p27<sup>Kip1</sup> and Cyclin D1 Are Necessary for Focal Adhesion Kinase Regulation of Cell Cycle Progression in Glioblastoma Cells Propagated in Vitro and in Vivo in the Scid Mouse Brain*  

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We have reported previously that the expression of focal adhesion kinase (FAK) is elevated in glioblastomas and that expression of FAK promotes the proliferation of glioblastoma cells propagated in either soft agar or in the C.B.17 severe combined immunodeficiency (scid) mouse brain. We therefore determined the effect of FAK on cell cycle progression in these cells. We found that overexpression of wild-type FAK promoted exit from G<sub>1</sub> in monolayer cultures of glioblastoma cells, enhanced the expression of cyclins D1 and E while reducing the expression of p27<sup>Kip1</sup> and p21<sup>Waf1</sup>, and enhanced the kinase activity of the cyclin D1-cyclin-dependent kinase-4 (cdk4) complex. Transfection of the monolayers with a FAK molecule in which the autophosphorylation site is mutated (FAK397F) inhibited exit from G<sub>1</sub> and reduced the expression of p27<sup>Kip1</sup> and p21<sup>Waf1</sup>. Small interfering RNA (siRNA)-mediated down-regulation of cyclin D1 inhibited the enhancement of cell cycle progression observed on expression of wild-type FAK, whereas siRNA-mediated down-regulation of cyclin E had no effect. siRNA-mediated down-regulation of p27<sup>Kip1</sup> overcame the inhibition of cell cycle progression observed on expression of FAK397F, whereas down-regulation of p21<sup>Waf1</sup> had no effect. These results were confirmed in vivo in the scid mouse brain xenograft model in which propagation of glioblastoma cells expressing FAK397F resulted in a 50% inhibition of tumor growth and inhibited exit from G<sub>1</sub>. Taken together, our results indicate that FAK promotes proliferation of glioblastoma cells by enhancing exit from G<sub>1</sub> through a mechanism that involves cyclin D1 and p27<sup>Kip1</sup>.

FAK<sup>1</sup> is a nonreceptor cytoplasmic tyrosine kinase. It has been shown to be activated on clustering of integrin receptors in the cell membrane or by the ligation of multiple growth factor receptors (1–5). Activation occurs through phosphorylation of Tyr<sup>397</sup>, either through autophosphorylation or Src-induced phosphorylation (4). Clustering of integrin receptors occurs during cell attachment, and the receptors localize to focal contacts (early adhesion complexes) where, in a temporally related manner, a signaling complex is assembled which includes FAK as well as cellular Src and other kinases, cytoskeletal proteins, and adaptor molecules (3, 5, 6). In nonmalignant and nontransformed cells, this localization of FAK to focal contacts or focal adhesions (mature adhesion complexes) has been shown to be necessary for its activation, indicating that FAK activation is regulated by integrin receptor engagement or cell adhesion in these cells. In contrast, in malignant or transformed cells, the activation of FAK does not appear to be regulated solely by cell adhesion or integrin receptor engagement. For example, we have shown the activation of FAK (phosphorylation of Tyr<sup>397</sup>) in glioblastoma cells propagated in suspension, suggesting that the mechanisms governing the activation of FAK in malignant or transformed cells may differ from those utilized by normal cells (7).

Previous studies examining the role of FAK in proliferation and regulation of the cell cycle have focused largely on NIH3T3 fibroblasts (8–10). In these cells, overexpression of wild-type FAK promotes cell proliferation, as assessed by bromodeoxyuridine (BrdUrd) labeling, and this is associated with promotion of the exit from G<sub>1</sub> by enhancement of the transcriptional activation of cyclin D1 (9). Specifically, FAK promotes the binding activity of an Ets transcription factor to the Ets B element in the cyclin D1 promoter and induces expression of the transcription factor, kruppel-like factor 8 (KLF8), which binds a GT box in the cyclin D1 promoter (9, 10). In addition, the activation of ERK was shown to be necessary for FAK-promoted cyclin D1 transcription in the NIH3T3 cells, because the MEK inhibitor PD98059, but not the JNK inhibitor curcumin, inhibited cyclin D1 transcription in cells overexpressing wild-type FAK (9). In contrast, expression of a truncated FAK (FAK-AC14), which

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1 The abbreviations used are: FAK, focal adhesion kinase; cdk, cyclin-dependent kinase; BrdUrd, bromodeoxyuridine; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; KLF8, kruppel-like factor 8; mAb, monoclonal antibody; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MHC, major histocompatibility complex; PCNA, proliferating cell nuclear antigen; PR, proline rich; PTEN, phosphatase and tensin homolog deleted on chromosome 10; RB, retinoblastoma protein; Rb [pSer<sup>795</sup>], anti-phosphospecific retinoblastoma protein at Ser<sup>795</sup>; RT, reverse transcription; siRNA, small interfering RNA; scid, severe combined immunodeficiency; TET, tetrasaccharide; TELC, N<sub>p</sub>-tosyl-l-lysine chloromethyl ketone; TUNEL, terminal nucleotidyl transferase-mediated UTP nick end labeling; G3PDH, anti-glyceraldehyde 3-phosphate dehydrogenase.
because of the lack of the carboxyl-terminal 14 residues does not localize to focal adhesions, inhibits BrdUrd labeling. Although the expression of FAK-ΔC14 in the NIH3T3 cells was found to enhance the expression of the cyclin-dependent kinase (cdk) inhibitor p21Waft, p21Waft was not necessary for FAK-ΔC14 inhibition of cell cycle progression in these cells. The expression of p27Kip1 was unaltered by the expression of FAK-ΔC14 in the NIH3T3 cells.

