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Inhibition of Focal Adhesion Kinase Expression or Activity Disrupts Epidermal Growth Factor-stimulated Signaling Promoting the Migration of Invasive Human Carcinoma Cells[1]

Christof R. Hauck, David J. Sieg, Datsun A. Hsia, Joseph C. Loftus, William A. Gaarde, Brett P. Monia, and David D. Schlaepfer[2]

The Scripps Research Institute, La Jolla, California 92037 [C. R. H., D. J. S., D. A. H., D. D. S.]; Mayo Clinic, Scottsdale, Arizona 85259 [J. C. L.]; and ISIS Pharmaceuticals, Carlsbad, California 92008 [W. A. G., B. P. M.]

ABSTRACT

Elevated focal adhesion kinase (FAK) expression in human tumor cells has been correlated with an increased cell invasion potential. In cell culture, studies with FAK-null fibroblasts have shown that FAK function is required for cell migration. To determine the role of elevated FAK expression in facilitating epidermal growth factor (EGF)-stimulated human adenocarcinoma (A549) cell motility, antisense oligonucleotides were used to reduce FAK protein expression >75%. Treatment of A549 cells with FAK antisense (ISIS 15421) but not a mismatched control (ISIS 17636) oligonucleotide resulted in reduced EGF-stimulated $p130^{Cas}$-Src complex formation, c-Jun NH_2-terminal kinase (JNK) activation, directed cell motility, and serum-stimulated cell invasion through Matrigel. Because residual FAK protein in ISIS 15421-treated A549 cells was highly phosphorylated at the Tyr-397/Src homology (SH)2 binding site, expression of the FAK COOH-terminal domain (FRNK) was also used as an inhibitor of FAK function. Adenoviral-mediated infection and expression of FRNK promoted FAK dephosphorylation at Tyr-397, resulted in reduced EGF-stimulated JNK as well as extracellular-regulated kinase 2 (ERK2) kinase activation, inhibited matrix metalloproteinase-9 (MMP-9) secretion, and potently blocked both random and EGF-stimulated A549 cell motility. Equivalent expression of a FRNK (S-1034) point-mutant that did not promote FAK dephosphorylation also did not affect EGF-stimulated signaling or cell motility. Dose-dependent reduction in EGF-stimulated A549 motility was observed with the PD98059 MEK1 inhibitor and the batimastat (BB-94) inhibitor of MMP activity, but not with the SB203580 inhibitor of p38 kinase. Finally, comparisons between normal, FAK-null, and FAK-reconstituted fibroblasts revealed that FAK enhanced EGF-stimulated JNK and ERK2 kinase activation that was required for cell motility. These data indicate that FAK functions as an important signaling platform to coordinate EGF-stimulated cell migration in human tumor cells and support a role for inhibitors of FAK expression or activity in the control of neoplastic cell invasion.

INTRODUCTION

EGFr/ErbB1[3] is a transmembrane protein with intrinsic PTK activity that is activated on ligand binding. EGFr activation involves homo- and heterodimerization with other EGFr family members such as ErbB2 (1), transphosphorylation of receptors, recruitment of various signaling proteins, and the activation of a number of different downstream signaling pathways (reviewed in Refs. 2 and 3). Initial connections between EGFr activation and tumor progression came from studies that showed that fibroblasts overexpressing the EGFr became transformed when grown in the presence of EGF (4). Subsequent studies have found that the EGFr is overexpressed in a variety of human cancers (5) with the level of EGFr expression correlated to the increased metastatic potential of the tumors (6).

One of the fundamental components promoting tumor cell invasion is increased cell migration. EGFr activation is believed to stimulate cell migration through receptor phosphorylation and the subsequent activation of downstream signaling pathways including phospholipase Cγ and the ERK/MAP kinase cascade (reviewed in Refs. 2 and 7). However, additional signaling inputs through extracellular matrix-integrin interactions also play important roles in modulating EGF-stimulated cell motility responses (8). Whereas integrins and EGFr can cocluster at the cell surface (9, 10) and can coactivate common intracellular signaling cascades (11, 12), these signals must become integrated to promote coordinated processes such as cell motility. In addition, cell-to-substratum (termed focal-contact) linkage sites mediated by integrin binding to matrix proteins must be regulated in order for cells to generate traction forces and to initiate directional movement (13). One protein that associates with both EGFr- and integrin-associated signaling complexes to coordinate and promote cell motility signaling events is FAK (14).

FAK is a nonreceptor PTK that localizes to focal contact sites and has been linked to the generation of cell survival, cell cycle progression, and cell motility signals (reviewed in Ref. 15). Significantly, FAK-null ($FAK^{-/-}$) fibroblasts exhibit an increased number of focal contact sites and are refractory to both integrin- and growth factor-stimulated migratory cues (16). Stable FAK reconstitution of $FAK^{-/-}$ cells promotes focal contact turnover (17) and reverses the motility defects of these cells (14, 18, 19). Mechanistically, the stimulated phosphorylation of FAK at Tyr-397 creates an SH2 binding motif (20) that is required for FAK function in promoting cell motility (14, 18, 19). FAK promotes both integrin- and growth factor-stimulated cell motility, in part through the recruitment of Src-family PTKs as well as adaptor proteins such as $p130^{Cas}$ and Grb7 into a focal contact-associated signaling complex (18, 21, 22). Elevated tyrosine phosphatase activity or expression of the FAK CT domain (termed FRNK) as a dominant-negative inhibitor promotes FAK dephosphorylation and inhibits FAK function in facilitating cell motility (23–26). The majority of these findings were obtained using normal fibroblasts, and it is unclear whether FAK functions in the same manner in human tumor cells.

Evaluation of human tumor samples and tumor-derived cell lines have shown that FAK expression is elevated during the processes of prostate (27), breast (28), colon (29), ovarian (30), oral (31), and thyroid (32) tumorigenesis. The *fak* gene on chromosome 8q has been

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[2] To whom requests for reprints should be addressed, at The Scripps Research Institute, Department of Immunology, IMM26, 10550 North Torrey Pines Road, La Jolla, CA 92037. Phone: (858) 784-8207; Fax: (858) 784-8227; E-mail: dschlaep@scripps.edu.

[3] The abbreviations used are: EGF, epidermal growth factor; EGFr, EGF receptor; β-Gal, β-galactosidase; CS, calf serum; CT, COOH-terminal; ERK, extracellular-regulated kinase; FAK, focal adhesion kinase; FRNK, FAK-related non-kinase; GAPDH, glyceraldhyde-3-phosphate dehydrogenase; HA, hemagglutinin; IP, immunoprecipitation; JNK, c-Jun NT kinase; MAP; mitogen-activated protein; MEK, MAP kinase kinase; MBP, myelin basic protein; MMP, matrix metalloproteinase; MOI, multiplicity/multiplicities of infection; NT, NH_2-terminal; P.Tyr, phosphotyrosine; PTK, protein-tyrosine kinase; SAP, stress-activated protein; SH, Src homology; uPA, urokinase-type plasminogen activator; WCL, whole cell lysate; mAb, monoclonal antibody; GST, glutathione *S*-transferase; IVK, *in vitro* kinase; FERM, band 4.1, ezrin, radixin, moesin; UTR, untranslated region; RIPA, radio-immune precipitation assay.

shown to be amplified in invasive squamous cell carcinomas (33). In cultured epithelial cells, overexpression of a chimeric and activated form of FAK promoted anchorage-independent cell growth and tumor formation in nude mice (34). Increased FAK expression has been correlated with the enhanced motility (35) and invasiveness of human tumor cells (36, 37) as well as with promoting increased cell proliferation (38). Despite these observations, the molecular mechanisms through which FAK facilitates these events in human tumor cells remains relatively undefined.

