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Inhibition of Focal Adhesion Kinase (FAK) Signaling in Focal Adhesions Decreases Cell Motility and Proliferation

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It has been proposed that the focal adhesion kinase (FAK) mediates focal adhesion formation through tyrosine phosphorylation during cell adhesion. We investigated the role of FAK in focal adhesion structure and function. Loading cells with a glutathione-S-transferase fusion protein (GST-Cterm) containing the FAK focal adhesion targeting sequence, but not the kinase domain, decreased the association of endogenous FAK with focal adhesions. This displacement of endogenous FAK in both BALB/c 3T3 cells and human umbilical vein endothelial cells loaded with GST-Cterm decreased focal adhesion phosphotyrosine content. Neither cell type, however, exhibited a reduction in focal adhesions after GST-Cterm loading. These results indicate that FAK mediates adhesion-associated tyrosine phosphorylation, but not the formation of focal adhesions. We then examined the effect of inhibiting FAK function on other adhesion-dependent cell behavior. Cells microinjected with GST-Cterm exhibited decreased migration. In addition, cells injected with GST-Cterm had decreased DNA synthesis compared with control-injected or noninjected cells. These findings suggest that FAK functions in the regulation of cell migration and cell proliferation.

INTRODUCTION

Cell adhesion to extracellular matrix (ECM) is mediated by heterodimeric transmembrane integrins (Hynes, 1992). In many cultured cells, integrins bridge between matrix proteins and the cytoskeleton at sites of tight juxtaposition between the cell surface and matrix, known as focal adhesions (Burridge *et al.*, 1988). These structures link integrin cytoplasmic domains with filamentous actin stress fibers (Horwitz *et al.*, 1986; DePasquale and Izzard, 1987, 1991; Otey *et al.*, 1990). Cell attachment and spreading on ECM is associated with the organization of a number of cytoskeletal proteins into focal adhesions. The association of integrins with cytoskeletal proteins seems to be important for integrin clustering in focal adhesions during cell adhesion (Marcantonio *et al.*, 1990; Chen *et al.*, 1995; Lewis and Schwartz, 1995). In some cells, the assembly of focal adhesions also accompanies migration (Izzard and Lochner, 1980; Nuckolls *et al.*, 1992; Herman, 1993). In addition, integrin-dependent adhesion at focal adhesions may serve a role in the regulation of cell growth and proliferation (Ingber *et al.*, 1990; Schwartz *et al.*, 1990), and similar structures may mediate cell-matrix interactions in vivo (Drenckhahn *et al.*, 1988).

During integrin-mediated cell adhesion, a number of signaling events are associated with focal adhesion formation (Burridge *et al.*, 1992a; Juliano and Haskill, 1993; Schwartz *et al.*, 1995). The work of many groups has shown that several focal adhesion proteins become tyrosine-phosphorylated in response to integrin-mediated adhesion, antibody-induced integrin clustering, and cell migration (Guan *et al.*, 1991; Kornberg *et al.*, 1991; Burridge *et al.*, 1992b; Lipfert *et al.*, 1992; Pelletier *et al.*, 1992; Romer *et al.*, 1994). Much evidence has indicated that the focal adhesion kinase (FAK) may be responsible for these tyrosine phosphorylation events (Richardson and Parsons, 1995). FAK is a cyto-

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plasmic tyrosine kinase that is localized to focal adhesions (Hanks *et al.*, 1992; Schaller *et al.*, 1992) via a focal adhesion targeting (FAT) sequence at the carboxy-terminus (Hildebrand *et al.*, 1993). FAK binds to several focal adhesion proteins, including paxillin (Hildebrand *et al.*, 1995), talin (Chen *et al.*, 1995), and the cytoplasmic domains of integrins (Schaller *et al.*, 1995). FAK is activated during cell adhesion and cell migration (Guan and Shalloway, 1992; Lipfert *et al.*, 1992; Schaller *et al.*, 1992; Romer *et al.*, 1994). Tyrosine kinase inhibitors have been found to block the tyrosine phosphorylation of FAK and other focal adhesion proteins, while preventing focal adhesion formation and cell motility (Burridge *et al.*, 1992b; Romer *et al.*, 1992; Chrzanowska-Wodnicka and Burridge, 1994; Ridley and Hall, 1994; Romer *et al.*, 1994). These data all support the concept of a role for FAK in cytoskeletal assembly. However, other data suggest that FAK activation may be downstream of focal adhesion and stress fiber formation. Thus, the tyrosine phosphorylation of FAK can be inhibited by cytochalasin D (Lipfert *et al.*, 1992; Pelletier *et al.*, 1992; Bockholt and Burridge, 1993; Haimovich *et al.*, 1993; Seufferlein and Rozengurt, 1994). Furthermore, integrin cytoplasmic domain mutants that do not target to focal adhesions (Marcantonio *et al.*, 1990) do not promote tyrosine phosphorylation or colocalization of FAK (Guan *et al.*, 1991; Lewis and Schwartz, 1995). These same integrin cytoplasmic domain mutants also fail to bind talin (Chen *et al.*, 1995). Together, these findings suggest that the association of integrins with the cytoskeleton is required for FAK activation by integrins.

FAK has been linked to an extensive network of signaling proteins that interface with growth regulation (for review, Clark and Brugge, 1995). These include $pp60^{src}$ (Schaller *et al.*, 1994; Xing *et al.*, 1994), the p85 subunit of PI-3 kinase (Chen and Guan, 1994; Guinebault *et al.*, 1995), the adaptor protein Grb-2 (Schlaepfer *et al.*, 1994), and Csk (Sabe *et al.*, 1994). In addition, paxillin, which interacts with FAK (Turner and Miller, 1994; Bellis *et al.*, 1995; Hildebrand *et al.*, 1995), can interact with the guanine nucleotide exchange factor C3G via the adaptor protein Crk (Birge *et al.*, 1993; Tanaka *et al.*, 1994; Schaller and Parsons, 1995). This evidence supports a role for FAK in downstream signaling after focal adhesion formation, and in the regulation of anchorage-dependent growth behavior.

To date, a direct role for FAK has yet to be established in the following processes: the tyrosine phosphorylation of focal adhesion components, the assembly of focal adhesions, and the regulation of anchorage-dependent growth. In this paper, we have inhibited FAK activity in focal adhesions. Microinjection or bead loading of cells with the FAT-containing carboxy-terminus of FAK, without the kinase domain, was used to competitively inhibit FAK entry into focal adhesions. We report the resulting effects on focal adhesion tyrosine phosphorylation, focal adhesion assembly, cell motility, and the regulation of DNA synthesis.

MATERIALS AND METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were harvested from fresh human umbilical veins using previously described techniques (Romer *et al.*, 1994). Segments of vessels were cannulated, gently irrigated to remove debris, and incubated with collagenase (1 mg/ml; Worthington Biochemical, Freehold, NJ) for 10 min. The irrigant was then agitated within the vessels and collected. Harvested cells were plated on tissue culture plastic that had been preincubated with 1% gelatin (Difco, Detroit, MI) in Dulbecco's PBS. HUVEC were cultured in medium 199 with Earle's salts, supplemented with vitamins and amino acids (Life Technologies, Gaithersburg, MD), 20% fetal calf serum, 100 μg/ml heparin, ~100 μg/ml endothelial cell growth factor isolated from bovine hypothalamus, 50 U/ml penicillin, 50 μg/ml streptomycin, and 4 μg/ml amphotericin B. HUVEC were used between passages 2 and 4. Cell identity was verified by morphology and by positive testing for von Willebrand's factor by immunofluorescence microscopy with polyclonal antibody obtained from Sigma Chemical (St. Louis, MO). BALB/c 3T3 cells were passaged every 3 d and cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Tissue culture coverslips were coated with human plasma fibronectin (Life Technologies; 75 μg/ml) in TBS (150 mM NaCl, 0.1% NaN3, 50 mM Tris-HCl, pH 7.6) at 37°C for 1 h. Before plating, coverslips were rinsed with serum-free Dulbecco's modified Eagle's medium.

Fusion Proteins

The fusion protein GST (29 kDa) was generated from the plasmid pGEX3x (Pharmacia, Piscataway, NJ). GST fusion protein (GST-Cterm) (~58 kDa) includes FAK amino acids 765 through 1052. This was generated by an in-frame insertion of the C-terminal fragment of wild-type *FAK* cDNA that included the FAT (Hildebrand *et al.*, 1993) sequence, but not the kinase domain, into the multiple cloning site of pGEX2TK. GST-ΔNE (~64 kDa) includes FAK amino acids 685 through 965 and 1012 through 1052 with a substitution of an alanine for the glutamic acid at FAK residue 965. This deletion mutant is thus missing a portion of the FAT sequence. FAK containing this mutation did not target to focal adhesions (Hildebrand *et al.*, 1993). Schematics of these fusion proteins and the intact FAK protein are displayed in Figure 1A. The FAK fusion proteins were generous gifts from Dr. Michael Schaller (University of North Carolina at Chapel Hill, Chapel Hill, NC) and Dr. Jeff Hildebrand (University of Washington).