FAK protein expression is elevated in a number of malignant tumors, including breast and colon cancer (11, 12). We and others have reported previously that FAK protein and activity (based on phosphorylation of Tyr397) are elevated in grades III and IV malignant astrocytoma tumors (anaplastic astrocytoma and glioblastoma, respectively), compared with the normal brain (7, 13). We have also shown that the overexpression of wild-type FAK promotes soft agar growth of glioblastoma cells (14), the expression of the mutant FAK397F inhibits soft agar growth of these cells (15), and that FAK is sufficient to promote Ras activity in glioblastoma cells propagated in aggregate suspension or as a monolayer (15). Because constitutively active Ras is known to promote soft agar growth of multiple cell types (16, 17), the ability of FAK to promote soft agar growth is likely caused, at least in part, by its activation of Ras.

Because FAK is known to promote proliferation by affecting the cell cycle in NIH3T3 cells, and as it is well established that the expression of molecules that regulate the cell cycle in cancer cells is frequently altered (18, 19), it is possible that the mechanism(s) by which FAK regulates the cell cycle is altered in cancer cells. Here, we report that wild-type FAK promotes the cell cycle progression of glioblastoma cells propagated both in vitro as a monolayer and in vivo in the C.B.17 scid mouse brain. Our results demonstrate that overexpression of wild-type FAK promotes exit from G1 in glioblastoma cells propagated both in vitro as a monolayer and in vivo in the scid mouse brain. In common with the previous findings examining the effects of expression of FAK on normal cells, we found that FAK enhancement of cell proliferation in the glioblastoma cells requires ERK activity, and the promotion of the exit from G1 requires cyclin D1; however, in glioblastoma cells we found that the FAK-mediated regulation of the exit from G1 also involves p27Kip1.

**EXPERIMENTAL PROCEDURES**

Reagents—PD98059, curcumin, and SB202247 were purchased from Calbiochem. The following purified rabbit polyclonal antibodies were purchased: anti-integrin α directed toward the cytoplasmic tail (Chemicon, Temecula, CA); anti-FAK (Upstate Biotechnology, Lake Placid, NY); and anti-phospho-ERK (Cell Signaling-New England Biolabs, Beverly, MA); anti-cyclin A, anti-cyclin B1, anti-p27Kip1, anti-p21Waft, anti-p57Kip2, and anti-ERK IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The following monoclonal antibodies (mAbs) were purchased: anti-actin (Sigma); anti-PCNA (Dako Corp., Carpinteria, CA); anti-c-Myc (Oncogene Research Products, Cambridge, MA); anti-glyceroldehyde-3-phosphate dehydrogenase (G3PDH) (Research Diagnostics, Inc., Flanders, NJ); anti-cdk4 (Chemicon International, Temecula, CA); anti-cyclin D1, anti-cyclin E, and anti-phospho-JNK IgG (Santa Cruz Biotechnology). The anti-CKLFA antiseraum has been described previously (10). Rabbit anti-phosphospecific retinoblastoma (Rb) (pSer780) IgG and anti-Rb IgG directed toward the carboxyl terminus were purchased (Cell Signaling).

**Cells and Constructs—**U-251MG human glioblastoma cells stably overexpressing wild-type FAK in the tetracycline (TET)-inducible system (OFAK5) and the U-251MG cells stably expressing mutant FAK397F (OFAK397F-clones 18 and 3) in the TET-inducible system, as well as the empty vector clones TRE2, have been described previously (14, 15).

**Analysis of Mouse Intracerebral Xenograft Tumors—**C.B.17 scid mice obtained from the Frederick Cancer Center at the National Cancer Institute were injected intracerebrally with stereotactic assistance at 6 weeks of age with 1 × 10⁶ cells of the OFAK5, OFAK397F-18, or the OFAK397F-3 cell clone, and the injected animals were divided randomly into two groups, as described previously (14). Doxycline (2 mg/ml in saline) or vehicle alone (1 ml of saline) were administered daily by intraperitoneal injection. At 8 days postinjection, mice were euthanized, and the brains were harvested and processed for either FACS analysis or isolation of human tumor cells by magnetic beads; snap frozen for homogenization and immunoblotting; or fixed, as described previously (14). Briefly, a single brain cell suspension for FACS analysis was prepared as follows. Xenograft brains were harvested, placed into Dulbecco’s modified Eagle’s medium with 1% bovine serum albumin and the following inhibitors: 100 μM phenylmethylsulfonyl fluoride, 10 μM aprotinin, 10 μM leupeptin, 100 μM sodium vanadate, and 20 μM TLCK, minced thoroughly (30 min/brain), passed through a 170-μm mesh, resuspended in the above buffer with fresh protease inhibitors; 500,000 cells were incubated with FITC-conjugated mAb anti-human specific MHC Class I IgG (Pharmingen) or with FITC-conjugated mouse IgG isotype control (Pharmingen) (30 min, 4 °C), homogenized subjected to FACS analysis. The mouse IgG isotype control was used to determine the background fluorescence. Magnetic bead isolation of human tumor cells was performed as follows. A single cell suspension of xenograft brain was prepared as above and incubated with mAb anti-human specific MHC Class I IgG that had been coupled to magnetic polystyrene Dynabeads, as recommended by the manufacturer for 30 min at 4 °C (Collecion Pan Mouse IgG kit, Dynal A.S., Oslo, Norway). The beads were washed, and the beads were harvested by DNase treatment, which cleaves the linker molecule, lysed in RIPA lysis buffer 1% deoxycholate, 1.0% Triton X-100, and 0.1% SDS in 0.01 M Tris base (pH 7.4) with 0.15 M NaCl with the above protease inhibitors, centrifuged (35,000 rpm, 4 °C, 1 h), and the protein concentration determined in the supernatant. Brains were snap frozen in liquid nitrogen for immunoblot analysis of total brain lysates and then homogenized using a Polytron (Kinematics, Luzern, Switzerland). Each tumor was harvested by DNase treatment, which cleaves the linker molecule, lysed in RIPA lysis buffer with the above protease inhibitors, the lysates centrifuged (35,000 rpm, 4 °C, 1 h), and the concentration of the protein in the supernatant determined. For histologic analysis, brains were fixed in 4% buffered paraformaldehyde (4 h, 4 °C) followed by immersion in 6% sucrose overnight, then frozen and maintained at −70 °C until sectioning. Tumor volume was assessed as described previously (14). Each brain was serially sectioned (10 μm per section) and stained every 40 μm with mAb anti-human specific MHC Class I IgG (10 μg/ml, 2 h, 4 °C) (mAb W6/32, Serotec, Inc., Raleigh, NC), followed by a multilink secondary antibody conjugated to biotin and horseradish peroxidase, followed by incubation with streptavidin, and then 3,3′-diaminobenzidine substrate, as per the instructions in the kit from BioGenex (San Ramon, CA). Digital images were imported into Adobe Photoshop (Adobe Systems, Inc., Mountain View, CA), and areas of interest were quantitated as a pixel number, and pixel numbers for all sections from each brain were summed to obtain a total pixel number. The proliferation index or PCNA index was determined as described previously (14); briefly, two sections of each brain were incubated with 1 μg/ml PCNA mAb (1 h, 22 °C), followed by the secondary antibody. The detection procedure was described above. The percentage of cells containing TUNEL-positive signals in each tumor was determined using a TUNEL assay, as described previously (14). Briefly, two tissue sections from each brain were reacted with biotinylated dNTP, plus terminal deoxynucleotidyltransferase enzyme, followed by biotin, streptavidin-horseradish peroxidase, and the 3,3′-diaminobenzidine substrate, according to the instructions provided with the terminal deoxynucleotidyltransferase-Frag EL DNA Fragmentation Detection kit (Oncogene Research Products).