In this report, we test the importance of FAK function in promoting EGF-stimulated cell motility through strategies of using antisense oligonucleotide treatment to reduce FAK expression or adenoviral-mediated expression of FRNK to promote FAK dephosphorylation in human A549 adenocarcinoma cells. We find that FAK antisense treatment and FRNK expression potently inhibit EGF-stimulated chemotaxis motility, in part by affecting the extent and duration of EGF-stimulated ERK2/MAP and JNK/SAP kinase activation. Both FAK antisense and FRNK expression inhibit MMP-9 secretion and prevent serum-stimulated A549 cell invasion through Matrigel. These A549 cell motility and signaling studies are supported by results obtained from EGF-stimulated FAK$^{-/-}$ and FAK reconstituted fibroblasts. Our results support the hypothesis that FAK promotes motility and focal contact remodeling events in part through the regulation of MMP secretion, and our studies support the further testing of inhibitors of FAK expression or activity in the control of human tumor cell dissemination.

MATERIALS AND METHODS

Reagents. The PD98059 MEK1 inhibitor, the SB203580 p38 inhibitor, and recombinant human EGF were purchased from Calbiochem (San Diego, CA). The BB-94 batimastat MMP inhibitor (British Biotechnology) was a generous gift from Dr. Vito Quaranta (Scripps Research Institute, La Jolla, CA), and other miscellaneous chemicals were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. A549 human adenocarcinoma cells (ATCC CCL-185) were cultured in DMEM containing 10% FCS supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), and sodium pyruvate (1 mM). Human 293T cells were cultured in DMEM with 10% CS as described previously (14). FAK wild-type, FAK-null (FAK$^{-/-}$), and FAK-reconstituted (DA2) primary mouse embryo fibroblasts were cultured as described previously (18). Before experimentation, cells were starved in DMEM containing 0.1% FCS for A549 cells, 0.5% FCS for mouse embryo fibroblasts, or 0.5% CS for 293T cells, for 16 h.

Antisense Treatment. Twenty different antisense oligonucleotides (data not shown) were designed to target the 5′ UTR, the coding region, and the 3′ UTR of the human FAK gene (GenBank accession no. L13616). All oligonucleotides contained 20 nucleotides synthesized using 2′-methoxyethyl-modified sugars on the 1–5 and 16–20 positions, and 2′-deoxy sugars on the 6–15 positions. These modifications enhance both the stability of the oligonucleotide and RNase H activation in cells (39). Oligonucleotide backbone chemistry was uniform phosphorothioate. Antisense oligonucleotides at 400 nM were screened in A549 cells by transfection using Lipofectin for 4–6 h in serum-free medium followed by incubation for an additional 20 h in growth medium. Total RNA was isolated (RNeasy; Qiagen) followed by Northern blot analysis to assess FAK mRNA expression. Antisense oligonucleotides that demonstrated the greatest target suppression in the screen were evaluated further by dose-response analyses. Briefly, A549 cells were treated with 25–400 nM of selected antisense oligonucleotides or 5-base mismatch control oligonucleotides to determine relative potency in terms of mRNA suppression.

ISIS 15421 FAK antisense 5′-TTT-CAA-CCA-GAT-GGT-CAT-TC-3′ or ISIS 17636 mismatched control 5′-TTT-TAA-TCA-TAT-TGT-TAT-TC-3′ oligonucleotides were selected based on potency and specificity results from the above studies. For cell motility and signaling studies, oligonucleotides were added at the indicated concentrations to A549 cells in 10-cm dishes containing 5 ml of OptiMem (Life Technologies, Inc., Gaithersburg, MD) with 15 μl of Lipofectin (Life Technologies, Inc.) per 100 nM oligonucleotide. This method yielded >80% transfection efficiency as determined by fluorescein-labeled oligonucleotides as described previously (40). Mock-treated cells were incubated with Lipofectin alone. After 6 h, the medium was changed to DMEM with 0.1% FCS. Cells were used 48 h after the addition of the oligonucleotide.

Northern Analyses. Total RNA was isolated using an Atlas Pure RNA isolation kit (Clontech, Palo Alto, CA). For Northern blots, RNA samples were electrophoresed through 1.2% agarose-formaldehyde gels and transferred to Hybond-N+ membranes from Pharmacia (Uppsala, Sweden) by capillary diffusion for 12–14 h. Immobilized RNA was membrane cross-linked by exposure to UV light using a Stratalinker (Stratagene, San Diego, CA) and hybridized with ^{32}P-labeled FAK- or GAPDH-specific cDNA probes prepared by random primer labeling (Prime-a Gene Labeling System; Promega, Madison, WI). FAK hybridization signals were quantitated using a Molecular Dynamics PhosphorImager (Sunnyvale, CA), and values were normalized to GAPDH mRNA levels.

Antibodies, IP, and Immunoblotting. Affinity-purified polyclonal antibodies to the FAK NT domain (A17), to JNK1 (C17), to ERK2 (C14), to MMP-2 (C19) and MMP-9 (C20), and to the EGFr (1005) were from Santa Cruz Biotechnology (Santa Cruz, CA). Affinity-purified polyclonal antibodies to dually-phosphorylated JNK (pT183/pY185) were from Promega. Site- and phospho-specific affinity-purified polyclonal antibodies to the FAK pY-397 SH2 binding site and to c-Src pY418 within the kinase domain were from QCB Biosource International (Hopkinton, MA). Affinity-purified polyclonal antibodies to the FAK NT domain (5904) or to the FAK CT(domain (5592) were prepared as described previously (41). mAbs 12CA5 to the HA-tag and mAbs B3B9 to ERK2 were a generous gifts from Jill Meisenhelder (The Salk Institute) and Mike Weber (University of Virginia, Charlottesville, VA), respectively. mAbs to P.Tyr (mAb 4G10) and to Shc were from Upstate Biotechnology (Lake Placid, NY), mAb to the HA-tag (16B12) was from Covance Research (Berkeley, CA), mAb to the flag-tag (clone M2) was from Sigma Chemical Co. (St. Louis, MO), and mAb to dually-phosphorylated ERK (pT202/pY204) was from New England Biolabs (Beverly, MA). mAb to p130^{Cas} (clone 21) and mAb to Pyk2 (clone 11) were from BD/Transduction Laboratories (Franklin Lakes, NJ).

Serum-starved cells were stimulated with 10 ng/ml EGF for the indicated times, washed with cold PBS, lysed at 4°C in modified RIPA buffer containing 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS, as described previously (42). For co-IP studies, cells on a 10-cm dish were lysed with 500 of μl RIPA buffer and were scraped and collected, diluted with 500 μl of HNTG buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol], sheared by passage through a 22-gauge needle, and precleared by incubation with agarose beads. Cell lysates for the IPs contained ~1 mg of total cell protein. Antibodies (3 μg of IgG) were incubated with lysates for 4 h at 4°C and were collected with 25 μl of either a Protein-A or a Protein G-plus (Oncogene Research Products) agarose bead slurry. Antibody-complexed proteins were washed at 4°C with Triton-only lysis buffer (41), followed by washes with HNTG buffer, and were analyzed by SDS-PAGE. WCLs were prepared by the addition of reducing sample buffer to the precleared lysates. Immunoblotting of proteins transferred to polyvinylidine difluoride membranes (Millipore, Bedford, MA) was performed with either 1 μg/ml mAbs or a 1:1000 dilution of polyclonal antibodies (in Tris-buffered saline containing 2% BSA and 0.05% Tween 20) for 2 h at room temperature and was visualized by enhanced chemiluminescent detection methods. Sequential reprobing of membranes was performed as described previously (41).