Escherichia coli containing these plasmids were grown in LB medium containing 100 μg/ml ampicillin to mid-log phase, induced to express FAK fusion proteins with the addition of 100 μM isopropyl-β-D-thiogalactopyranoside, and allowed to grow for an additional 2 h in a shaking incubator at 37°C. Bacterial pellets were isolated by centrifugation at 5000 × *g* for 10 min at 27°C, solubilized in PBS with 1% Triton X-100/0.5 mM EDTA, and lysed by sonication for 30 s in the presence of 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Fusion proteins were purified from bacterial lysates on glutathione agarose (Sigma) and eluted from the agarose with 50 mM Tris-HCl, pH 8.0, containing 5 mM glutathione.

For studies of focal adhesion formation, fusion proteins were mixed with coumarin-conjugated bovine serum albumin (BSA). BSA at 10 mg/ml in 0.1 M sodium carbonate, pH 9.0, was labeled overnight with 7-diethylamino-3-(4′-isothiocyanatophenyl)-4-meth-

ylcoumarin (CPI, Molecular Probes, Eugene, OR) and then dialyzed into microinjection buffer (75 mM KCl, 10 mM potassium phosphate buffer, pH 7.5). The coumarin-labeled BSA was added to fusion proteins before microinjection.

SDS-PAGE

Fusion protein solutions were combined with Laemmli sample buffer, boiled for 3 min, and electrophoresed on 10% polyacrylamide gels (Laemmli, 1970) containing SDS and a bisacrylamide concentration of 0.19%. After electrophoresis, gels were stained with Coomassie blue.

Microinjection

FAK fusion proteins were prepared for microinjection by dialysis against microinjection buffer (see above) and concentrated to 2 mg/ml (all fusion proteins were microinjected at this concentration except where otherwise noted). Microinjection was done using previously described techniques (Nuckolls *et al.*, 1992). Needles were prepared from Kwik-fil glass capillary tubes (World Precision Instruments, Sarasota, FL) pulled on a Brown-Flaming micropipette puller (Sutter Instrument, San Francisco, CA). Microinjection was done using a Leitz Diavert microscope with a ×32 phase-contrast objective and a Leitz micromanipulator (Leitz, Deerfield, IL).

Bead Loading

Fusion proteins were prepared for bead loading by dialysis against microinjection buffer (see above), or Dulbecco's PBS, and concentrated to 2 mg/ml (all fusion proteins were bead-loaded at this concentration). Pluronic F-68 (Sigma) was added to all fusion protein solutions to a final concentration of 2% (wt/vol) to enhance macromolecular transit across the plasma membrane (Clarke and McNeil, 1992). Fusion proteins were loaded into cells using previously described techniques (McNeil and Warder, 1987). Cells adherent to fibronectin-coated coverslips were washed with PBS/2% Pluronic F-68 for 2 min. Cells were then incubated with fusion protein solutions for 30 s and then covered with glass beads (450–600 μm, Sigma) for 30 s. Cells were then returned to serum-free growth medium.

Phosphotyrosine and Focal Adhesion Formation Assays

BALB/c 3T3 cells or HUVEC were plated on fibronectin-coated coverslips 1 h before microinjection. Coverslips with injected cells were then incubated with 1 mM EDTA at 27°C for ~5 min under continuous inspection with phase microscopy until most of the cells had rounded, but not detached. Serum-free or serum-containing growth medium (as designated in the text below) was then returned to the cell cultures, and the cells were allowed to respread for 1 h before preparation for immunofluorescence.

Migration Assays

Wounds were made in confluent monolayers of either HUVEC or BALB/c 3T3 cells 12 h after plating by clearing the cells from one-half of the coverslip with a Teflon spatula. Cells immediately adjacent to the wound were microinjected. After 24 h, cells were prepared for immunofluorescence microscopy. Microinjected cells were assigned to one of the following three categories based on their final position in the microscopic field: baseline (those remaining at the wound edge); migrating pack (those that migrated but did not reach the leading edge); and leading edge (cells at the forefront of the migrating pack).

DNA Synthesis Assays

Cells were serum-starved overnight to synchronize the cell cycle and then plated on fibronectin-coated coverslips for 1 h in serum-free media. Cells were then microinjected, incubated for 24 h in medium containing 100 μM bromodeoxyuridine (BrdU; Sigma), and prepared for fluorescence microscopy.

Fluorescence Microscopy and Antibodies

Cells were fixed in 3.7% formaldehyde in Dulbecco's PBS for 10 min, rinsed in (TBS), and permeabilized for 5 min in TBS containing 0.5% Triton X-100 before staining for fluorescence microscopy. Some coverslips were then incubated for 30 min with py20 (ICN Biochemicals, Costa Mesa, CA) and anti-GST polyclonal antisera (Molecular Probes) in TBS. The coverslips were then rinsed extensively in TBS and stained with combined fluorophore-conjugated, affinity-purified, donkey anti-mouse and anti-rabbit IgG antibodies in TBS (Chemicon International, Temecula, CA) for 30 min at 37°C. For visualization of FAK, coverslips were incubated with a monoclonal anti-FAK antibody (Transduction Laboratories, Lexington, KY; epitope is not in the carboxy-terminus) and stained with a second antibody as described above. Other coverslips were incubated with polyclonal anti-talin (N681; gift of Dr. Keith Burridge, University of North Carolina), monoclonal anti-vinculin (7F9; gift of Dr. Alexey Belkin, University of North Carolina), or anti-paxillin (Transduction Laboratories), along with either polyclonal anti-phosphotyrosine (Transduction Laboratories) or monoclonal py20, and then stained with second antibodies as described above. For visualization of filamentous actin, coverslips were incubated with rhodamine-conjugated phalloidin (Molecular Probes) or with coumarin-conjugated phalloidin (Sigma) for 30 min at 37°C. For visualization of nuclear DNA, coverslips were incubated with Hoechst reagent (Molecular Probes) after fixation and permeabilization as above. For visualization of BrdU incorporation, coverslips were incubated with DNAse (0.1 U/μl; Promega, Madison, WI) for 30 min at 37°C, and then with monoclonal anti-BrdU antibody (Sigma) for 30 min at 37°C. After the antibody incubations, the coverslips were washed in TBS, rinsed in deionized water, and mounted with gelvatol or mowiol. Coverslips were viewed on a Zeiss Axiophot microscope equipped for epifluorescence. Fluorescence micrographs were taken on T-max 400 film (Eastman Kodak, Rochester, NY).

Statistical Methods and Study Design

All findings were confirmed in both cell types. Data from migration experiments were analyzed by using ANOVA for repeated measures. Data from focal adhesion FAK content, focal adhesion phosphotyrosine, focal adhesion vinculin, and DNA synthesis experiments were analyzed using the Pearson chi-square test. Experiments on focal adhesion phosphotyrosine, focal adhesion formation, and cell motility (including dose–response studies) were carried out blind, with the identity of the microinjected proteins not known to the investigators until after the data were analyzed.

RESULTS

GST-Cterm Localizes to Focal Adhesions and Displaces Endogenous FAK

GST fusion proteins, including portions of the FAK carboxy-terminus, were prepared as described above (see MATERIALS AND METHODS). Schematics of the proteins GST and GST-Cterm are shown in Figure 1A. The purity of representative aliquots of each fusion protein is shown in the Coomassie blue–stained gel in Figure 1B. The localization of fusion proteins after loading and the effects on endogenous FAK were