**Cell Cycle Analysis—**Cells were harvested with trypsin, washed with cold phosphate-buffered saline, fixed in 70% ethanol, and kept at 4 °C; overnight. Fixed cells were washed with phosphate-buffered saline and resuspended in 40 μg/ml RNase A (Sigma) and 40 μg/ml propidium iodide (Sigma) in phosphate-buffered saline (30 min, 22 °C). The cell suspension was then analyzed using a FACScan for DNA content (BD Biosciences, FACS Calibur).

**BrdUrd Labeling—**Cells were treated with doxycycline or vehicle for 4 days and then replated onto vitronectin-coated glass coverslips in serum-containing medium, minus/minus doxycycline and labeled overnight (18 h) with 10 μM BrdUrd. The next morning, cells were fixed, reacted with mAb anti-BrdUrd followed by a secondary horseradish peroxidase-conjugated antibody and substrate according to the manufacturer’s instructions (Oncogene Research Products). The percent of cells with dark brown nuclei were counted in six fields at 10× magnification.

**Annexin V Apoptosis Assay—**The Annexin V assay with propidium iodide labeling was performed on cells in culture as per the manufacturer’s instructions (Pharmingen).

**Immunoblotting—**Cells were lysed in 1% Nonidet P-40 lysis buffer containing 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Nonidet P-40,
and 20 mM Tris-HCl, pH 8.0, with the above protease inhibitors (4 °C, 60 min), centrifuged (35,000 rpm, 1 h, 4 °C), and the protein concentration determined in the supernatant (20). Equivalent μg of protein from each lysate were electrophoresed on a 12% SDS-PAGE, transferred to Immobion-P membrane (Millipore Corp., Bedford, MA), blocked with 5% buffered bovine serum albumin (3 h, 22 °C), and then reacted with primary antibody overnight at 4 °C, followed by 0.025 μg/ml horseradish peroxidase-conjugated secondary antibody (1 h, 22 °C), and the membrane was developed with the ECL system (Amersham Biosciences). For semiquantitative analysis of band intensity, a specific band on the autorad from three different exposures was subjected to densitometric analysis, and the densitometric readings were averaged; the background densitometric reading on the autorad was subtracted.

**Soft Agar Growth Assays**—Anchorage-independent growth assays were performed as described previously (14). Briefly, cells were treated with doxycycline or vehicle for 4 days, harvested and resuspended in a 0.3% agar (plus/minus doxycycline), poured onto plates containing a layer of 0.5% agar (plus/minus doxycycline), and then maintained at 37 °C, 5% CO2 for 14 days. The resulting colonies were counted with a colony being defined as >10 cells. Samples were assayed in replicas of three, and the experiment was repeated twice.

**siRNA Studies**—Commercially available siRNA duplexes directed toward p27Kip1, p21Waf1, p57Kip2, as well as cyclin D1, or cyclin E were purchased from Santa Cruz Biotechnology. siRNA duplexes directed toward cyclin E (Dharmacon) was used to optimize the transfection conditions and efficiency, as described previously (21). Additional controls of nonspecific siRNA duplex or vehicle alone were evaluated and found not to alter p27Kip1, p21Waf1, p57Kip2, cyclin D1, or cyclin E mRNA, and protein expression at the concentration of siRNA found to reduce the specific target message and protein optimally.

**Immunofluorescence Analysis**—The cellular localization of the mutant FAK397F was assessed using immunofluorescence analysis as described previously (21). Briefly, cells were propagated in complete medium with 2 μg/ml doxycycline or vehicle for 4 days, harvested with buffered EDTA, resuspended in adhesion assay buffer with 1 mM MgCl2 and 100 μM MnCl2 with 1% bovine serum albumin, and 40,000 cells were plated onto a coverslip coated previously with 5 μg/ml rat anti-Rb IgG, stripped, and reprobed with rabbit anti-phosphospecific Rb [pSer795] IgG, incubated with 12% SDS-PAGE, and immunoblotted with rabbit anti-Rb IgG.