Adenovirus Production and Infection. Production of β-gal (LacZ)-expressing adenoviruses were as described previously (43). Coding sequence for human FRNK was amplified by PCR using cDNA prepared from WI-38 human lung fibroblasts (ATCC CCL-75) and coding sequence for HA-tagged murine FRNK S-1034 was removed from pCDNA3.1 by *Afl*II/*Xba*I digestion (18). FRNK and FRNK S-1034 were subcloned into the adenoviral shuttle vector pShuttle-CMV to prepare recombinant E1-deleted adenovirus using the Ad-Easy system (44). All of the viruses were propagated in 293 cells, clonally isolated, and titered using plaque assays. A549 cells were infected at matched MOI between 2 and 5 plaque-forming units/cell and were analyzed after 2 days. Staining for LacZ activity in infected cells was performed as described previously (18), and antibodies to the FAK CT domain (5592) were used to detect FRNK (M_r ~43,000) or triple-HA-tagged FRNK S-1034 (M_r ~50,000) expression after infection.

Migration and Invasion Assays. For chemotaxis assays, modified Boyden chambers (Millicell, 8-μm pore size, 12-mm diameter; Millipore, Bedford, MA) were coated for 24 h at 4°C with 5 μg/ml rat tail collagen (Boehringer Mannheim, Indianapolis, IN), washed with PBS, air-dried, and placed into 24-well chambers containing 0.4 ml of migration media (DMEM with 0.5% BSA) with or without EGF at the indicated concentrations. For invasion assays, growth factor-reduced Matrigel (Collaborative Biomedical Products, Bedford, MA) was diluted in 4°C water and the indicated microgram amount was added to the membrane topside in 100-μl total volume. The Matrigel was allowed to polymerize for 1 h at 37°C, the chambers were air-dried for 16 h, the Matrigel barrier (~1 mm) was reconstituted with 100 μl DMEM for 2 h at 37°C, and chambers were placed into 24-well dishes containing 0.4 ml of migration media with or without 10% FCS. Serum-starved cells (0.1% FCS for A549 and 0.5% FCS for fibroblasts) were added to the upper compartment (1×10^5 cells in 0.3 ml of migration media) and after 6 h (chemotaxis migration) or 48 h (invasion) at 37°C, cells on the upper membrane surface were removed by a cotton tip applicator, chambers were washed with PBS, and migratory cells on the lower membrane surface were fixed by treatment with 2% formaldehyde/0.5% glutaraldehyde. Cells were stained [0.1% crystal violet, 0.1 M borate (pH 9.0) and 2% ethanol] and migration values were determined either by dye elution and absorbance measurements at 600 nm or by counting five high-power (×40) fields/chamber. Mean values were obtained from three individual chambers for each experimental point per assay. Pharmacological inhibitors or DMSO were preincubated (30 min) with suspended cells and were also included in the migration assay at the indicated concentrations.

IVK Assays. Assays were performed with lysates from either serum-starved or EGF-stimulated (10 ng/ml for 10 min) cells. Briefly, 10 μCi of [γ-^{32}P]ATP were added to immuno-isolated proteins and incubated at 37°C for 15 min in kinase buffer [20 mM HEPES (pH 7.4), 10% glycerol, 10 mM $MgCl_2$, and 150 mM NaCl]. Labeled proteins were visualized by autoradiography after SDS-PAGE. To measure ERK2 kinase activity, polyclonal ERK2 IPs were made from 500 μg of total cell lysate, washed in Triton Lysis buffer, followed by HNTG buffer, and then in ERK2 kinase buffer [25 mM HEPES (pH 7.4) and 10 mM $MgCl_2$]. MBP (2.5 μg) was added to each IP as a substrate. Kinase reactions (~35 μl total volume) were initiated by ATP addition (5 μl, final concentration 20 μM ATP, 10 μCi/nmol [γ-^{32}P]ATP), incubated at 32°C for 10 min, and stopped by the addition of 2× SDS-PAGE sample buffer. Cotransfection of 293T cells with a flag-tagged JNK-1 reporter (1 μg) and either HA-FRNK or HA-FRNK S-1034 in pCDNA3.1 (2.5 μg), followed by measurements of JNK IVK activity toward GST-c-Jun, were performed as described previously (45). Cotransfection of 293T cells with a flag-tagged ERK2 reporter (1 μg) and either HA-FRNK or HA-FRNK S-1034 in pCDNA3.1 (2.5 μg) followed by measurements of ERK2 IVK activity toward MBP were performed as described (41).

Zymography. A549 cells (~80% confluent) were incubated in serum-free DMEM at 37°C for 18 h, and the conditioned medium was collected. After clarification by centrifugation (5 min at 2000 × *g*), the medium was diluted in 4× nonreducing sample buffer [0.25 M Tris/HCl (pH 6.8), 8% SDS, 40% glycerol, and 0.1 mg/ml bromphenol blue] and electrophoresed in 10% SDS polyacrylamide gels containing 0.1% (w/v) gelatin for MMP detection or containing 0.1% (w/v) casein with 10 μg/ml plasminogen for uPA detection. Gels were washed repeatedly in 2.5% Triton X-100 followed by washes in 10 mM Tris (pH 8.0) at 4°C before they were incubated at 37°C for 20 h in 50 mM Tris (pH 8.0), 5 mM $CaCl_2$ (MMP detection) or in 100 mM Tris (pH 7.5), 0.15 NaCl (uPA detection). The gels were then stained with Coomassie Brilliant Blue and destained until white zones on a dark background appeared. Purified human MMP-2 and MMP-9 were purchased from Chemicon (Temecula, CA) and were used as migration standards for gelatin zymography.

Statistical Analyses. Ordinary one-way ANOVA was used to determine the overall significance within data groups. If a significant result was obtained by ANOVA, the Tukey-Kramer multiple-comparisons *t* test was also used to determine significance between individual groups.

RESULTS

Elevated FAK Expression in Human Adenocarcinoma Cells. High levels of FAK protein expression have been documented in tissue samples and cell lines derived from malignant human tumors (28). FAK protein expression is greatly elevated in human A549 human adenocarcinoma cells compared with the level of FAK in normal ($FAK^{+/+}$) and FAK-reconstituted (DA2) murine fibroblasts (Fig. 1*A*). This is in contrast to equal levels of FAK-associated $p130^{Cas}$ adaptor protein expression in FAK-null ($FAK^{-/-}$), $FAK^{+/+}$, DA2, and A549 cells (Fig. 1*A*). Previous studies with $FAK^{+/+}$ and DA2 primary fibroblasts have shown that FAK associates with an activated EGFr complex and that this linkage is important for the promotion of EGF-stimulated cell motility (14). The EGFr is expressed in A549 cells, and on EGF stimulation (10 ng/ml for 10 min), EGFr and a number of other signaling proteins such as Shc and ERK2/MAP kinase exhibit increased tyrosine phosphorylation (Fig. 1*B*). FAK was highly tyrosine-phosphorylated in starved as well as EGF-stimulated A549 cells and FAK associated with a M_r ~190,000 tyrosine-phosphorylated protein on EGF stimulation of A549 cells by co-IP analyses (Fig. 1*C*). This FAK-associated protein was confirmed to be activated EGFr by reblotting the membrane with anti-EGFr antibodies (Fig. 1*C*, *Lanes 2* and *4*). These results show that the mechanisms promoting the stimulated association of FAK with an activated EGFr complex in normal murine fibroblasts remain intact in transformed A549 human epithelial carcinoma cells and are consistent with findings of a stimulated FAK/EGFr complex formed in neuroblastoma (46) and A431 cells (47).