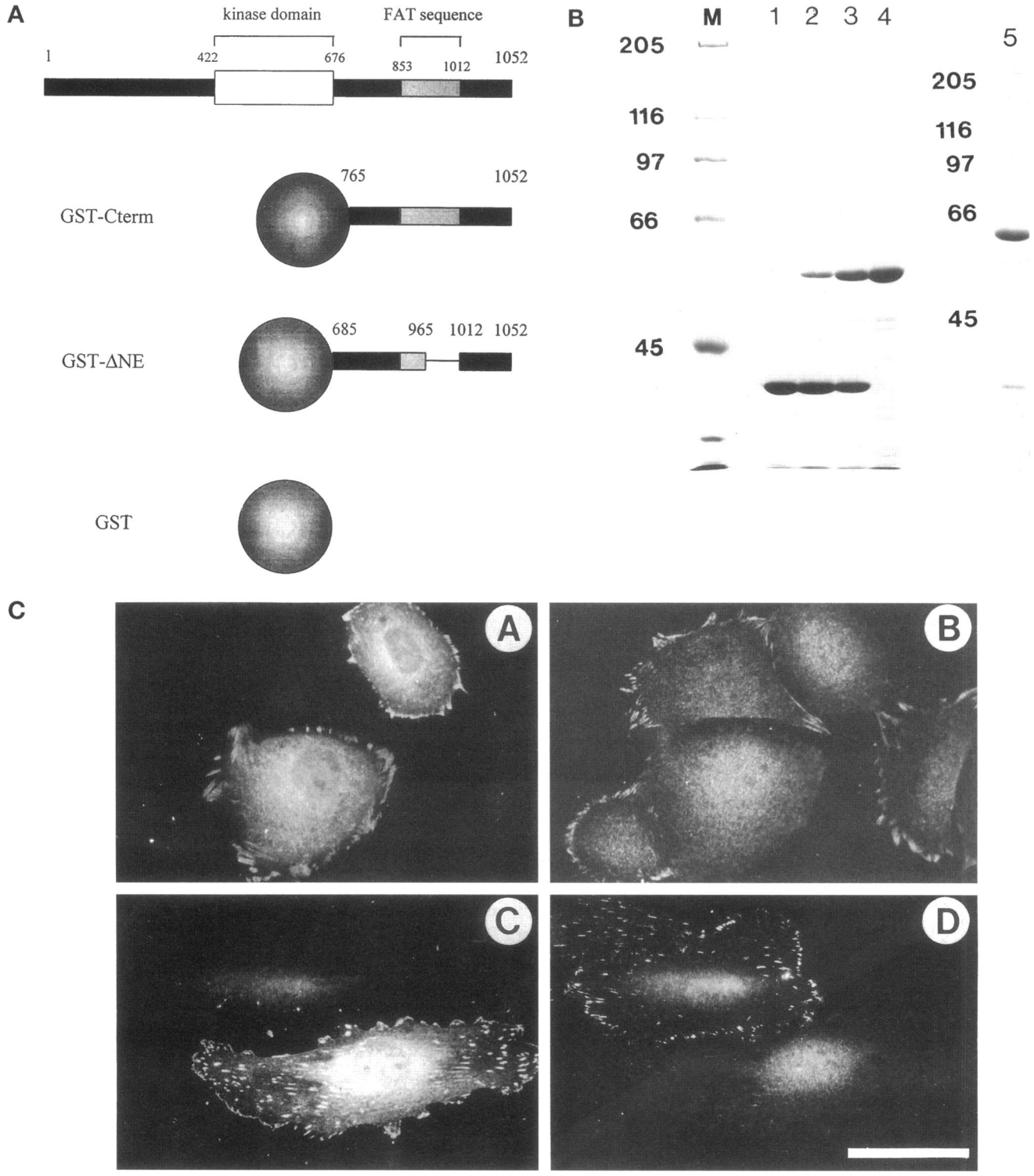

Figure 1. (A) Schematic representation of FAK and GST-FAK fusion proteins relative to the complete FAK sequence. Indicated are the kinase domain (amino acids 422–676) and the FAT sequence (amino acids 853–1012). The amino acids encoded by each of the fusion proteins are indicated. Fusion protein constructs were made as described in MATERIALS AND METHODS. (B) Expression and purification of GST-FAK fusion proteins for microinjection and dilutions for dose-dependent experiments. Lane 1, 2 mg/ml GST; lane 2, 0.25 mg/ml GST-Cterm + 1.75 mg/ml GST; lane 3, 1 mg/ml GST-Cterm + 1 mg/ml GST; lane 4, 2 mg/ml GST-Cterm; lane 5, 2 mg/ml GST-ΔNE. Note that the second set of molecular mass markers and lane 5 were taken from a separate gel. (C) Bead-loaded GST-Cterm localizes to focal

studied by immunofluorescence (Figure 1). After injection or bead loading, the cells were rounded (but not detached) with 1 mM EDTA in PBS. Cells were then induced to respread by addition of serum-free medium for 1 h and prepared for immunofluorescence. Injected cells were identified with an anti-GST polyclonal antibody. GST-Cterm localized to focal adhesions in both BALB/c 3T3 cells and HUVEC (Figure 1C, panels A and C). GST alone did not localize to focal adhesions. Endogenous FAK localization was studied in cells loaded with fusion proteins using a monoclonal antibody (mAb) that recognizes an epitope that is not in the carboxy-terminus. This anti-FAK mAb did not recognize the injected GST-Cterm. Cells microinjected or bead-loaded with GST-Cterm showed decreased endogenous FAK in focal adhesions (Figure 1C, panels B and D). Cells injected or bead-loaded with GST alone did not exhibit a change in endogenous FAK association with focal adhesions. The competitive exclusion of endogenous FAK from the focal adhesions of cells loaded with GST-Cterm was found to be statistically significant in large samples of both BALB/c 3T3 cells and HUVEC (Table 1).

Loading of GST-Cterm Results in Reduced Focal Adhesion Phosphotyrosine

We wished to investigate the effects of preventing endogenous FAK localization to focal adhesions during adhesion and spreading on fibronectin. BALB/c 3T3 cells and HUVEC were plated onto fibronectin-coated coverslips and allowed to spread for 1 h. Cells were then bead-loaded with either GST or GST-Cterm (Figure 1). The cells were then rounded and allowed to respread as above, fixed, and examined by immunofluorescence. Coverslips were triple-labeled with antibodies against GST and phosphotyrosine, and with coumarin-conjugated phalloidin (Figure 2). Cells loaded with GST-Cterm were well-spread. However, GST-Cterm loading resulted in the reduction of focal adhesion phosphotyrosine to undetectable levels in both BALB/c 3T3 cells (Figure 2, A and B), and HUVEC (Figure 2, G and H). The prevention of focal adhesion phosphotyrosine detected by immunofluorescence in cells loaded with GST-Cterm was found to be statistically significant in large samples of both BALB/c 3T3 cells and HUVEC (Table 1). In contrast, loading of GST alone had no effect on the accumulation of phosphotyrosine in focal adhesions in either cell type (Figure 2, D, E, J, and K). Neither fusion protein altered the actin cytoskeleton in either cell type (Figure 2, C, F, I, and L). The data shown in Figures 1C, 2, and Table 1 indicate that microinjected or bead-loaded GST-Cterm localized to focal adhesions, prevented endogenous FAK entry into focal adhesions, and inhibited tyrosine phosphorylation in focal adhesions.

Table 1. Fusion protein effects on FAK, phosphotyrosine (PY), and vinculin in bead-loaded cells

Cell type	Balb/c 3T3		HUVEC	
Fusion protein	GST-Cterm	GST	GST-Cterm	GST
% positive FAK	10.3 (194)	97.6 (127)	6.2 (402)	96.0 (202)
p value FAK	<0.0001	—	<0.0001	—
% positive PY	15.6 (282)	98.4 (187)	5.5 (401)	98.7 (229)
p value PY	<0.0001	—	<0.0001	—
% + vinculin	96.8 (186)	98.8 (172)	97.5 (203)	98.0 (200)
p value vinculin	0.839	—	0.753	—

% positive = % of total loaded cells (# counted in parentheses). p values are for differences from GST controls.

The effects of GST-Cterm on focal adhesion tyrosine phosphorylation were examined in both microinjected and bead-loaded cells under a variety of conditions. Anti-phosphotyrosine staining was also reduced to undetectable levels in cells that were not rounded but were allowed to remain spread. This demonstrated that the effect was not restricted to newly forming focal adhesions. Decreased phosphotyrosine in focal adhesions was also seen in cells that were injected with GST-Cterm, rounded, and then allowed to respread in the presence of serum. The injection of GST-Cterm at lower concentrations was associated with a concentration-dependent reduction of focal adhesion phosphotyrosine staining. This further suggests that FAK is important for the tyrosine phosphorylation in focal adhesions in adherent cells.

We examined the specificity of the effect observed with GST-Cterm by loading the fusion protein GST-ΔNE, which lacks a portion of the FAT sequence (see Figure 1A and MATERIALS AND METHODS). Cells loaded with GST-ΔNE were rounded and respread on fibronectin in serum-free medium as above. Anti-phosphotyrosine and anti-GST staining were compared in BALB/c 3T3 cells bead-loaded with GST-Cterm or GST-ΔNE (Figure 3, A and B, and C and D, respectively). Only GST-Cterm–loaded cells showed reduced tyrosine phosphorylation in focal adhesions. The fusion protein made from the carboxy-terminal deletion construct GST-ΔNE did not localize to focal adhesions and had no effect on anti-phosphotyrosine

(Figure 1 cont.) adhesions and prevents endogenous FAK entry into focal adhesions. BALB/c 3T3 cells (A and B) and HUVEC (C and D) were plated on fibronectin-coated coverslips and allowed to spread for 1 h before bead loading with 2 mg/ml GST-Cterm. Cells were then rounded and allowed to respread. Cells were immunostained with a polyclonal anti-GST antibody to visualize bead-loaded cells (A and C), and with an anti-FAK mAb (B and D; epitope is not in GST-Cterm). GST-Cterm–loaded cells demonstrated localization of the fusion protein to focal adhesions and displacement of endogenous FAK. Bar, 20 μm.