**Statistical Analysis**—Data were analyzed using an unpaired t test (SigmaPlot 2000, SPSS, Inc.). A p value < 0.05 was considered statistically significant. All experiments were repeated two to three times with consistent results.
doxycycline-inducible mutant FAK (FAK397F) of cells grown in monolayer cultures. The percentage of cells in each phase of the cell cycle was estimated using FACS analysis of propidium iodide-stained cells. These studies were initially performed after serum starvation to synchronize the cell cycle. Under conditions of serum starvation, in which the cells were starved of serum for 2 days (0.4% fetal bovine serum) prior to doxycycline or vehicle administration and then cultured for 4 days in reduced serum (4% fetal bovine serum), the percentage of cells in S phase was higher in doxycycline-induced cells expressing wild-type FAK (26%) than vehicle controls (21%), and the percentage of cells in G0/G1 was lower (48% versus 54%) (Fig. 1, A and B). Under conditions of serum starvation, the percentage of cells in S phase was lower in doxycycline-induced cells expressing FAK397F (10% versus 22%), and the percentage of cells in G0/G1 was higher (74% versus 56%) (Fig. 1, C and D). Similar results were observed when the experiments were performed in complete medium (10% fetal bovine serum). The percentage of cells in S phase was higher on expression of wild-type FAK (46% versus 33%), and the percentage of cells in G0/G1 was lower (40% versus 49%) (Fig. 2, A and B), whereas the percentage of cells in S phase was lower on expression of FAK397F (11.5% versus 31.5%) and the percentage of cells in G0/G1 was higher (73% versus 52%) (Fig. 2, C–F; values for the two mutant FAK397F clones averaged for the histogram in E and F). The percentage of cells in G2/M was not altered on expression of either wild-type or mutant FAK in cells cultured under serum-starved conditions or in complete medium (Figs. 1 and 2). These results are consistent with those of Zhao et al. (8), who also found that serum starvation did not significantly alter the effect of FAK on cell cycle progression in NIH3T3 cells and indicate that FAK promotes exit from G1 in glioblastoma cells in monolayer culture. This conclusion was supported by the results obtained on analysis of BrdUrd labeling of cells cultured in complete medium, which showed that expression of wild-type FAK resulted in a 58% higher level of BrdUrd labeling than in cells that were not treated with doxycycline (p = 0.001) (Fig. 3) and that expression of FAK397F resulted in a 70% reduction in BrdUrd labeling compared with the cells not treated with doxycycline (p < 0.001) (Fig. 3).
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Expression of mutant FAK (FAK397F) in glioblastoma cells failed to alter the levels of early apoptotic cells in vitro

FAK397F-18 and -3, OFAK5, and TRE2 (control) clones were administered 2 μg/ml doxycyline (Dox) or vehicle in complete medium for 4 days and then harvested and stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer’s instructions (BD Biosciences-Clontech, ApoAlert Annexin V-FITC kit).

<table>
<thead>
<tr>
<th>Dox</th>
<th>% PI-negative and annexin V-positive (mean ± S.E.)</th>
<th>% PI-positive and annexin V-positive (mean ± S.E.)</th>
</tr>
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<tbody>
<tr>
<td>OFAK397F-18</td>
<td>0.64 ± 0.04</td>
<td>1.73 ± 0.11</td>
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<tr>
<td>OFAK397F-18</td>
<td>0.3 ± 0.05</td>
<td>3.29 ± 0.09</td>
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<tr>
<td>OFAK397F-3</td>
<td>0.48 ± 0.06</td>
<td>1.62 ± 0.08</td>
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<tr>
<td>OFAK397F-3</td>
<td>0.49 ± 0.04</td>
<td>2.7 ± 0.07</td>
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<tr>
<td>OFAK5</td>
<td>0.53 ± 0.06</td>
<td>1.84 ± 0.21</td>
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<tr>
<td>OFAK5</td>
<td>0.55 ± 0.02</td>
<td>1.44 ± 0.28</td>
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<tr>
<td>TRE2</td>
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<tr>
<td>TRE2</td>
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p27Kip1 Is Necessary for the Inhibition of Cell Cycle Progression Found in Glioblastoma Cells Expressing Mutant FAK397F—To determine whether p27Kip1 and/or p21Waf1 is necessary for the inhibitory effect of the mutant FAK397F on the cell cycle progression of glioblastoma cells, we down-regulated p27Kip1, p21Waf1, and p57Kip2 individually with duplex siRNA and then examined the cell cycle by analyzing DNA content after propidium iodide labeling. The conditions for down-regulation of each of the three cdk inhibitor mRNAs and proteins were determined, and the optimal concentrations of siRNA were found to be: 150 nM siRNA for down-regulation of p27Kip1, 200 nM siRNA for p21Waf1, and 250 nM siRNA for p57Kip2 (Fig. 6, A and B). No change in cell viability was seen at these concentrations of siRNA over the time frame of the assay. Down-regulation of p27Kip1 (Fig. 6, C and D) blocked the ability of FAK397F to inhibit glioblastoma cell cycle progression, suggesting that p27Kip1 is involved in FAK regulation of the cell cycle. Down-regulation of neither p21Waf1 (Fig. 6, C and D) nor
p57Kip2 (data not shown) relieved the inhibitory effect of FAK397F on cell cycle progression.

Cyclin D1 Is Necessary for FAK Promotion of Cell Cycle Progression in Glioblastoma Cells Expressing Wild-type FAK—We used a similar approach to determine whether cyclins D1 and/or E are necessary for the cell cycle progression observed on expression of wild-type FAK in glioblastoma cells. The optimal concentration of siRNA was established as 200 nM siRNA for cyclin D1 and 150 nM siRNA for cyclin E (Fig. 7, A and B), and the cell viability was unaffected at these concentrations of siRNA over the time frame of the assay. Down-regulation of cyclin D1, but not of cyclin E, blocked the ability of wild-type FAK to promote cell cycle progression (Fig. 7, C and D), suggesting that cyclin D1 is necessary for FAK promotion of cell cycle progression in glioblastoma cells.