Reduction of A549 FAK Expression by Antisense Oligonucleotide Treatment Inhibits EGF-stimulated Cell Motility. To address the question as to whether FAK is required for EGF-stimulated A549

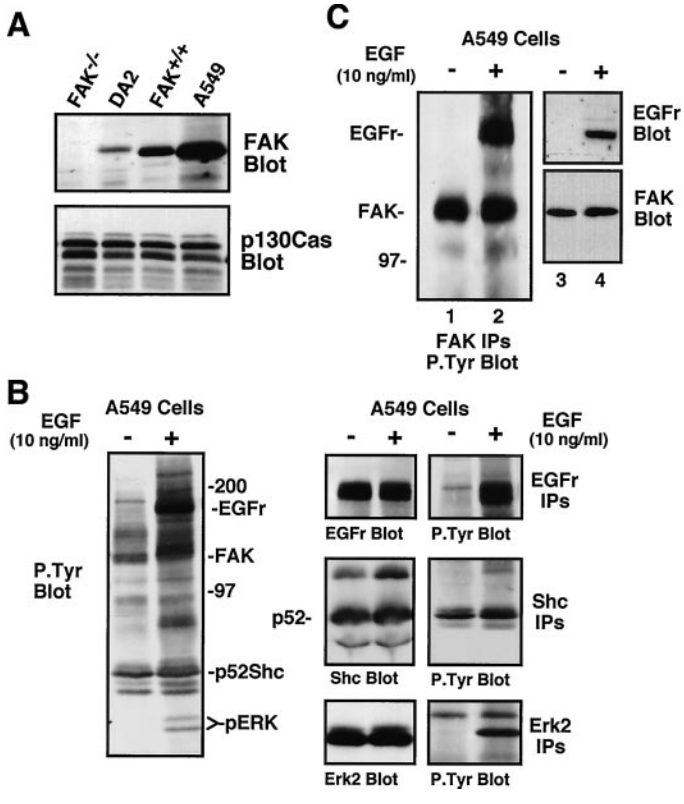

Fig. 1. Elevated FAK expression and the stimulated association of FAK with an activated EGFr complex in human A549 adenocarcinoma cells. In *A*, equal total protein (~100 μg) lysates from $FAK^{-/-}$ fibroblasts, FAK-reconstituted (*DA2*) fibroblasts, $FAK^{+/+}$ fibroblasts, and A549 human adenocarcinoma cells were sequentially analyzed by anti-FAK and anti-$p130^{Cas}$ antibody blotting. *B*, anti-P.Tyr antibody blotting analyses of proteins lysates from serum-starved or EGF-stimulated (10 ng/ml, 10 min) A549 cells. EGFr, Shc, and ERK2 IPs were analyzed by either anti-P.Tyr or the respective EGFr, Shc, and ERK2 antibodies. *C*, anti-P.Tyr blotting of FAK IPs made from lysates of serum-starved (*Lane 1*) or EGF-stimulated A549 cells (*Lane 2*). Reprobing of the membrane either by anti-EGFr or by anti-FAK blotting (*right panels*, *Lanes 3* and *4*) was used to confirm the presence of a FAK-EGFr complex after EGF-stimulation.

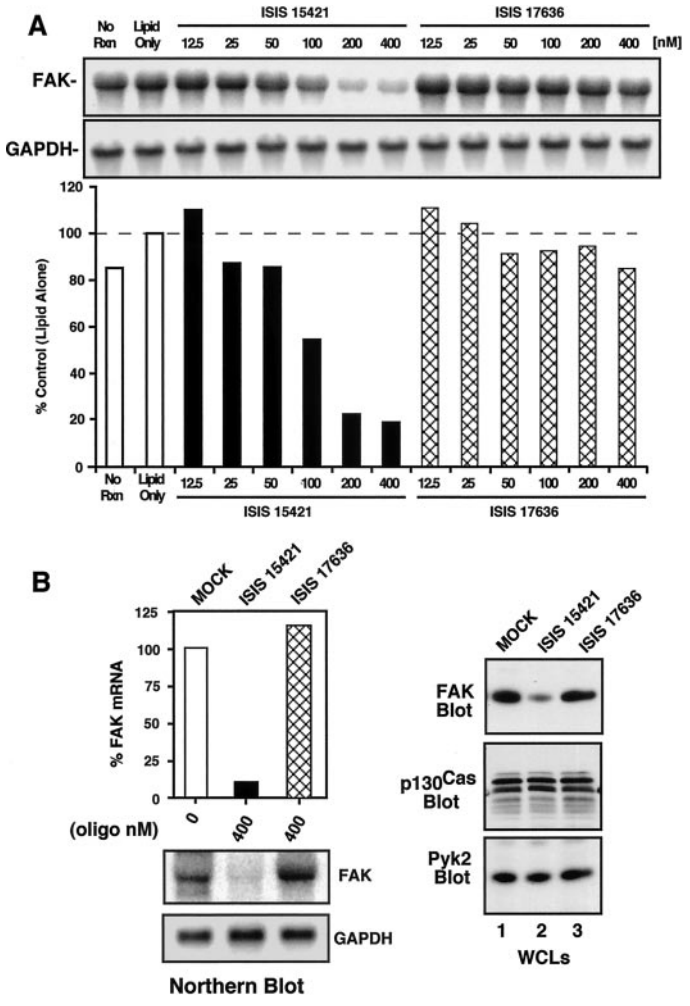

Fig. 2. Specific reduction of A549 FAK expression by antisense oligonucleotide treatment. In *A, bottom*, A549 cells either were mock-treated with transfection reagents (*open bars*) or were treated for 36 h with the indicated concentrations (nM) of ISIS 15421 FAK antisense (*closed bars*) or the mismatched ISIS 17636 control (*hatched bars*) oligonucleotide. FAK and GAPDH mRNA expression was quantified by Northern blot analyses of the same membrane (*A, top*). Graph represents percentage FAK mRNA levels compared with mock-treated cells (set to 100) and normalized to GAPDH. In *B*, A549 cells were either mock-transfected (*open bar*) or treated with 400 nM of FAK antisense ISIS 15421 (*closed bar*) or with ISIS 17636 (*hatched bar*) oligonucleotides and were analyzed after 48 h by Northern blot analyses (*left panel*) or antibody Western blotting of WCLs (*right panels*). Anti-FAK blotting was sequentially followed by anti-p130^{Cas} and anti-Pyk2 blotting of the same membrane.

cell migration, an antisense oligonucleotide directed to the 3′ untranslated region of FAK mRNA (ISIS 15421) was used to block FAK mRNA production (Fig. 2). Treatment of A549 cells for 36 h with ISIS 15421 reduced FAK mRNA levels in a dose-dependent manner (80% reduction at 200 nM), whereas treatment of A549 cells with equal concentrations of a mismatched control oligonucleotide (ISIS 17636) did not reproducibly alter FAK mRNA levels as analyzed by Northern blot analyses and normalized to GAPDH mRNA levels (Fig. 2*A*). Maximal inhibition of FAK mRNA levels (90% reduction) occurred with 400 nM ISIS 15421 treatment for 48 h (Fig. 2*B*).