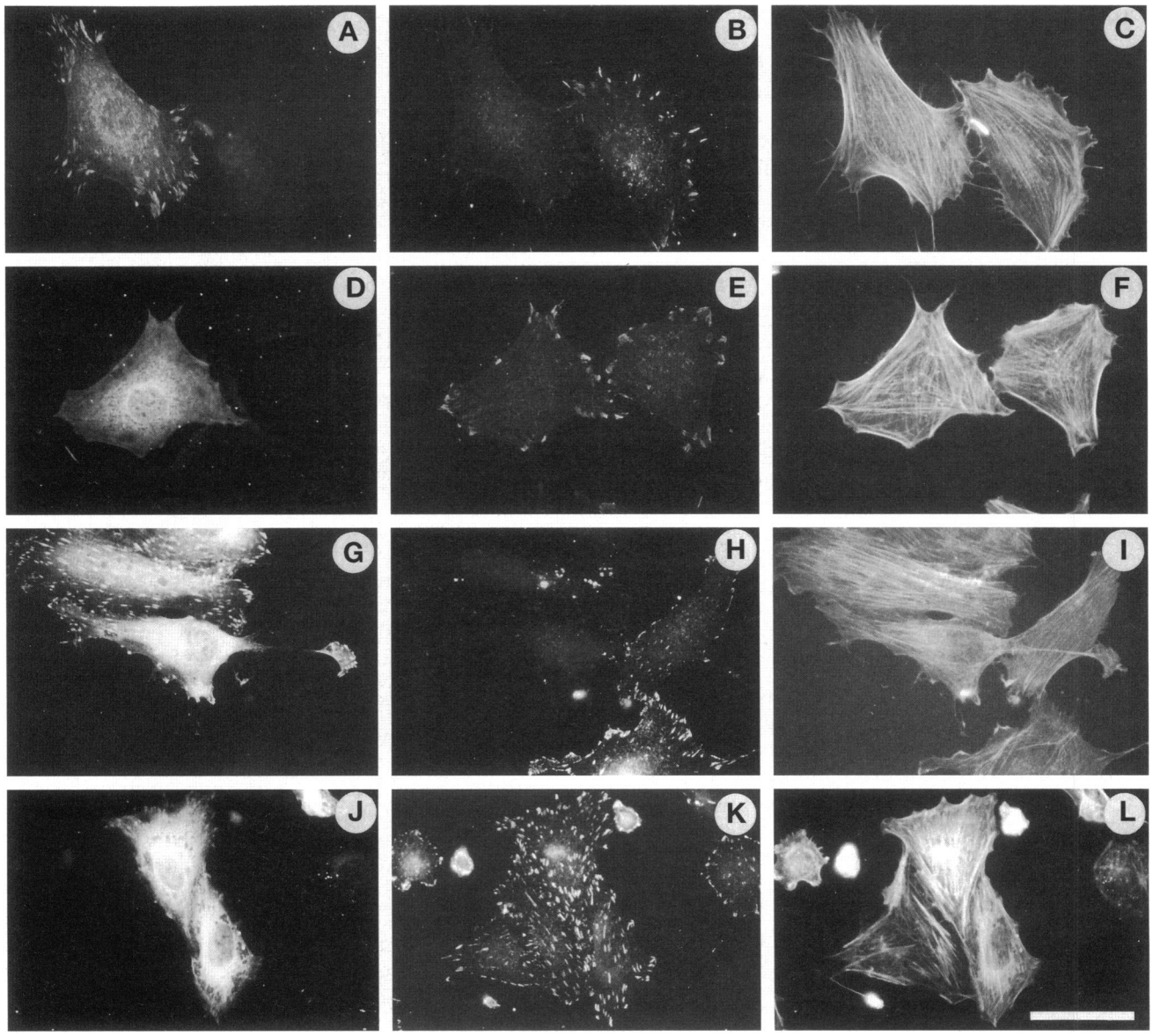

Figure 2. Effects of GST-Cterm and GST on focal adhesion phosphotyrosine. BALB/c 3T3 cells (A–F) and HUVEC (G–L) were bead-loaded with either GST-Cterm (A–C, G–I) or GST (D–F, J–L). Cells were then rounded and allowed to respread. Cells were triple-labeled with a polyclonal anti-GST antibody (A, D, G, and J) to visualize bead-loaded cells, anti-phosphotyrosine mAb py20 (B, E, H, and K), and coumarin-conjugated phalloidin (C, F, I, and L). Bar, 20 μm.

staining. This indicates that a complete FAT sequence is required to compete with endogenous FAK.

Formation of Focal Adhesions in GST-Cterm–injected Cells

Previous studies have demonstrated that tyrosine kinase inhibitors, such as tyrphostin AG 213 and herbimycin A, can prevent the formation of focal adhesions when cells adhere and spread on ECM proteins (Burridge *et al.*, 1992b; Romer *et al.*, 1992, 1994). To determine whether focal adhesions could form in the absence of FAK-dependent tyrosine phosphorylation, the localization of several cytoskeletal proteins was examined after injection, rounding, and respreading on fibronectin-coated coverslips. GST-Cterm was coinjected with coumarin-labeled BSA (see MATERIALS AND METHODS) to identify injected cells. This provided the means to examine the effects of GST-Cterm on both tyrosine phosphorylation and focal adhesion protein localization within the same cell.

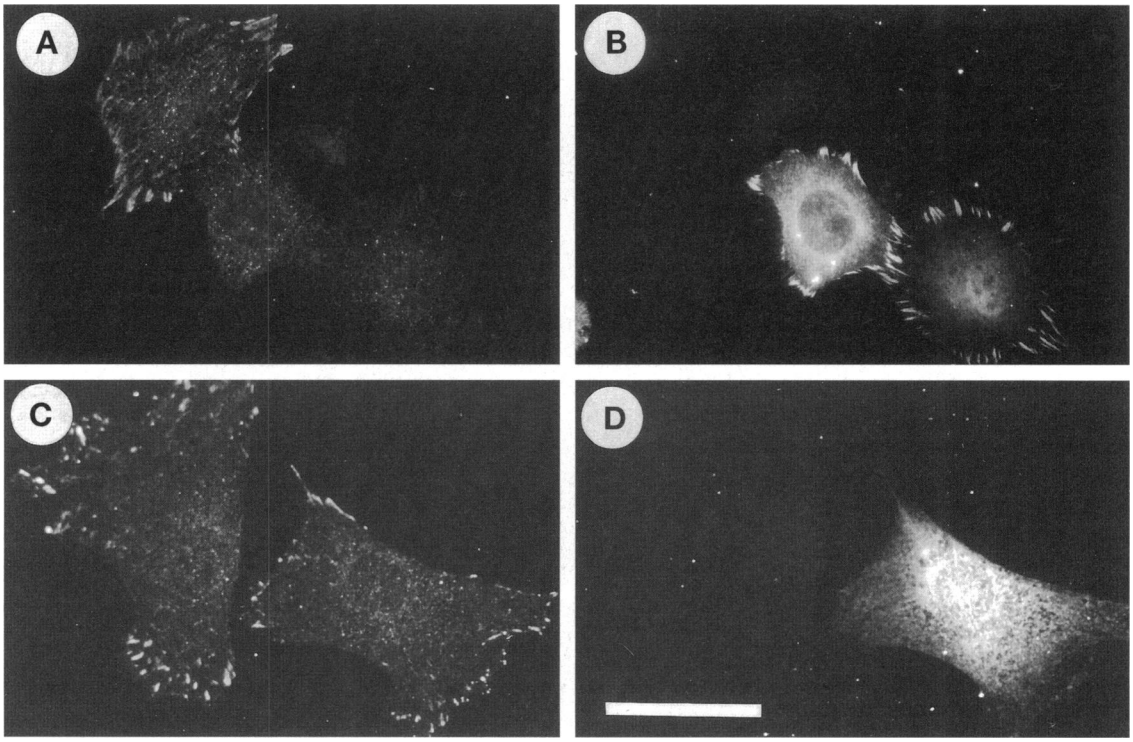

Figure 3. Effect of FAK deletion variant on focal adhesion phosphotyrosine. BALB/c 3T3 cells plated on fibronectin-coated coverslips were bead-loaded with GST-Cterm (A and B) or GST-ΔNE (C and D). Cells were then rounded and allowed to respread. Coverslips were double-immunostained with anti-phosphotyrosine mAb py20 (A and C) and a polyclonal anti-GST antibody (B and D) to visualize bead-loaded cells. Bar, 20 μm.