Expression of Wild-type FAK Enhances the Activity of the Cyclin D1-cdk4 Complex in Glioblastoma Cells—Elevated levels of cyclin D1 typically promote deregulated S phase progression and loss of the G1 checkpoint (18, 19, 23). An important role of cyclin D1 is to bind cdk4 and form an active complex that can phosphorylate the Rb protein at Ser795 (18, 19, 23). Therefore, we examined whether the enhanced cyclin D1 levels with expression of wild-type FAK promoted the activity of the cyclin D1-cdk4 complex using a standard nonradioactive cdk4 immunoprecipitation kinase assay as described previously (22). We found an estimated 4–5-fold increase in the level of phosphorylation at Ser795 of a recombinant truncated Rb protein in the lysate of glioblastoma cells overexpressing wild-type FAK (Fig. 8, A and B, lane 2 and 3). The phosphospecific anti-Rb [pSer795] IgG typically detects a single species on immunoblotting (22). A similar elevation in the level of phosphorylation of Ser795 on the recombinant Rb protein was detected when the lysate of glioblastoma cells overexpressing wild-type FAK was immunoprecipitated with mAb anti-cyclin D1 and subjected to the cdk4 kinase assay (data not shown). As a control to assess the quality of the lysate, equivalent μg of the lysate from the whole brain lysate of animals was electrophoresed on SDS-PAGE and immunoblotted with mAb anti-actin (Fig. 9C).

Expression of TET-inducible Mutant FAK397F Inhibits Glioblastoma Tumor Cell Proliferation When Propagated in the Scid Mouse Brain—We have previously reported that the overexpression of wild-type FAK using the TET-inducible system promotes the proliferation of glioblastoma cells when propagated in the C.B.17 scid mouse brain (14). To substantiate further a role for FAK in promoting glioblastoma cell proliferation, we examined the effect of expression of the mutant FAK397F on glioblastoma cell proliferation in vivo using the TET-inducible system. The FAK397F-18 or -3 clones were injected into the scid mouse brain with stereotactic assistance and allowed to propagate for 8 days in the animals. The in vivo expression of the c-Myc-tagged mutant FAK397F was verified by immunoprecipitating whole brain lysate with rabbit anti-FAK IgG, followed by SDS-PAGE and blotting for c-Myc. The detection of a band migrating with a relative molecular mass of 125-kDa, consistent with the relative migration of c-Myc-tagged mutant FAK397F, in the lysates of brains from animals administered doxycycline, and its absence in the lysates of brains from animals administered vehicle, confirmed the expression of FAK397F (Fig. 9A). On stripping and reprobing the membrane with rabbit anti-FAK IgG, a band at 125 kDa was detected in the brain lysates (Fig. 9B). Densitometric analysis indicated that the intensity of this 125 kDa band was 4-fold greater in the lysates of brains from animals administered doxycycline than the lysates of brains from animals administered vehicle (Fig. 9B). This level of construct induction on doxycycline administration is similar to that achieved in our previous studies in which we used doxycycline administration to induce expression of wild-type FAK in this animal model (14). As a control to assess the quality of the lysate, equivalent μg of lysate from the whole brain lysate of animals was electrophoresed on SDS-PAGE and immunoblotted with mAb anti-actin (Fig. 9C).

Expression of FAK397F significantly reduced (50%) the percentage of tumor cells detected in the scid mouse brain after 8 days of propagation (Fig. 9, D and E) (clone 18, 12.2 ± 3.2% (mean percent ± S.E.) tumor cells with vehicle administration, and 6.1% ± 2.1% tumor cells with doxycycline administration, p = 0.03; and clone 3, 12.4% ± 3.1% tumor cells with vehicle administration, and 6.5% ± 1.9% tumor cells with doxycycline administration, p = 0.04). A corresponding statistically signif-
FIG. 6. Down-regulation of p27<sup>Kip1</sup> blocks the inhibitory effect of mutant FAK397F on the cell cycle progression of glioblastoma cells. Parallel cultures of the OFAK397F-18 clone propagated in complete medium were administered 2 μg/ml doxycycline (Dox) or vehicle for 4 days and then transfected with duplex siRNA directed toward p27<sup>Kip1</sup>, p21<sup>Waf1</sup>, or p57<sup>Kip2</sup>, as described under “Experimental Procedures.” A, post-transfection (48 h) the cells were harvested and RNA extracted for real time RT-PCR using forward and reverse primers directed specifically toward p27<sup>Kip1</sup>, p21<sup>Waf1</sup>, or p57<sup>Kip2</sup>. The relative expression of these mRNAs was normalized to actin. B, cells were detergent lysed and the lysate subjected to immunoblotting with the indicated antibodies. C and D, the cells were harvested, propidium iodide labeled, and the DNA content analyzed by FACS analysis as described in the legend for Fig. 1. In D, the percentage of cells in G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M phases is plotted as a histogram, and the statistical significance of differences in each phase when cells were doxycycline treated versus vehicle treated is indicated by the p value; p < 0.05 was considered significant.
A significant decrease (80%) in mean tumor volume was found in animals injected with the OFAK397F-18 clone and administered doxycycline (n/H11005/6) compared with animals administered vehicle (n/H11005/6) (mean tumor volume/H11005/286,000 pixels/H11006/116,000 with vehicle; and mean tumor volume/H11005/41,000 pixels/H11006/29,000 with doxycycline administration, p/H11005/0.002). We showed previously in this animal model that doxycycline administration had no effect on the proliferation of control vector-transfected glioblastoma cells (TRE4) or of the wild-type U-251MG cells (14). These results indicate that expression of the mutant FAK397F inhibits tumor growth in vivo and suggest that FAK is necessary for tumor cell proliferation.