To address the question as to whether the reduction in FAK mRNA levels was accompanied by lower FAK protein expression, lysates of mock-treated A549 cells or cells treated with 400 nM ISIS 15421 or 400 nM ISIS 17636 were analyzed by blotting for FAK, p130^{Cas}, or the FAK-related PTK, Pyk2 (Fig. 2*B*). ISIS 15421 treatment resulted in a ~75% reduction of FAK protein expression compared with mock- or ISIS 17636-treated A549 cells. Importantly, ISIS 15421 treatment did not affect the expression of FAK-associated proteins, such as p130^{Cas}, or FAK-related proteins, such as Pyk2 (Fig. 2*B, Lane 2*). Whereas elevated levels of cell apoptosis after FAK antisense oligonucleotide treatment have been reported (48), a 400-nM ISIS 15421 addition to A549 cells for 2 days had no increased toxic effect compared with mock-treated cells as measured either by trypan blue exclusion, annexin V binding, or propidium iodide staining (data not shown).

To determine the effect of reduced FAK expression on EGF-stimulated A549 cell motility, mock-, ISIS 15421-, or ISIS 17636-treated cells were evaluated in modified Boyden chamber chemotaxis assays (Fig. 3*A*). Compared with the high basal level of A549 cell motility measured in the absence of EGF addition, maximal EGF-stimulated directed cell motility (~3-fold increase) occurred at low EGF concentrations (2.5–10 ng/ml). Notably, ISIS 15421-mediated reduction in FAK expression potently inhibited EGF-stimulated A549 cell motility without significantly affecting random A549 cell motility in the absence of EGF (Fig. 3*A*). ISIS 15421-mediated inhibition of EGF-stimulated cell motility occurred at low but not at high (50 ng/ml) EGF concentrations (data not shown). Anti-P.Tyr blotting analyses of lysates from serum-starved ISIS 15421-treated cells re-

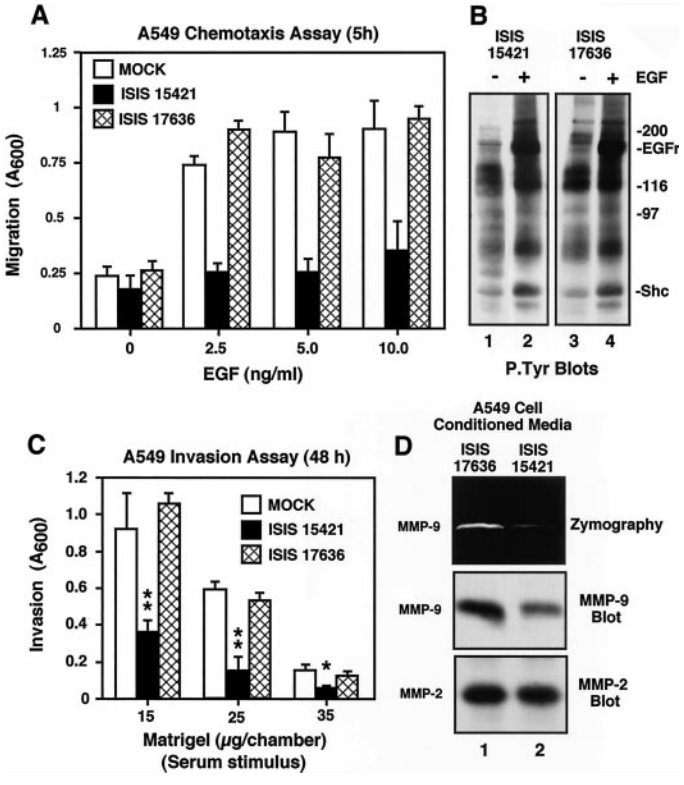

Fig. 3. ISIS 15421-mediated reduction in FAK expression inhibits EGF-stimulated A549 motility, serum-stimulated invasion through Matrigel, and MMP-9 secretion. In *A*, A549 cells either were mock-transfected (*open bars*) or were treated with 400 nM of FAK antisense ISIS 15421 (*closed bars*) or with ISIS 17636 (*hatched bars*) oligonucleotides and, 48 h later, were used in a modified Boyden chamber chemotaxis assay using either BSA or the indicated concentrations of EGF as stimuli for 6 h. Values are means ± SD from three independent experiments. In *B*, equal total protein lysates from either 400 nM ISIS 15421- (*Lanes 1* and *2*) or 400 nM ISIS 17636-treated (*Lanes 3* and *4*) A549 cells were made after serum starvation or EGF stimulation (10 ng/ml, 10 min) as indicated and were analyzed by anti-P.Tyr blotting. In *C*, A549 cells were transfected as described above, seeded on top of the indicated amount of a reconstituted basement membrane (Matrigel), and evaluated for cell invasion after 48 h. Values are means ± SD from two independent experiments. Invasion of ISIS 1542-treated A549 cells at 15 and 25 μg of Matrigel is significantly different ($P < 0.001$), and, at 35 μg of Matrigel, is significantly different ($P < 0.05$) from the invasion of mock- and ISIS 17636-treated A549 cells. In *D*, A549 cells were transfected with either 400 nM ISIS 15421 (*Lane 1*) or 400 nM ISIS 17636 (*Lane 2*), and 48 h later, serum-free medium was added for 16 h and collected. MMP activity was detected by gelatin zymography analyses. MMP protein expression was detected by either MMP-9 or MMP-2 blotting as indicated.

vealed the absence of M_r ~116,000–130,000 tyrosine phosphorylated proteins compared with control ISIS 17636-treated cells (Fig. 3*B*, *Lanes 1* and *3*). However, after EGF stimulation, the protein P.Tyr pattern was similar in both ISIS 15421 and ISIS 17636-treated cells (Fig. 3*B*, *Lanes 2* and *4*) with equivalent levels of activated EGFr phosphorylation (data not shown). These results show that antisense-mediated reduction in FAK expression leads to a similar motility-defective cell phenotype as observed in FAK-null fibroblasts (14). Our results also support the conclusion that motility signaling is blocked at a point downstream of EGFr activation.

ISIS 15421 FAK Antisense Treatment Inhibits Serum-stimulated A549 Matrigel Invasion and MMP-9 Secretion. Because cell motility is an important component promoting an invasive phenotype of transformed cells, *in vitro* cell invasion assays through an ~1-mm Matrigel barrier were performed (Fig. 3*C*). Because an EGF chemotactic gradient (2–10 ng/ml) was not sufficient to promote A459 cell invasion through Matrigel, a serum stimulus was used to stimulate cell invasion. FAK mRNA reduction by ISIS 15421 treatment of A549 cells strongly inhibited cell invasion after 48 h compared with either mock- or control ISIS 17636-treated A549 cells. Notably, the extent of total A549 cell invasion decreased with increasing Matrigel concentrations, and cell invasion was abolished at Matrigel concentrations of >50 μg/chamber (Fig. 3*C* and data not shown). These results demonstrate that the Matrigel barrier was functional and that FAK plays a role in promoting the invasive phenotype of A549 adenocarcinoma cells.

Because elevated expression, activation, and/or secretion of MMPs in transformed cells can potentiate an invasive phenotype (49, 50), gelatinase zymography comparisons were made between ISIS 15421- and ISIS 17636-treated A549 cells (Fig. 3*D*). Analyses of A549 conditioned media revealed the reduced secretion of a M_r ~90,000 MMP protein in ISIS 15421-treated cells (Fig. 3*D*, *Lane 2*). This band comigrated with purified MMP-9 (data not shown), and immunoblotting results confirmed the reduced secretion of MMP-9 into A549 conditioned medium after ISIS 15421 treatment (Fig. 3*D*, *Lane 2*). Notably, only minor changes in the secretion of MMP-2 were observed for ISIS 15421-treated compared with ISIS 17636-treated A549 cells (Fig. 3*D*). These results are consistent with recent studies showing a positive role for FAK in mediating MMP-9 secretion in other cell types (51, 52) and support the conclusion that FAK enhances *in vitro* A549 cell invasion activity, in part by promoting both increased cell motility and MMP secretion.