Cells were injected with GST-Cterm, rounded with EDTA as above, and allowed to respread for 1 h on fibronectin-coated coverslips. The localization of three prominent focal adhesion proteins, vinculin, talin, and paxillin, was determined. GST-Cterm injection did not prevent the localization of any of these three proteins to focal adhesions (Figure 4, A, D, and G, respectively), despite the fact that phosphotyrosine staining was undetectable in these cells by immunofluorescence staining (Figure 4, B, E, and H). The formation of vinculin-containing focal adhesions in cells loaded with GST-Cterm was confirmed in large samples of both BALB/c 3T3 cells and HUVEC (Table 1). The effects of GST-Cterm injection on focal adhesion formation were independent of the presence of serum in the growth medium. Identical results were obtained with cells injected during the initial stages of spreading after plating on fibronectin. The localization of the focal adhesion proteins β1 integrin and α-actinin were also not altered by GST-Cterm loading. Although the methods used in these studies cannot rule out low levels of phosphotyrosine in the focal adhesion proteins of GST-Cterm–injected cells, the localization of paxillin to focal adhesions did not appear to correlate with its tyrosine phosphorylation. Paxillin localized to focal adhesions that had no detectable phosphotyrosine by immunofluorescence.

The Effects of GST-Cterm Microinjection on Cell Migration

Previous studies have suggested a role for FAK in the dynamic turnover of focal adhesions during cell movement (Romer *et al.*, 1994). We used GST-Cterm microinjection and an in vitro wound-healing model to examine the role of FAK in cell migration. HUVEC or BALB/c 3T3 cells were grown to confluence on fibronectin-coated glass coverslips. Cells were then cleared from one-half of the coverslip with a Teflon spatula. Cells immediately contiguous to the wound edge were injected with GST-Cterm or with GST alone. The progress of cell movement is illustrated in Figure 5. Microinjected cells were assigned to one of three categories (defined in MATERIALS AND METHODS): baseline (BL), migrating pack (MP), or leading edge (LE). Data from 1266 microinjected HUVEC are shown in Figures 5 and 6 and in Table 2. GST-injected HUVEC showed a normal distribution between the three categories, with 53.8% of cells in the migrating pack, 22.2% of cells remaining at the wound

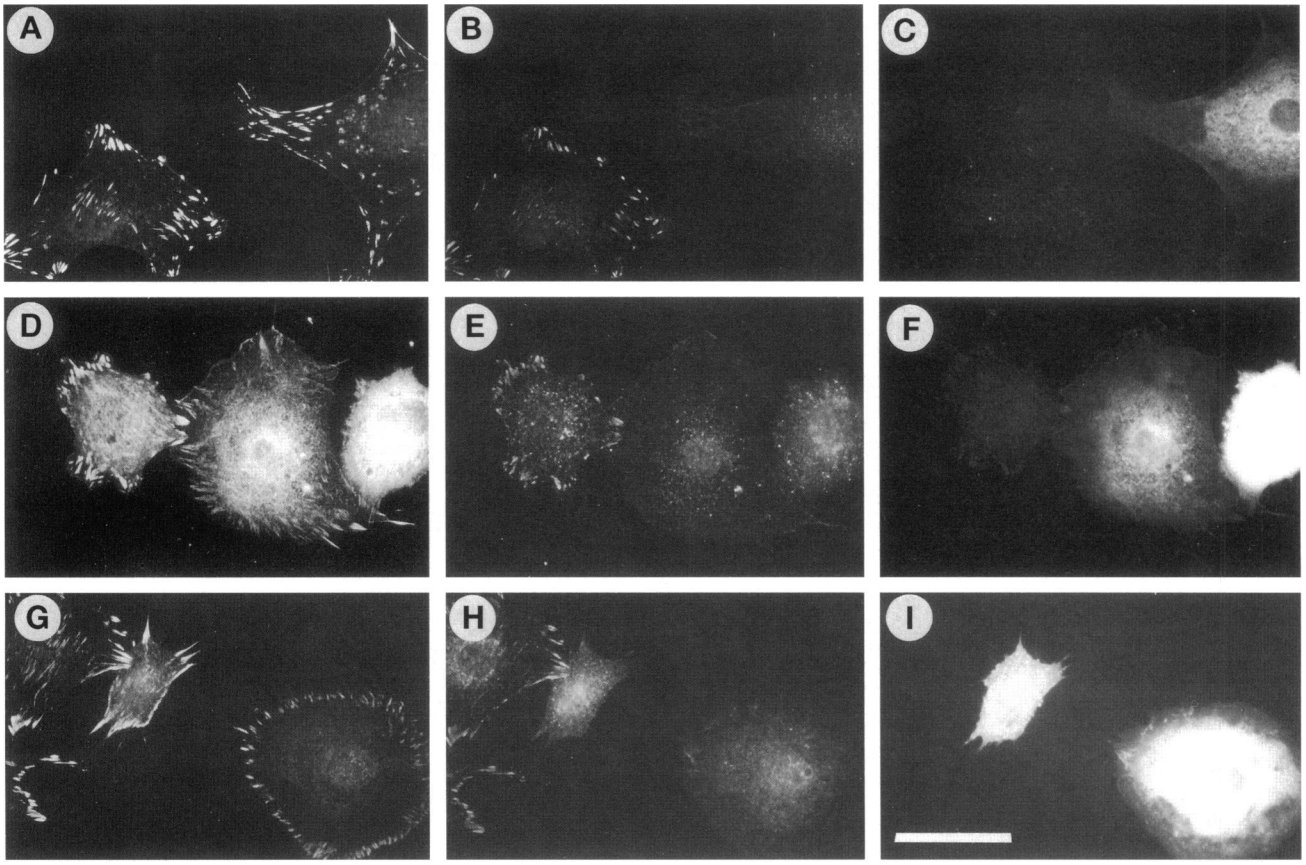

Figure 4. Effect of GST-Cterm microinjection on focal adhesion formation. BALB/c 3T3 cells plated on fibronectin were injected with GST-Cterm and coumarin-conjugated BSA. Cells were then rounded, allowed to respread, and processed for immunofluorescence. Coumarin-BSA was used to identify injected cells (C, F, and I). Cells were immunostained for phosphotyrosine with polyclonal antibody (B and H) or the mAb py20 (E), along with either anti-vinculin mAb (A), anti-talin polyclonal antibody (D), or anti-paxillin mAb (G). Bar, 20 μm.

baseline, and 24% of cells achieving the leading edge. In contrast, HUVEC injected with GST-Cterm demonstrated decreased movement (Figure 5). The majority of GST-Cterm–injected HUVEC remained at the wound baseline (63.9%) or just entered the migrating pack (33.3%). Microinjection with GST-Cterm similarly inhibited the motility of BALB/c 3T3 cells. HUVEC injected with GST-ΔNE did not exhibit a change in motility compared with GST-injected cells. These data indicate that GST-Cterm microinjection and the consequent inhibition of endogenous FAK interactions with the cytoskeleton are associated with decreased cell motility.

The effects of lower concentrations of GST-Cterm on HUVEC motility were measured (Figure 1B, lanes 1–4; Figure 6; Table 2). GST-Cterm injection was associated with a dose-dependent effect on the ability of HUVEC to migrate in this in vitro wound-healing model. As the concentration of GST-Cterm increased from 0.25 mg/ml to 2 mg/ml, the number of GST-Cterm–microinjected HUVEC at the leading edge declined (from 10.5 to 2.8%), and the number of GST-Cterm–microinjected HUVEC at the wound baseline increased (from 50.7 to 63.9%). The percentages of GST-Cterm–microinjected HUVEC at BL and LE were significantly changed from control values by all of the studied GST-Cterm concentrations (by ANOVA; $p < 0.001$).