To determine whether the decrease in tumor cell number and tumor volume detected on expression of the mutant FAK397F in glioblastoma cells propagated in the scid mouse brain is the result of inhibition of cell proliferation, the number of proliferating cells in two sections from each animal brain was estimated using immunohistochemical staining of PCNA. In animals injected with the OFAK397F-18 clone and administered doxycycline, a 54% reduction in PCNA-positive tumor nuclei was found compared with the percentage of PCNA-positive tumor nuclei in the animals administered vehicle (mean percent positive PCNA-labeled tumor nuclei/H11005/30%/H11006/3.1% in animals administered vehicle; and mean percent positive tu-
FAK Regulation of Cell Cycle Progression in Glioblastoma

**Expression of wild-type FAK promotes an enhanced activity of the cyclin D1-cdk4 complex in glioblastoma cells.** Parallel cultures of the OFAK5 clone were propagated in complete medium and administered 2 μg/ml doxycycline (Dox) or vehicle for 4 days and then lysed. A and B, equivalent amount of lysate (700 μg) was immunoprecipitated (IP) with mAb anti-cdk4 or mouse IgG, washed, the immunoprecipitated protein subjected to the cdk4 kinase assay, followed by 12% SDS-PAGE and immunoblotting with anti-Rb (pSer795) IgG (A), stripped, and reprobed with anti-total Rb IgG (B), as described under "Experimental Procedures." C, an equivalent amount of lysate (30 μg) was subjected to SDS-PAGE and immunoblotted with mAb anti-glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as a control for the quality of the lysate.

<table>
<thead>
<tr>
<th>- Dox</th>
<th>+ Dox</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IP</strong></td>
<td></td>
</tr>
<tr>
<td>A. anti-Rb (pSer-795) IgG blot</td>
<td></td>
</tr>
<tr>
<td>anti-Rb (pSer-795) IgG blot</td>
<td></td>
</tr>
<tr>
<td>anti-Rb IgG blot</td>
<td>Total Rb</td>
</tr>
<tr>
<td>B. anti-Rb IgG blot</td>
<td>38 kDa</td>
</tr>
<tr>
<td>C. mAb anti-G3PDH</td>
<td>36 kDa</td>
</tr>
</tbody>
</table>

**Fraction of TUNEL-positive cells in the two groups of animals (mean from each animal brain indicated no change in the percentage isolated from mouse brains after intracerebral propagation of the OFAK5 clone) as a control for antigenicity of the MOAb anti-glyceraldehyde-3-phosphate dehydrogenase (G3PDH).**

**Expression of Mutant FAK397F Inhibits ERK Activity in Glioblastoma Cells Propagated in Vitro and in Vivo in the Scid Mouse Brain—To determine whether FAK regulates ERK activity in the glioblastoma cells, we examined the effect of expression of the wild-type FAK (OFAK5 clone) and the mutant FAK (OFAK397F-18 clone) on ERK activity. In vitro propagation of the OFAK5 clone for 4 days with doxycycline administration or vehicle in complete medium followed by detergent lysis demonstrated higher ERK activity (2-fold increase) compared with the cells administered vehicle, whereas there was no change in JNK activity (Fig. 14B). This conclusion was based on summing the densitometric reading of the p42- and p44-kDa phospho-ERK bands and normalizing this value to the sum of the densitometric reading of the 42- and 44-kDa bands on the total ERK blot. In vitro propagation of the OFAK397F-18 clone with doxycycline administration in complete medium for 4 days followed by detergent lysis demonstrated lower ERK activity by 200% compared with the cells administered vehicle, whereas there was no significant difference in JNK activity (Fig. 14B). Analysis of ERK activity in the lysate from the human tumor cells isolated from six control mice injected with the wild-type FAK clone (OFAK5) and administered doxycycline showed a 2-fold greater ERK activity compared with the lysate of human tumor cells isolated from the brains of mice injected with the OFAK397F-18 clone and administered doxycycline compared with the lysate of human tumor cells isolated from six control mice.
mice (Fig. 14). JNK activity was not altered with the expression of the wild-type FAK or the mutant FAK397F in these tumor cell clones propagated in vivo (Fig. 14, C and D). These results, taken together with the results obtained for in vitro soft agar growth, suggest that FAK regulates ERK activity in glioblastoma cells and support our hypothesis that FAK signaling to ERK is likely necessary for FAK promotion of cell cycle progression.

**DISCUSSION**

In this paper we demonstrate that FAK promotes cell cycle progression of glioblastoma cells (grade IV malignant astrocytoma) when propagated in vitro as a monolayer and in vivo in the scid mouse brain by promoting exit from G1. Furthermore, we show that FAK regulation of cell cycle progression in these cells requires p27Kip1 and cyclin D1 and that FAK induces expression of the KLF8 transcription factor, which is known to promote cyclin D1 transcription. As a functional correlate of these observations, we show that FAK promotes the activity of the cyclin D1-cdk4 complex. Lastly, we demonstrate that the promotion of proliferation by wild-type FAK in glioblastoma cells requires ERK activity. These findings confirm prior studies examining FAK regulation of the cell cycle, which have shown that FAK promotes exit from G1 in NIH3T3 cells by promoting cyclin D1 transcription (8–10) and extend these studies by identifying the involvement of a cdk inhibitory protein and demonstrating FAK promotion of cell cycle progression in vivo.

