEF expressing ts LA29 v-Src and either Y397F-FAK (lower panels) or wt-FAK (upper panels) were maintained at 41 °C or were shifted to 35 °C for 24 h. Altered cell morphology was visualized by phase-contrast microscopy. Cells were also fixed and stained with mouse anti-paxillin mAb or phallolidin-TRITC for visualization of actin stress fibers (small double panels). Size bars indicate 25 µm.
Tyrosine 397-independent Phosphorylation of FAK by v-Src

To date, there have been multiple reports of Tyr397-independent phosphorylation, with the most rigorous being the recent works of Tepel and colleagues (1) and others (2). Our results demonstrate that neither the FAK-Tyr 397/Src association nor Tyr397 phosphorylation, and responsible for integrin-induced focal adhesion assembly, is unclear, it is noteworthy that it is not only the Src family kinases that binds to phosho-Tyr397. Also binding via this site are the p85 regulatory subunit of phosphoinositol 3-kinase (40), which is required for FAK-dependent cell motility (41), and the tumor suppressor protein phosphatase, PTEN (42), that is able to de-phosphorylate FAK in response to placing cells in suspension (43–45). In addition, the adapter protein Grb7 also binds to FAK via Tyr397 (46). The increasing number of signaling proteins now shown to bind FAK in a Tyr397-dependent manner indicates that there is unlikely to be a single effector pathway activated as a result of integrin-induced phosphorylation at this site.

Our results demonstrate that neither the FAK-Tyr397/Src-SH2 domain interaction, nor prior phosphorylation of FAK-Tyr397, is required for v-Src to induce tyrosine phosphorylation of FAK (Fig. 4), or focal adhesion turnover that accompanies transformation (Fig. 5). However, although there is no absolute requirement, the kinetics of Src family kinase-dependent phosphorylation of FAK that occurs at later times after integrin engagement may be influenced by the state of FAK-Tyr397 phosphorylation (Fig. 4).

In conclusion, although the downstream consequences of individual FAK phosphorylation events are not fully understood, we have shown that integrin- and activated Src-induced phosphorylation of FAK are at least partly distinct, with only integrin engagement stimulating Tyr397 phosphorylation. The specific phosphorylation of FAK induced by these stimuli, together with their biological correlates, suggests that FAK phosphorylation may determine the balance of focal adhesion assembly and disassembly, and consequently the rate of integrin-dependent cell motility.

Acknowledgment—We thank John Wyke for interest in this work and helpful comments on the manuscript.

REFERENCES
1. Andre, E., and Becker-Andre, M. (1995) Biochem. Biophys. Res. Commun. 190, 140–147
2. Grant, S. G., Karl, K. A., Kielbier, M. A., and Kandel, E. R. (1995) Genes Dev. 9, 1999–1921
3. Hanks, S. K., and Polte, T. R. (1997) Bioessays 19, 137–145
4. Richardson, A., and Parsons, J. T. (1994) Bioessays 16, 229–236
5. Schaller, M. D., and Parsons, J. T. (1994) Curr. Opin. Cell. Biol. 6, 705–710
6. Schaller, M. D., Bergman, C. A., Cobb, B. S., Vines, R. B., Reynolds, A. B., and Parsons, J. T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5182–5186
7. Hanks, S. K., Calabia, M. B., Harper, M. C., and Patel, S. K. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8487–8491
8. Schaller, M. D., Otea, C. A., Hildebrand, J. D., and Parsons, J. T. (1995) J. Cell Biol. 130, 1181–1187
9. Danker, K., Gabriel, B., Heidrich, C., and Reutter, W. (1998) Exp. Cell Res. 239, 326–331
10. Hildebrand, J. D., Taylor, J. M., and Parsons, J. T. (1996) Mol. Cell. Biol. 16, 3169–3178
11. Cary, L. A., Han, D. C., Polte, T. R., Hanks, S. K., and Guan, J. L. (1998) J. Cell Biol. 140, 211–221
Tyrosine 397-independent Phosphorylation of FAK by v-Src