The Effects of GST-Cterm Microinjection on DNA Synthesis

Wound healing in in vitro models has been shown to be the product of both cell motility and cell proliferation (Coomber and Gotlieb, 1990). The observations that GST-Cterm injection inhibited wound healing and motility led us to examine the effects of displacing FAK from focal adhesions on DNA synthesis in adherent cells. BALB/c 3T3 cells or HUVEC were synchronized overnight by serum starvation and plated in serum-free medium on fibronectin-coated coverslips the next morning. Cells on each coverslip were then microinjected with either GST-Cterm or with GST alone. After injection, cells were incubated in serum-containing medium with BrdU (100 μM) for 24 h and

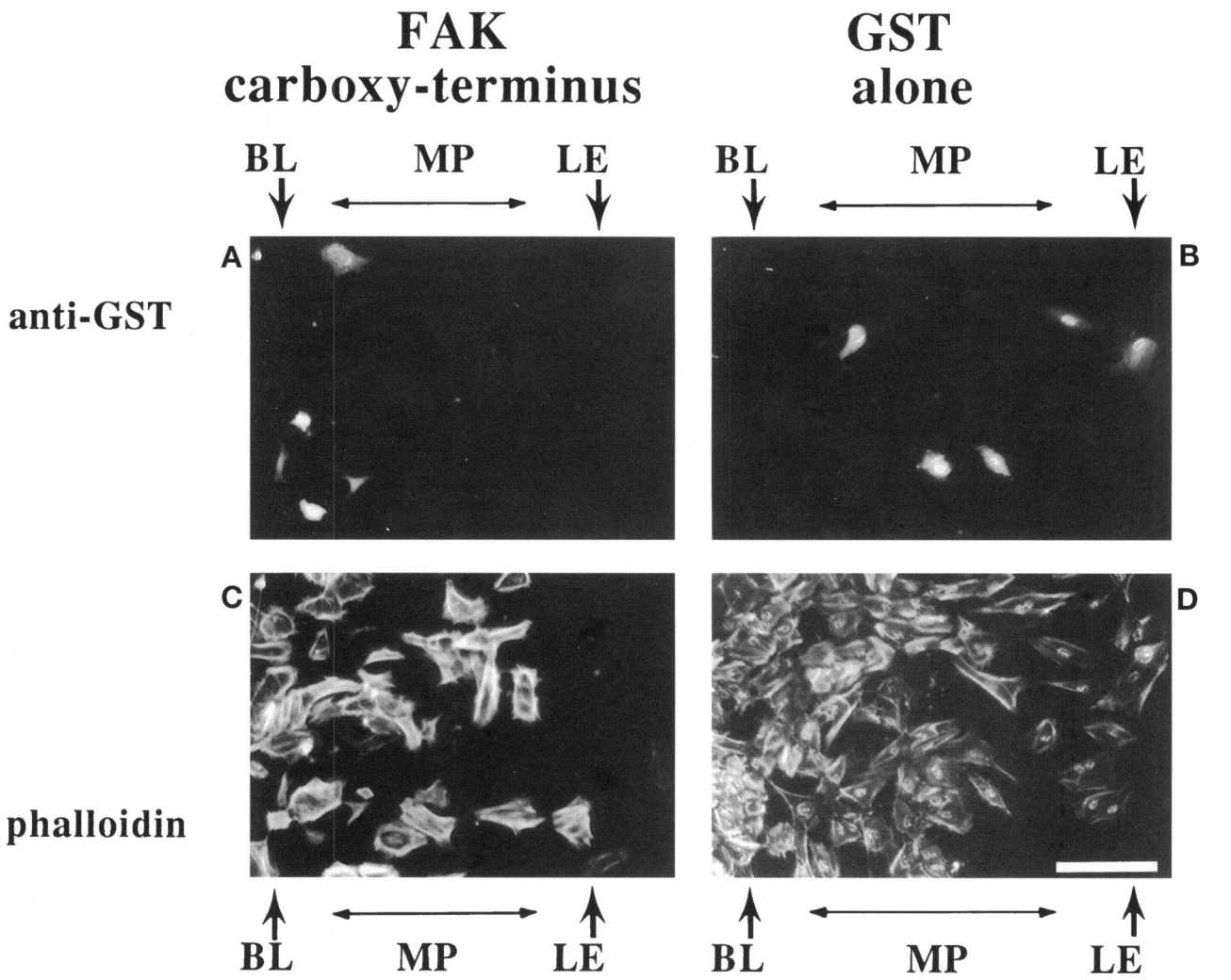

Figure 5. Effect of GST-Cterm microinjection on cell migration. HUVEC were grown to confluence on coverslips. Cells were cleared from one-half of the coverslip. Cells along the wound edge were injected with either GST-Cterm (A and C) or GST alone (B and D), allowed to migrate for 24 h, and prepared for immunofluorescence. Injected cells were visualized by staining with an anti-GST polyclonal antibody (A and B). Coverslips were stained with rhodamine-conjugated phalloidin to visualize all cells (C and D). Cells were scored for migration in three groups: those remaining at the wound baseline (BL), those within the migrating pack (MP), and those at the leading edge of the migrating cells (LE). Bar, 150 μm.

then prepared for immunofluorescence microscopy. Cells were triple-labeled after fixation using an anti-GST antibody to identify injected cells, an anti-BrdU antibody to identify BrdU incorporation, i.e., new DNA synthesis (Olson *et al.*, 1995), and Hoechst reagent to highlight all of the nuclei in the microscopic field. Representative data from experiments with HUVEC are shown in Figure 7, A–F. The majority of GST-injected and noninjected HUVEC demonstrated BrdU incorporation after 24 h (Figure 7, D and E). In contrast, GST-Cterm injection was associated with a marked decrease in the number of nuclei that stained positively for BrdU (Figure 7, A and B). All of the nuclei in each of these two microscopic fields are shown by Hoechst staining in Figure 7, C and F.

BrdU incorporation data were analyzed quantitatively and subjected to statistical analysis as shown in Figure 7, G and H. HUVEC microinjected with GST alone demonstrated a BrdU incorporation rate of 59%, compared with 52.5% for noninjected cells. Similarly, HUVEC microinjected with GST-ΔNE showed no difference in BrdU incorporation when compared with noninjected cells ($p = 0.089$ by Pearson chi-square analysis). However, BrdU incorporation fell sharply in GST-Cterm–injected HUVEC to 15.4% (Figure 7G; $p < 0.0001$ by Pearson chi-square analysis). DNA synthesis

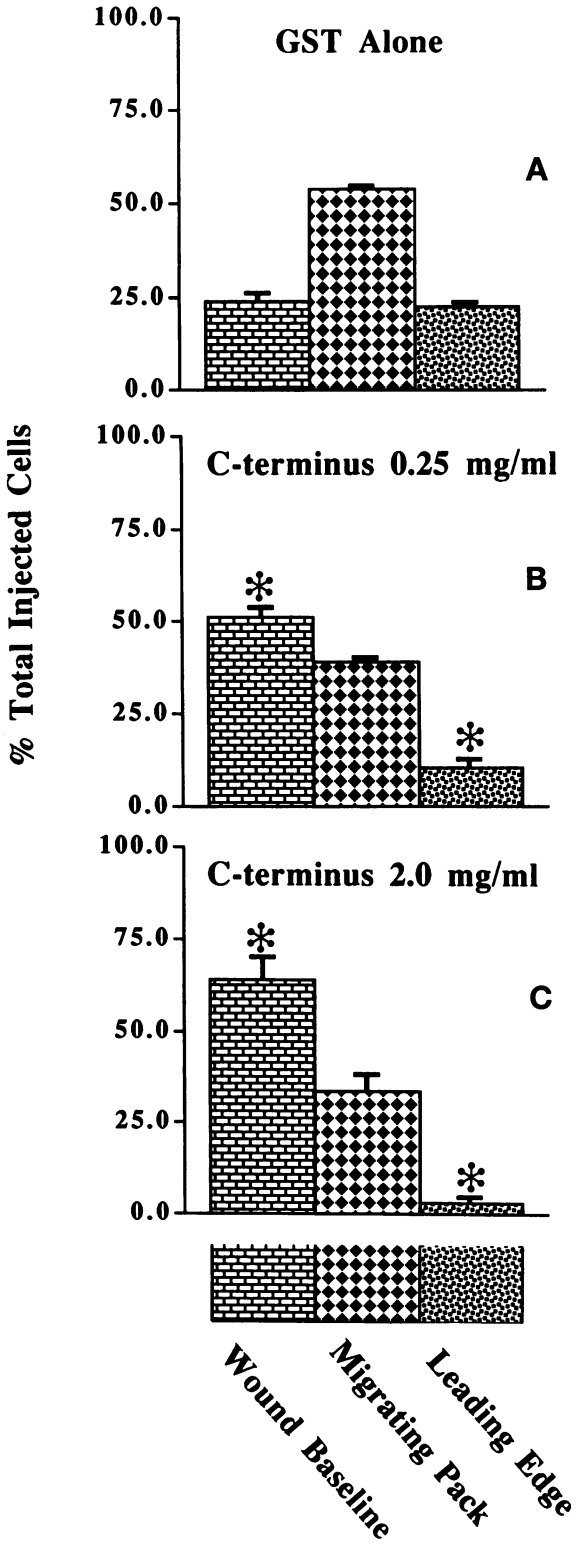

Figure 6. The effect of GST-Cterm microinjection on cell migration is dose-dependent. HUVEC monolayers were wounded and injected with various dilutions of GST-Cterm from 0.25 mg/ml to 2 mg/ml or with GST alone. Cells were scored as outlined in MATERIALS AND METHODS and Figure 5, and the results are represented as the percentage of cells in each of the three regions. (A) 2 mg/ml GST; (B) 0.25 mg/ml GST-Cterm; (C) 2 mg/ml GST-Cterm. Both concentrations of GST-Cterm increased the percentage of injected cells at the baseline and decreased the percentage of injected cells at the leading edge, compared with GST-injected cells. Asterisks, $p < 0.001$ by ANOVA.