A requirement for p27Kip1 in the inhibitory effect of FAK397F on the cell cycle progression of glioblastoma cells is supported by several lines of evidence, including that expression of FAK397F increased the level of p27Kip1 protein expression, the expression of wild-type FAK reduced the level of p27Kip1 protein, and down-regulation of p27Kip1 with siRNA blocked the inhibitory effect of FAK397F. Deregulation of p27Kip1 protein is a common finding in cancer cells and can be associated with altered proteolytic degradation, mislocalization in the cytoplasm, or altered protein binding (18, 26). Deletion of the p27Kip1 gene typically does not account for the low levels of p27Kip1 protein (18, 26). Loss of PTEN function in malignant or cancer cells can affect p27Kip1 levels, with PTEN regulating the
ubiquitin-dependent degradation of p27Kip1 through the ubiquitin E3 ligase SCFSKP2 (27). Reexpression of the PTEN gene in glioblastoma cells has been shown to inhibit S phase entry by recruitment of p27Kip1 into a cyclin E-cdk2 complex (28). Overexpression of p27Kip1 in U-251MG glioblastoma cells propagated in vitro suppresses cell growth (29), and, in astrocytoma biopsies, the levels of p27Kip1 protein are correlated inversely with tumor grade and are a strong predictor of survival (30–32). Our finding of an involvement of p27Kip1 differs from those reported by Zhao et al. (8), who did not find a change in the level of p27Kip1 protein on expression of wild-type FAK or FAK-H9004C14 in the NIH3T3 cells. Although this difference may suggest some cell type specificity in the mechanism by which FAK regulates the cell cycle, several factors, including mutation of the p53 gene, deletion of the ink4B(p15) and ink4A(p16) tumor suppressor genes, and mutation of the PTEN gene impairing function in the U-251MG glioblastoma cells (33–35), could all contribute to differences in the mechanisms whereby FAK regulates the cell cycle in the U-251MG glioblastoma cells compared with the NIH3T3 cells.

Although expression of FAK397F and wild-type FAK altered the expression of p21Waf1 protein in the glioblastoma cells, p21Waf1 was not necessary for the inhibitory effect of FAK397F on the cell cycle progression of the glioblastoma cells, as siRNA directed toward p21Waf1 did not block this inhibitory effect. Similarly, Zhao et al. (8) reported decreased levels of p21Waf1 protein on expression of wild-type FAK and increased levels on expression of FAK-H9004C14 in the NIH3T3 cells, but they found that p21Waf1 was not necessary for the inhibitory effect of FAK-H9004C14 on the cell cycle in fibroblasts because expression of this construct in p21-null fibroblasts resulted in inhibition of cell cycle progression.

We also found that cyclin D1 is necessary for FAK promotion of cell cycle progression in the glioblastoma cells because expression of wild-type FAK increased, and expression of the mutant FAK397F decreased cyclin D1 protein levels, and down-regulation of cyclin D1 with siRNA inhibited the ability of wild-type FAK to promote cell cycle progression. Down-regulation of cyclin E had no effect. Other investigators have shown that cyclin D1 is necessary for FAK promotion of cell cycle progression in NIH3T3 cells and that FAK induces the expression of the KLF8 transcription factor and promotes cyclin D1 transcription through the binding of KLF8 to the GT box in the cyclin D1 promoter (10). Based on the latter findings, we examined KLF8 protein levels in the glioblastoma cells and found that the expression of wild-type FAK induced KLF8 expression, and the expression of the mutant FAK397F inhibited KLF8 expression in cells propagated both in vitro and in

### Table II

FAK promotes cell cycle progression of glioblastoma cells propagated in vivo in the intracerebral scid mouse xenograft model

<table>
<thead>
<tr>
<th>Mouse number</th>
<th>Dox</th>
<th>G0/G1 (mean ± S.E.)</th>
<th>S (mean ± S.E.)</th>
<th>G2/M (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OFAK5 (6 mice)</td>
<td>-</td>
<td>65.5% ± 3.5</td>
<td>12.1% ± 0.7</td>
<td>22.4% ± 3.5</td>
</tr>
<tr>
<td>OFAK5 (6 mice)</td>
<td>+</td>
<td>31.6% ± 10.2</td>
<td>37.7% ± 15.8</td>
<td>30.67% ± 5.2</td>
</tr>
<tr>
<td>OFAK397F-18 (6 mice)</td>
<td>-</td>
<td>73.6% ± 3.9</td>
<td>14.4% ± 7.9</td>
<td>12.0% ± 5.7</td>
</tr>
<tr>
<td>OFAK397F-18 (6 mice)</td>
<td>+</td>
<td>83.0% ± 1.8</td>
<td>4.1% ± 1.7</td>
<td>12.9% ± 1.0</td>
</tr>
</tbody>
</table>

![Image](http://www.jbc.org/)
**FIG. 12.** Increased expression of p27Kip1 and decreased expression of cyclin D1 with expression of mutant FAK397F in glioblastoma cells propagated in the scid mouse brain. C.B.17 scid mice were injected intracerebrally with the OFAK397F-18 clone (1 x 10^6 cells), the tumors allowed to propagate for 8 days, and the animals were administered doxycycline (Dox) or vehicle daily, euthanized, the brains harvested, a single cell suspension isolated which was reacted with mAb anti-human specific MHC Class I IgG coupled to magnetic beads and the human tumor cells lysed in RIPA buffer with protease inhibitors. Equivalent µg of lysate from each animal brain were subjected to disulfide-reduced SDS-PAGE and immunoblotted with the indicated antibodies, as described under “Experimental Procedures.”