ISIS 15421 FAK Antisense Treatment Inhibits EGF-stimulated Signaling to JNK. To address the molecular mechanism(s) through which ISIS 15421-reduced FAK expression inhibited EGF-stimulated cell motility, blotting comparisons of serum-starved or EGF-stimulated (10 ng/ml, 10 min) A549 cells (Fig. 4*A*) were performed. EGF-stimulated EGFr tyrosine phosphorylation was not affected by ISIS 15421 treatment compared with ISIS 17636-treated cells (Fig. 4*A*, *Lanes 2* and *4*). Because downstream targets such as the ERK/MAP and JNK/SAP kinase cascades are activated by EGF, lysates of A549 cells were analyzed with antibodies directed either to phosphorylated and activated JNK-1 (P-JNK-1) or to ERK2. In control ISIS 17636-treated A549 cells, EGF stimulated the activation of both JNK-1 and ERK2 (Fig. 4*A*). Activated ERK2 was detected by the appearance of a slower-migrating form of ERK2, which represents the phosphorylated (P-ERK2) and activated form (Fig. 4*A*, *Lane 2*). By densitometry analyses, ISIS 15421-treated A549 cells exhibited a 6-fold reduction in EGF-stimulated JNK-1 activation and a 2-fold reduction in ERK2 activation compared with ISIS 17636-treated cells (Fig. 4*A*, *Lanes 2* and *4*). Results from JNK-1 or ERK2 IVK assays paralleled the blotting results (data not shown) whereby ISIS 15421-mediated reduction in FAK expression had modest inhibitory effects

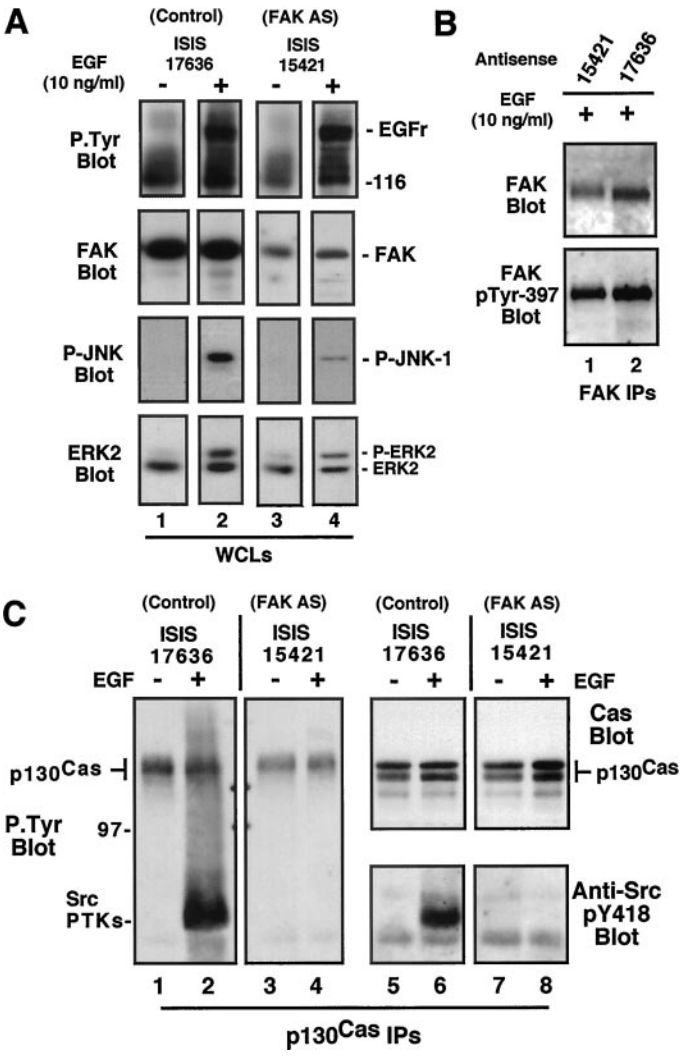

Fig. 4. Reduced FAK expression by ISIS 15421 treatment results in decreased EGF-stimulated JNK-1 activation and p130^{Cas}-Src-family PTK signaling complex formation. In *A*, A549 cells were treated with 400 nM ISIS 17636 (*Lanes 1* and *2*) or 400 nM ISIS 15421 (*FAK AS*, *Lanes 3* and *4*), were serum-starved (*Lanes 1* and *3*), or were stimulated with EGF (*Lanes 2* and *4*). WCLs were prepared and analyzed by anti-P.Tyr (*top panel*), anti-FAK (*upper middle panel*), phospho-specific anti-JNK (*P-JNK*, *lower middle panel*), and anti-ERK2 (*bottom panel*) antibody blotting. In *B*, A549 cells were treated with 400 nM ISIS 15421 (*Lane 1*) or 400 nM ISIS 17636 (*Lane 2*) and stimulated with EGF, and FAK IPs were sequentially analyzed by anti-FAK and anti-FAK pTyr-397 phospho-specific antibody blotting. *C*, A549 cells were treated with 400 nM ISIS 17636 (*Lanes 1* and *2*) or 400 nM ISIS 15421 (*FAK AS*, *Lanes 3* and *4*), were serum-starved (*Lanes 1* and *3*), or were stimulated with 10 ng/ml EGF for 10 min (*Lanes 2* and *4*). The p130^{Cas}-associated proteins were visualized by anti-P.Tyr blotting (*Lanes 1–4*), and the membrane was sequentially reblotted with anti-p130^{Cas} (*Lanes 5–8*, *top panels*) or anti-Src pTyr-418 phospho-specific (*Lanes 5–8*, *lower panels*) antibodies.

on EGF-stimulated ERK2 activation and potent inhibitory effects on JNK activation.

Interestingly, whereas ISIS 15421-treated A549 cells contained less FAK (Fig. 4*B*), the residual FAK protein in these cells exhibited a slower migration and was highly phosphorylated at Tyr-397 and Tyr-925 (data not shown) as detected by site- and phospho-specific antibodies (Fig. 4*B*, *Lane 1*). Because FAK Tyr-397 phosphorylation positively contributes to serum and platelet-derived growth factor-stimulated signaling to ERK2 (26, 53), it is possible that residual FAK protein in ISIS 15421-treated cells may still functionally contribute to EGF-stimulated ERK2 activation.

ISIS 15421-reduced FAK Expression Disrupts the Formation of an EGF-stimulated p130^{Cas}-Src Family PTK Signaling Complex. Because enhanced signaling through the JNK/SAP kinase pathway has been positively linked to the promotion of cell migration (54, 55)

and FAK connections to adaptor proteins such as p130^{Cas} and paxillin have been linked to JNK activation (56, 57), EGF-stimulated paxillin and p130^{Cas} tyrosine phosphorylation were investigated in ISIS antisense-treated A549 cells (Fig. 4*C*). Whereas no detectable differences were observed for paxillin tyrosine phosphorylation (data not shown), densitometry analyses revealed that both basal and EGF-stimulated p130^{Cas} tyrosine phosphorylation levels were reduced 2- to 3-fold in ISIS 15421-treated compared with control ISIS 17636-treated cells (Fig. 4*C*). The co-IP of a M_r ~60,000 tyrosine phosphorylated protein with antibodies to p130^{Cas} after EGF stimulation was disrupted after ISIS 15421-mediated reduction in FAK expression (Fig. 4*C, Lanes 2* and *4*). This M_r ~60,000 protein was not recognized with antibodies to the Crk adaptor protein but was reactive to antibodies directed against Src-family PTKs (data not shown).