Table 2. Dose-dependent decreases in HUVEC motility

	Wound baseline	Migrating pack	Leading edge
Injectate	(% of total injected cells ± SEM		
GST	24.0 (1.7)	53.8 (0.6)	22.2 (1.2)
CT 0.25 mg/ml	50.7 (2.5)	38.8 (1.2)	10.5 (1.8)
CT 1.0 mg/ml	51.5 (2.6)	41.1 (3.3)	6.8 (1.0)
CT 2.0 mg/ml	63.9 (5.8)	33.3 (4.4)	2.8 (1.4)

was also decreased in BALB/c 3T3 cells that had been injected with GST-Cterm (Figure 7H). GST-injected and noninjected BALB/c 3T3 cells demonstrated BrdU incorporation rates of 86 and 76.3%, respectively. In GST-Cterm–injected BALB/c 3T3 cells, BrdU incorporation fell to 30% ($p < 0.0001$ by Pearson chi-square analysis). These data indicate that FAK signaling in focal adhesions serves a role in the regulation of DNA synthesis and cell cycle progression in adherent cells.

DISCUSSION

We have examined the effects of displacing FAK from the focal adhesions of fibroblasts and endothelial cells. Previous work has shown that tyrosine phosphorylation of the focal adhesion proteins FAK, paxillin, tensin, and $p130^{cas}$ accompany cell adhesion and the assembly of focal adhesions and stress fibers (Burridge *et al.*, 1992b; Guan and Shalloway, 1992; Hanks *et al.*, 1992; Kornberg *et al.*, 1992; Romer *et al.*, 1992; Schaller *et al.*, 1992; Bockholt and Burridge, 1993; Nojima *et al.*, 1995; Petch *et al.*, 1995; Vuori and Ruoslahti, 1995). The inhibition of tyrosine kinase activity has been associated with decreased focal adhesion formation and decreased tyrosine phosphorylation of FAK and paxillin (Burridge *et al.*, 1992b; Romer *et al.*, 1992, 1994). In addition, increased FAK activity has been shown to correlate with new focal adhesion formation in migrating endothelial cells (Romer *et al.*, 1994). These previous data provided correlative evidence that FAK activation might be necessary for cytoskeletal organization during integrin-mediated cell adhesion; however, the precise role of FAK in focal adhesion formation and function has remained unclear. Indeed, FAK-independent focal adhesion formation has been demonstrated in murine aortic

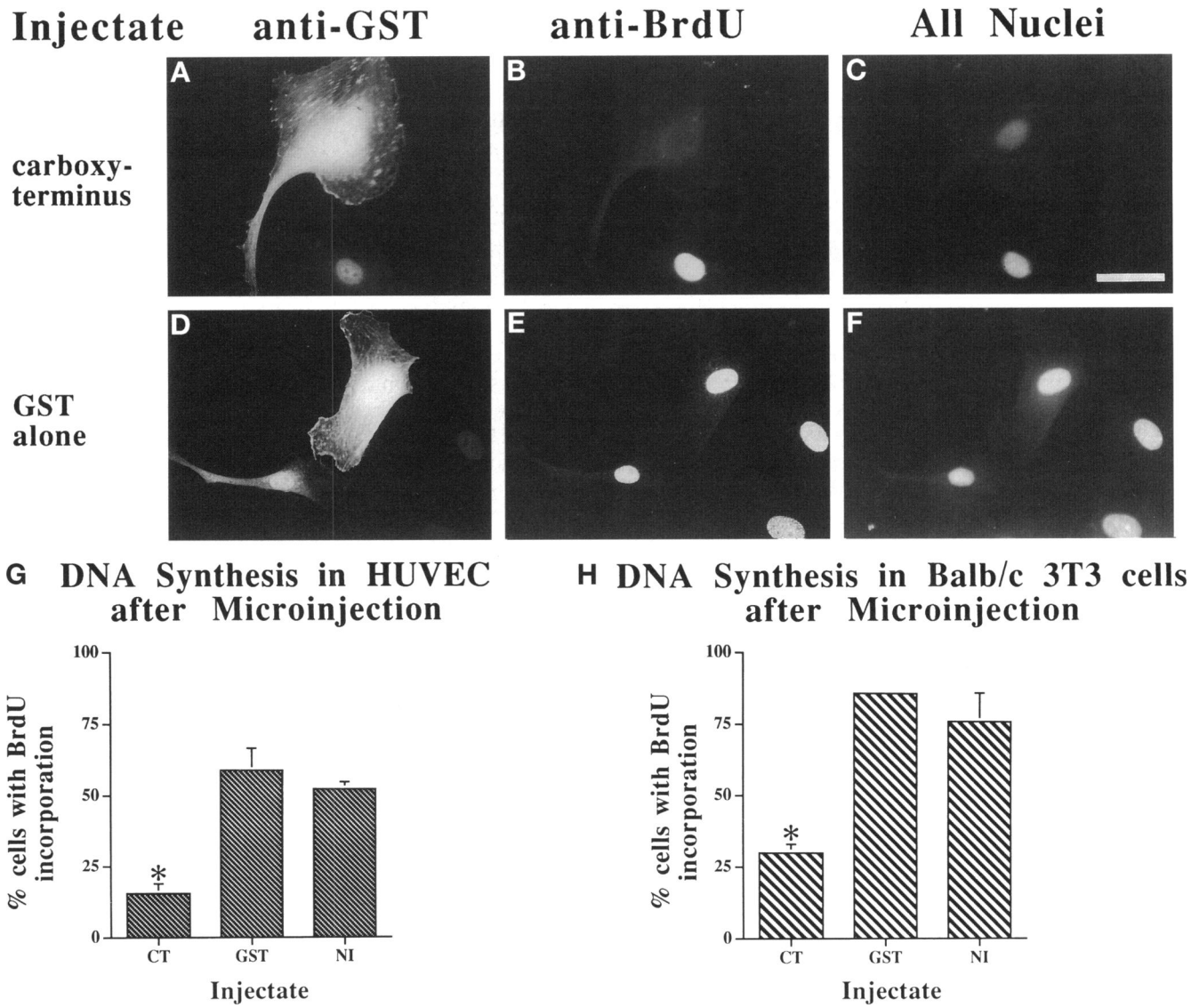

Figure 7. Effect of GST-Cterm or GST microinjection on DNA synthesis. HUVEC grown on coverslips were serum-starved overnight, injected with GST-Cterm (A–C) or GST alone (D–F), and incubated for 24 h in the presence of serum and BrdU. Cells were then stained with anti-GST polyclonal antibody (A and D), anti-BrdU mAb (B and E), and Hoechst reagent to visualize all nuclei (C and F). Bar, 40 μm. (G) Quantification of inhibition of DNA synthesis by GST-Cterm in HUVEC. The percentage of cells showing any nuclear staining with the anti-BrdU mAb is shown for GST-Cterm–injected cells (CT), GST-injected cells (GST), and noninjected cells (NI). Cells were incubated with BrdU for 24 h after injection. Data were obtained from four experiments and a total of 623 cells. Bars, SEM; asterisks, $p < 0.0001$ by Pearson chi-square analysis. (H) Quantification of GST-Cterm inhibition of DNA synthesis in BALB/c 3T3 cells. The percentage of cells showing any nuclear staining with the anti-BrdU mAb is shown for GST-Cterm–injected cells (CT), GST-injected cells (GST), and noninjected cells (NI). Data were obtained from three experiments and a total of 228 cells. Cells were incubated with BrdU for 24 h after injection. Bars, SEM; asterisks, $p < 0.0001$ by Pearson chi-square analysis.

smooth muscle cells (Wilson *et al.*, 1995). Our data demonstrate that displacement of FAK from focal adhesions decreases the level of phosphotyrosine in these structures (Figure 2). The finding that focal adhesion formation still occurred in this system (Figure 4) indicates that FAK-mediated tyrosine phosphorylation of focal adhesion constituents may not be essential for focal adhesion assembly. This implies that FAK activation and FAK-mediated tyrosine phosphorylation of focal adhesion proteins are downstream events during integrin-mediated cell adhesion. Previously reported changes in focal adhesion assembly after tyrosine kinase inhibitor treatment may, therefore, have been because of effects on kinases that modulate cytoskeletal organization but do not reside in focal adhesions.