**FIG. 13.** Elevated expression of the transcription factor KLF8 with expression of wild-type FAK in glioblastoma cells propagated in vitro and in vivo. A and B, parallel cultures of the following clones OFAK5, OFAK397F-18, and TRE2 propagated in complete medium were administered 2 µg/ml doxycycline (Dox) or vehicle for 4 days, and then detergent lysed. C–F, C.B.17 scid mice were injected intracerebrally with the OFAK5 or the OFAK397F-18 clone (1 x 10^6 cells), administered doxycycline or vehicle for 8 days, the animals euthanized, the brains harvested, a single cell suspension isolated that was reacted with mAb anti-human-specific MHC Class I IgG coupled to magnetic beads, and the human tumor cells were lysed in RIPA buffer with protease inhibitors. Equivalent µg of lysate were subjected to SDS-PAGE and immunoblotted with the indicated antibodies, as described under “Experimental Procedures.”
FAK phosphorylation on Tyr397, the site of Src SH2 domain binding, H18528 FAK complexes in cells because of the inhibition of FAK with endogenous Src adhesions (37). FRNK expression also disrupts the formation of focal contacts/focal adhesions (3). The FRNK construct, which contains the FAT domain, inhibits FAK signaling by competing for FAK promotion of glioblastoma (grade IV malignant astrocytoma) and in glioblastoma (grade IV malignant astrocytoma) tumor biopsies compared with the normal brain (7, 36). The elevation of cyclin D1 protein is consistent with the altered transcription and the role of the Ets transcription factors. We and others have shown previously that the level of cyclin D1 protein is elevated in anaplastic astrocytoma (grade III malignant astrocytoma) and in glioblastoma (grade IV malignant astrocytoma) tumor biopsies compared with the normal brain (7, 36). The elevation of cyclin D1 protein is consistent with the altered regulation of cell proliferation seen in these tumors and our observation that FAK also promotes the activity of the cyclin D1-cdk4 complex.

FAK signaling in vitro is thought to require its localization to focal contacts/focal adhesions (3). The FRNK construct, which corresponds to the carboxyl-terminal region of FAK and contains the FAT domain, inhibits FAK signaling by competing with endogenous FAK for localization to the Ets transcription factor to the cyclin D1 promoter in the NIH3T3 cells (8). A focus of future work is to determine whether FAK induction of KLF8 expression in the glioblastoma cells promotes cyclin D1 transcription and the role of the Ets transcription factors. We and others have shown previously that the level of cyclin D1 protein is elevated in anaplastic astrocytoma (grade III malignant astrocytoma) and in glioblastoma (grade IV malignant astrocytoma) tumor biopsies compared with the normal brain (7, 36). The elevation of cyclin D1 protein is consistent with the altered regulation of cell proliferation seen in these tumors and our observation that FAK also promotes the activity of the cyclin D1-cdk4 complex.

Expression of the mutant FAK397F in the glioblastoma cells did not induce apoptosis. Down-regulation of FAK expression with antisense oligonucleotides can induce apoptosis in non-gliarial derived cancer cells (38). In anchorage-dependent p53 wild-type cells FAK promotes cell survival by suppressing p53-mediated apoptosis (39). Furthermore, integrin αβ1 engagement can send a prosurvival signal through FAK to JNK which does not require ERK in anchorage-dependent, p53 wild-type cells (40). In the latter two studies, expression of the FAT domain of FAK was used to inhibit the endogenous FAK signaling and serum starvation to induce apoptosis. FRNK expression in these cells did not increase apoptosis induced by serum starvation. The FRNK construct includes the proline-rich (PR) regions, PR1 and PR2, whereas the FAT construct does not contain either PR1 or PR2 (41). A docking molecule p130CAS binds to PR1 of FAK through its SH3 domain (42). To determine whether p130CAS and the PR1 region of FAK were important in the different effects seen with expression of FAT or FRNK, the SH3 domain of p130CAS was mutated, and expression of this construct increased apoptosis induced by serum starvation. This along with other data lead to the conclusion that the interaction of p130CAS with FAK through the PR1 domain of FAK may play a role in protecting anchorage-dependent cells from apoptosis induced by serum starvation. Therefore, the absence of apoptosis with expression of the mutant FAK397F in the glioblastoma cells is likely the result of the function of the PR1 domain in the mutant FAK397F, although the anchorage-independence and p53 mutant status of these cells also may contribute to this effect.

FAK promotion of cell cycle progression in NIH3T3 cells requires integrin engagement and ERK activity (9). Integrin engagement can activate ERK through FAK as well as through a FAK-independent mechanism (43–45). We show here that the promotion of glioblastoma cell proliferation with the expression of wild-type FAK in cells propagated in soft agar requires ERK activity, but not JNK activity. In soft agar growth integrin receptors are likely not engaged in cell-matrix
interactions; thus, the cell proliferation promoted by FAK and ERK signaling in the soft agar growth of glioblastoma cells is independent of integrin engagement. p27\(\text{Kip1}\) can be regulated by ERK and also by signaling molecules upstream of ERK, such as Ras and MEK (46–48). We have reported recently that FAK promotes Ras activity in the glioblastoma cells when propagated in suspension or as a monolayer (15). Furthermore, we show here that expression of the wild-type FAK increased ERK activity, but not JNK activity, in the glioblastoma cells, whereas the expression of mutant FAK397F decreased ERK activity, but not JNK activity, in the glioblastoma cells, suggesting that FAK regulates p27\(\text{Kip1}\) protein levels in the glioblastoma cells, independent of integrin engagement. p27\(\text{Kip1}\) can be regulated by ERK and also by signaling molecules upstream of ERK, such as Ras and MEK (46–48). We have reported recently that FAK regulates p27\(\text{Kip1}\) protein levels in the glioblastoma cells through a mechanism that is at least in part independent of ERK, and this is a focus of future work. The increased ERK activity we observed on expression of wild-type FAK in the glioblastoma cells likely promotes binding of an Ets transcription factor to the cyclin D1 promoter because the Ets transcription factors are nuclear effectors of the Ras-mitogen-activated protein kinase pathway (49).

In summary, our results taken together with those reported in the literature suggest a model in which elevated FAK expression in malignant cells promotes Ras activity and thereby ERK activity, reduces p27\(\text{Kip1}\) protein level in a mechanism yet to be defined, and induces expression of the KLF8 transcription factor and thereby promotes cyclin D1 transcription. Together, these effects result in exit from G1.

Acknowledgement—We thank Jo Self for assistance in preparing this manuscript.

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p27\(^{\text{Kip1}}\) and Cyclin D1 Are Necessary for Focal Adhesion Kinase Regulation of Cell Cycle Progression in Glioblastoma Cells Propagated \textit{in Vitro} and \textit{in Vivo} in the Scid Mouse Brain

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