12. Hildebrand, J. D., Schaller, M. D., and Parsons, J. T. (1993) *J. Cell Biol.* **123**, 993–1005
13. Schaller, M. D., Bergman, C. A., and Parsons, J. T. (1993) *Mol. Cell. Biol.* **13**, 785–791
14. Richardson, A., and Parsons, T. (1996) *Nature* **380**, 538–540
15. Calalb, M. B., Polte, T. R., and Hanks, S. K. (1995) *Mol. Cell. Biol.* **15**, 954–963
16. Calalb, M. B., Zhang, X., Polte, T. R., and Hanks, S. K. (1996) *Biochem. Biophys. Res. Commun.* **228**, 662–668
17. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994) *Nature* **372**, 786–791
18. Schaller, M. D., and Hunter, T. (1996) *Mol. Cell. Biol.* **16**, 5623–5633
19. Guan, J. L., and Shalloway, D. (1992) *Nature* **358**, 690–692
20. Burridge, K., Turner, C. E., and Romer, L. H. (1992) *J. Cell Biol.* **119**, 893–903
21. Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, J. W., Vines, R. R., and Parsons, J. T. (1994) *Mol. Cell. Biol.* **14**, 1680–1688
22. MacAuley, A., and Cooper, J. A. (1989) *Mol. Cell. Biol.* **9**, 2648–2656
23. Sieg, D. J., Ilic, D., Jones, K. C., Damsky, C. H., Hunter, T., and Schlaepfer, D. D. (1998) *EMBO J.* **17**, 5933–5947
24. Chen, H. C., and Guan, J. L. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 10148–10152
25. Guinebault, C., Payrastre, B., Racaud-Sultan, C., Mazarguil, H., Breton, M., Mauco, G., Plantavid, M., and Chap, H. (1995) *J. Cell Biol.* **129**, 831–842
26. Welham, M. J., and Wyke, J. A. (1988) *J. Virol.* **62**, 1698–1706
27. Fincham, V. J., and Frame, M. C. (1998) *EMBO J.* **17**, 81–92
28. McLean, G. W., Owsianka, A. M., Subak-Sharpe, J. H., and Marsden, H. S. (1991) *J. Immunol. Methods* **137**, 149–157
29. Fincham, V. J., Chukleigh, A., and Frame, M. C. (1999) *J. Cell Sci.* **112**, 947–956
30. Marsden, H. S., Stow, N. D., Preston, V. G., Timbury, M. C., and Wilkie, N. M. (1978) *J. Virol.* **26**, 624–642
31. Fincham, V. J., Wyke, J. A., and Frame, M. C. (1995) *Oncogene* **10**, 2247–2252
32. Eide, B. L., Turck, C. W., and Escobedo, J. A. (1995) *Mol. Cell. Biol.* **15**, 2819–2827
33. Aplin, A. E., Howe, A., Alahari, S. K., and Juliano, R. L. (1998) *Pharmacol. Rev.* **50**, 197–263
34. Owens, D. W., McLean, G. W., Wyke, A. W., Paraskeva, C., Parkinson, E. K., Frame, M. C., and Brunton, V. G. (2000) *Mol. Cell. Biol.* **20**, 51–64
35. Fincham, V. J., Ulnu, M., Brunton, V. G., Pitts, J. D., Wyke, J. A., and Frame, M. C. (1996) *J. Cell Biol.* **135**, 1531–1544
36. Kanner, S. B., Reynolds, A. B., Vines, R. R., and Parsons, J. T. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 3238–3332
37. Guan, J. L., Trevithick, J. E., and Hynes, R. O. (1991) *Cell Regul.* **2**, 951–964
38. Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatani, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995) *Nature* **377**, 539–544
39. Schaller, M. D., Hildebrand, J. D., and Parsons, J. T. (1999) *Mol. Biol. Cell* **10**, 3489–3505
40. Chen, H. C., Appeddu, P. A., Isoda, H., and Guan, J. L. (1996) *J. Biol. Chem.* **271**, 26329–26334
41. Reiske, H. R., Kao, S. C., Cary, L. A., Guan, J. L., Lai, J. P., and Chen, H. C. (1999) *J. Biol. Chem.* **274**, 12361–12366
42. Tamura, M., Gu, J., Danen, E. H., Takino, T., Miyamoto, S., and Yamada, K. M. (1999) *J. Biol. Chem.* **274**, 20693–20703
43. Tamura, M., Gu, J., Matsuno, K., Aota, S., Parsons, R., and Yamada, K. M. (1998) *Science* **280**, 1614–1617
44. Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsoo, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P., and Mak, T. W. (1998) *Cell* **95**, 29–39
45. Maehama, T., and Dixon, J. E. (1998) *J. Biol. Chem.* **273**, 13375–13378
46. Han, D. C., and Guan, J. L. (1999) *J. Biol. Chem.* **274**, 24425–24430