Our observation that focal adhesion tyrosine phosphorylation is inhibited by preventing FAK localization to focal adhesions (Figure 2) is in accordance with data from a fibroblast-dominant negative model using pp41/43^{FRNK} overexpression. pp41/43^{FRNK} (FAK-related nonkinase) is a naturally occurring truncation that consists of the carboxy-terminal noncatalytic domain of FAK (Schaller *et al.*, 1993). Recent studies have shown that pp41/43^{FRNK} overexpression correlates with decreased tyrosine phosphorylation of focal adhesion proteins (Richardson and Parsons, 1996). However, other investigators that have disrupted the gene encoding FAK in mice observed no reduction in focal adhesion phosphotyrosine in fibroblasts from embryonic material (Ilic *et al.*, 1995). This divergence in findings in part may be because FAK is one of a family of nonreceptor tyrosine kinases that contains at least two recently described FAK homologues, PYK2 and CAKβ (Lev *et al.*, 1995; Sasaki *et al.*, 1995). PYK2 has exhibited rapid phosphorylation in response to stimuli that cause intracellular calcium elevation (Lev *et al.*, 1995). Intracellular calcium elevation has been associated with adhesion-mediated tyrosine phosphorylation (Pelletier *et al.*, 1992). Although PYK2 and CAKβ are not normally expressed in fibroblasts, selective pressure may induce FAK null cells to express these or other FAK homologues. This compensation may be sufficient to support the survival of cells in culture, but insufficient to fully replace the functions of FAK in a whole mouse, such that death occurs after embryonic stage E8.5, as reported by Ilic *et al.* (1995).

Our data indicated that focal adhesions containing vinculin, talin, and paxillin formed in the absence of endogenous FAK association and in the setting of significantly diminished tyrosine phosphorylation (Figure 4). These findings agree with data from fibroblasts that overexpress pp41/43^{FRNK}, exhibit decreased focal adhesion phosphotyrosine, and still form focal adhesions. Furthermore, the observations of other investigators support the concept that focal adhesion formation precedes the association of FAK with focal adhesions. Thus, the talin-binding domain of FAK is located within the FAT region of FAK (Chen *et al.*, 1995). In addition, a single segment of the β1 integrin cytoplasmic domain is associated with both integrin localization to focal adhesions and with FAK colocalization with antibody-clustered integrins (Marcantonio *et al.*, 1990; Lewis and Schwartz, 1995). This same region of the integrin cytoplasmic domain also supports tyrosine phosphorylation (Guan *et al.*, 1991; Lewis and Schwartz, 1995). Furthermore, tyrosine phosphorylation after integrin clustering was found to be dependent on the integrity of the F-actin cytoskeleton (Miyamoto *et al.*, 1995). This suggests that FAK activity is downstream of the formation of focal adhesions and stress fibers. Many events that trigger FAK activation also lead to the formation of focal adhesions and stress fibers in adherent cells. These include integrin-mediated cell adhesion, neuropeptide stimulation, and exposure to lysophosphatidic acid (Burridge *et al.*, 1992b; Guan and Shalloway, 1992; Zachary *et al.*, 1992; Barry and Critchley, 1994; Chrzanowska-Wodnicka and Burridge, 1994; Ridley and Hall, 1994). Some of these agonists act through the small GTP-binding protein rho (Ridley and Hall, 1992; Kumagai *et al.*, 1993). All of these findings are consistent with a sequence of events in which focal adhesion assembly precedes FAK activation.

FAK may have a role in focal adhesion remodeling during cell motility. This was suggested by our observations that GST-Cterm–injected cells showed decreased migration in an in vitro wound-healing model (Figure 5). These results concur with the finding that fibroblasts from FAK null mutant murine embryos showed decreased motility (Ilic *et al.*, 1995). In previous work, we have demonstrated that migrating endothelial cells in culture exhibit increased FAK tyrosine phosphorylation compared with stationary cells, and that tyrosine kinase inhibitors decrease both tyrosine phosphorylation and migration (Romer *et al.*, 1994). In the current work, the specific inhibition of FAK localization to focal adhesions by loading of GST-Cterm resulted in dose-dependent decreases in cell motility. The mechanism by which FAK regulates cell migration and wound healing is not clear. "Nodes" of talin have been documented at the edges of spreading lamellae in fibroblasts (Depasquale and Izzard, 1991). Because FAK binds directly to talin (Chen *et al.*, 1995), FAK may associate with these talin-containing nodes. Indeed, FAK is found in the "focal complexes" described by Nobes and Hall (1995) in response to rac stimulation. These "focal complexes" may be equivalent to the talin-containing nodes at the leading edge of spreading lamellipodia. The presence of FAK at these sites may be needed for the propagation of a signaling cascade at the spreading edge of motile cells. GST-Cterm injection could displace endogenous FAK from these sites just as it prevents FAK entry into focal adhesions. Alternatively, FAK may affect cell migration by downstream signaling from focal adhesions. This downstream FAK signaling may affect both cell motility and proliferation. Both of these processes are important parts of wound healing in vitro (Coomber and Gotlieb, 1990), and both are affected by decreased FAK in focal adhesions (Figures 5 and 7).

It has long been known that most types of normal cells require attachment to ECM for normal growth, and that this regulatory mechanism is lost in the process of malignant transformation (Folkman and Moscona, 1978). Guadagno *et al.* (1993) showed that anchorage-dependent cells grown in suspension arrest in the G1 phase of the cell cycle, and this was linked to the adhesion-dependent expression of cyclin A in late G1. We found that the inhibition of focal adhesion

tyrosine phosphorylation by microinjection of the carboxy-terminus of FAK resulted in a decrease in the number of cells that synthesized new DNA in the 24 h after injection. Normal amounts of serum and growth factors were used in the culture medium for the BrdU incorporation experiments described in this paper. Our results suggest that FAK activation in focal adhesions may be a prerequisite for normal, adherent cells to enter the cell cycle, even in the presence of many growth factor–related signals. Other investigators have reported that cytoskeletal integrity is required for cell cycle progression in the presence of growth factors (Böhmer *et al.*, 1996). The inhibition of FAK function in focal adhesions in our current model may signal that detachment has occurred and that growth should halt.

Several downstream effectors of FAK have been identified that suggest mechanisms whereby FAK may signal to the nucleus and regulate cell proliferation (Clark and Brugge, 1995; Schwartz *et al.*, 1995). Recent data have linked integrin-mediated adhesion and FAK phosphorylation with the Ras signal transduction pathway (Kapron-Bras *et al.*, 1993) and mitogen-activated protein (MAP) kinase activation (Chen *et al.*, 1994; Schlaepfer *et al.*, 1994; Morino *et al.*, 1995; Zhu and Assoian, 1995). These findings are in concert with our observation that the inhibition of FAK-mediated focal adhesion tyrosine phosphorylation prevents cell cycle progression (Figure 7). Our data regarding the effects of decreased focal adhesion association of FAK on cell proliferation are in conflict with those of Ilic *et al.* (1995). These investigators found no changes in growth behavior in FAK null fibroblasts. The discrepancy between these findings and ours may be explained by the p53 mutation that was introduced into the FAK null cells by that group to promote the growth of FAK null cells in culture. Indeed, the p53 mutation that they used has been shown to enhance the proliferation and survival of cultured cells (Tsukada *et al.*, 1993), and, therefore, may have counteracted the antiproliferative effects caused by the absence of FAK.

Data from our studies are consistent with FAK involvement in signaling pathways that support the survival of adherent cells. Although the design of our experiments has precluded a definitive apoptosis study to date, we observed that up to 40% of cells that were microinjected with GST-Cterm for the BrdU experiments either detached or died during the 24 h after injection. Because GST-Cterm–injected cells formed new focal adhesions when they were allowed to respread, the decrease in cell survival was probably not because of decreased adhesion. Preliminary observations also suggest that cell survival after GST-Cterm microinjection is greater in the presence of serum than in serum-free conditions. It is therefore possible that the inhibition of FAK-mediated phosphotyrosine signaling within focal adhesions by GST-Cterm microinjection may induce apoptosis. Indeed, others have documented an increased rate of DNA fragmentation in cells with decreased FAK availability after downregulating FAK expression with antisense oligonucleotides or decreasing FAK availability by antibody microinjection (Xu *et al.*, 1996; J. Hungerford and C. Otey, personal communication).

Our findings and those of others suggest that the biological role of FAK includes the initiation of signaling pathways from focal adhesions to the nucleus that regulate anchorage-dependent cell behavior (Richardson and Parsons, 1995). FAK may principally serve as a signaling protein in focal adhesions, rather than as a mediator of focal adhesion assembly.

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