The M_r 60,000 Src-family PTK reacted strongly with site- and phospho-specific antibodies directed to Tyr-418 within the c-Src kinase domain (Fig. 4*C, Lane 6*), which is phosphorylated on c-Src activation (58). Because signaling downstream of p130^{Cas} has been linked to EGF-stimulated JNK activation (56), our combined results suggest that reduced FAK expression in ISIS 15421-treated cells does not directly interfere with EGFr activation but disrupts the EGF-stimulated formation of p130^{Cas} and Src-family PTK signaling complex.

Adenoviral Delivery of FRNK as a Dominant-Negative Inhibitor of FAK Function in A549 Cells. To support the observed connections between ISIS 15421-mediated reduction in FAK expression with the inhibition of EGF-stimulated signaling and motility events, expression of the FAK CT domain (FRNK) was used as a dominant-negative inhibitor of endogenous FAK function (59). Lipid-mediated transfection of A549 cells with plasmid DNA yielded only low transfection efficiencies (data not shown), which was not optimal to conduct cell motility studies. Because a recombinant type 5 Lac-Z-containing adenovirus could infect >90% of A549 cells as detected by β-Gal staining (Fig. 5*A*), human FRNK and a triple-HA-tagged point-mutant (S-1034) of murine FRNK were cloned into recombinant adenoviruses and used to infect A549 cells (Fig. 5*B*). By blotting with a FAK CT domain-directed antibody, the FRNK-containing adenovirus produced a M_r 44,000 protein, whereas triple HA-tagged FRNK S-1034 expression was detected as a M_r 52,000 protein (Fig. 5*B, Lanes 1–3*). In EGF-stimulated A549 cells, adenoviral-mediated expression of FRNK promoted the dephosphorylation of endogenous FAK at Tyr-397 compared with uninfected and HA-FRNK S-1034-infected A549 cells (Fig. 5*B, Lanes 4–6*). Previous studies have shown that HA-FRNK S-1034 expression in fibroblasts and smooth muscle cells does not localize to focal contact sites, does not promote FAK dephosphorylation, and does not block FAK-mediated cell migration as did HA-FRNK expression (14, 18, 26).

FRNK Blocks Both Random and EGF-stimulated A549 Cell Motility. Whereas FRNK expression at low MOI potently inhibited FAK tyrosine phosphorylation, FRNK expression did not affect EGFr activation after EGF-stimulation of A549 cells (data not shown). To determine whether FRNK expression functioned in a similar manner as FAK antisense ISIS 15421 treatment, A549 cells were infected with adenoviruses expressing Lac-Z, or FRNK, or HA-FRNK S-1034 at low MOI and then were assayed for EGF-stimulated cell migration in Boyden chamber chemotaxis assays (Fig. 5*C*). Lac-Z infection of A549 cells resulted in the elevation of random cell motility in the presence of BSA, and this may be attributable to the fact that adenoviral uptake by cells can stimulate p130^{Cas} tyrosine phosphorylation and phosphatidylinositol 3′-kinase activation (60). However, adenoviral-mediated FRNK expression potently inhibited both random and EGF-stimulated A549 cell motility responses (Fig. 5*C*) without detectable effects on cell adhesion to the collagen matrix substrate (data not shown). HA-FRNK S-1034 expression did not inhibit EGF-stimulated A549 cell motility as did FRNK and direct blotting analyses of excess A549 cells not used in the migration assay showed equivalent levels of FRNK and HA-FRNK S-1034 expression (Fig. 5*C*).

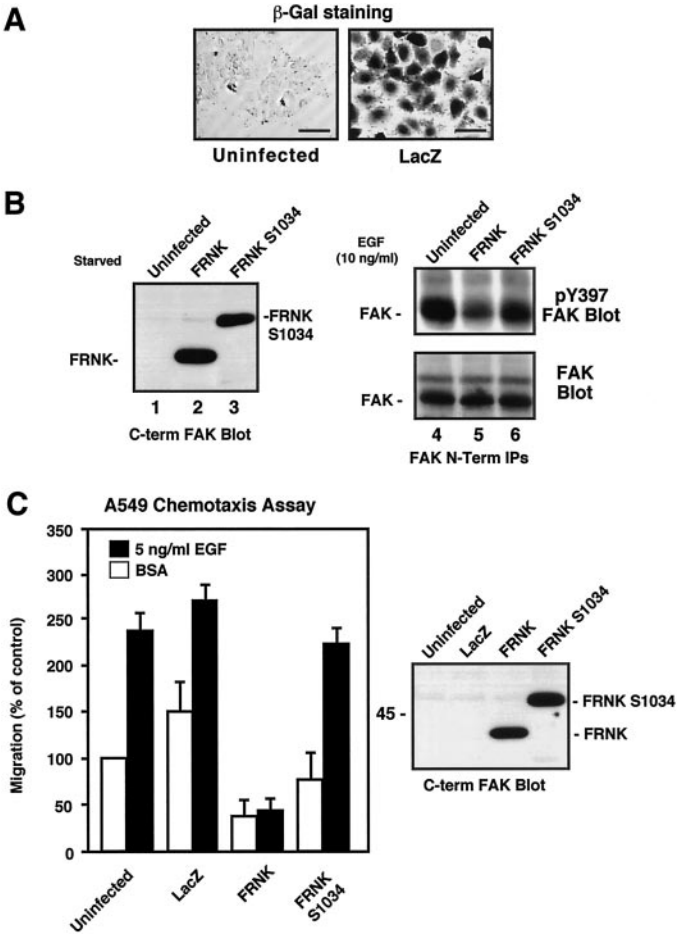

Fig. 5. Adenoviral-mediated expression of FRNK, but not HA-FRNK S-1034, promotes FAK dephosphorylation and inhibits A549 cell motility. In *A*, A549 cells were fixed and processed for β-gal activity using X-gal as a substrate 24 h after control (*Uninfected*) or recombinant LacZ-containing adenovirus infection (MOI, 2). In *B*, FRNK expression was detected by anti-FAK CT domain antibody blotting of control (*Uninfected*), adenoviral FRNK (*Lane 2*), or adenoviral HA-FRNK S-1034 (*Lane 3*)-infected (MOI, 5) A549 cells. Infected and serum-starved A549 cells were stimulated with EGF (10 ng/ml, 10 min), and NT antibody-directed FAK IPs from control (*Lane 4*), adenoviral FRNK (*Lane 5*), or adenoviral HA-FRNK S-1034 (*Lane 6*)-infected A549 cells were sequentially evaluated by anti-FAK and anti-FAK pTyr-397 phospho-specific antibody blotting. In *C*, A549 cells were infected (MOI, 5) with recombinant LacZ, FRNK, or HA-FRNK S-1034 adenoviruses or left uninfected for 36 h, serum-starved, and then used in a modified Boyden chamber chemotaxis assay using either BSA (*open bars*) or 5 ng/ml EGF (*closed bars*) for 6 h. Values were normalized to the level of random BSA-stimulated cell migration in uninfected A549 cells (set to 100). Results are means ± SD from two independent experiments. FRNK or HA-FRNK S-1034 expression was verified by anti-FAK blotting of excess cells not used in the migration assay (*right panel*).

FRNK Expression Inhibits EGF-stimulated Signaling to Both JNK-1 and ERK2. To determine the effect of FRNK expression on downstream signaling events, phospho-specific antibodies to JNK (P-JNK) and to ERK (P-ERK) were used to analyze the activation time course of these targets after EGF-stimulation of A549 cells (Fig. 6*A*). Compared with identical results obtained between uninfected and HA-FRNK S-1034 infected A549 cells, adenoviral-mediated FRNK expression inhibited the magnitude and duration of EGF-stimulated (10 ng/ml) JNK activation from 5 min to 2 h. Analyses of EGF-stimulated ERK activity revealed that FRNK but not FRNK S-1034 expression inhibited signaling to ERK2 between 5 and 30 min but did not totally block the 2-h duration of EGF-stimulated ERK2 activation (Fig. 6*A*).

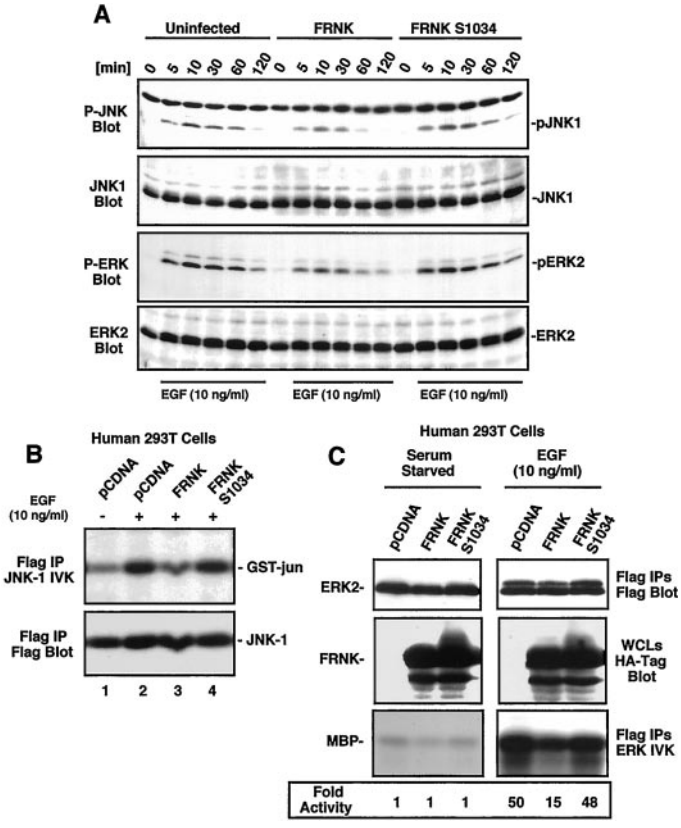

Fig. 6. Adenoviral-mediated FRNK expression inhibits the extent and duration of EGF-stimulated JNK-1 and ERK2 activation. In *A*, A549 cells were infected (MOI, 5) with recombinant FRNK or HA-FRNK S-1034 adenoviruses or were left uninfected for 36 h, serum-starved, and then stimulated with 10 ng/ml EGF for the indicated times. WCLs were analyzed by phospho-specific anti-JNK (*pJNK1*, *top panel*) or phospho-specific anti-ERK (*pERK2*, *lower middle panel*) antibody blotting, which was sequentially followed by anti-JNK1 (*upper middle panel*) or anti-ERK2 (*bottom panel*) blotting of the same membranes, respectively. In *B*, human 293T cells were transiently cotransfected with flag-tagged JNK1 along with either control vector (*pCDNA*, *Lanes 1* and *2*), HA-FRNK (*Lane 3*), or HA-FRNK S-1034 (*Lane 4*); and lysates were prepared from either serum-starved or EGF-stimulated (10 ng/ml, 10 min) cells. Flag-tagged JNK-1 IVK activity was measured in flag-tag IPs by the phosphorylation of GST-c-Jun and visualized by autoradiography (*top panel*). Expression of flag-tagged JNK-1 was visualized by flag-tag blotting of the same membrane (*bottom panel*). In *C*, human 293T cells were transiently cotransfected with flag-tagged ERK2 along with either control vector (*pCDNA*), HA-FRNK, or HA-FRNK S-1034, and lysates were prepared from either serum-starved or EGF-stimulated (10 ng/ml, 10 min) cells. Flag-tagged ERK2 expression was verified by flag-tag blotting (*top panel*) and HA-FRNK expression was visualized by HA-tag blotting (*middle panel*). Flag-tagged ERK2 IVK activity was measured in flag-tag IPs by the phosphorylation of MBP, visualized by autoradiography (*bottom panel*), and quantified by Cerenkov counting. Values indicate fold ERK2-activity over serum-starved cells.

To determine directly the effects of FRNK expression on either EGF-stimulated JNK-1 or ERK2 activation, human 293T cells were transiently cotransfected with either a flag-tagged JNK-1 reporter (Fig. 6*B*) or with a flag-tagged ERK2 reporter (Fig. 6*C*) along with plasmid expression vectors for HA-tagged FRNK or HA-FRNK S-1034. Flag-tagged JNK-1 exhibited elevated IVK activity toward GST-c-Jun in lysates from control-transfected (pCDNA) cells after EGF-stimulation (Fig. 6*B*, *Lanes 1* and *2*). Coexpression of HA-FRNK but not HA-FRNK S-1034 inhibited the activation of JNK-1 after EGF-stimulation of 293T cells (Fig. 6*B*, *Lanes 3* and *4*). ERK2 IVK activity toward MBP was also activated in pCDNA-transfected cells after EGF stimulation compared with serum-starved cells (Fig. 6*C*). Coexpression of HA-FRNK inhibited EGF-stimulated ERK2 activation 3-fold, whereas HA-FRNK S-1034 expression did not significantly affect EGF-stimulated ERK2 *in vitro* activity compared with control transfected cells (Fig. 6*C*). The combined ISIS antisense and FRNK inhibitory results support the conclusion that FAK expression and tyrosine phosphorylation positively contribute to EGF-stimulated signaling leading to JNK-1 and ERK2 activation.

The Importance of ERK Activation for Random and EGF-stimulated A549 Cell Motility. A549 cells were treated with the PD98059 pharmacological MEK-1 inhibitor of the ERK2/MAP kinase pathway to test whether ERK inhibition affected EGF-stimulated chemotaxis (Fig. 7). Whereas treatment of A549 cells with the PD98059 MEK-1 inhibitor did not affect EGF-stimulated EGFr activation or FAK tyrosine phosphorylation (Fig. 7*A* and data not shown), 10–50 μM PD98059 reduced EGF-stimulated ERK activation in a dose-dependent manner as measured by phospho-ERK blotting (Fig. 7*A*). A549 cells also exhibit a high basal ERK activity level under serum-starved conditions as detected by phospho-ERK blotting (Fig. 7*A*, *Lane 1*). Studies in glioblastoma cells have shown that increased ERK activity contributes to elevated random cell motility and treatment of A549 cells with 10–20 μM PD98059 inhibited both random and EGF-stimulated cell motility (Fig. 7*B*). Similar concentrations of the p38/MAP kinase inhibitor SB 203580 did not affect A549 cell motility responses (Fig. 7*B*).

FRNK Expression Blocks A549 MMP-9 Secretion. In accordance with the inhibitory effects on cell motility, adenoviral-mediated FRNK expression also inhibited serum-stimulated A549 cell invasion through Matrigel (data not shown). To determine whether FRNK

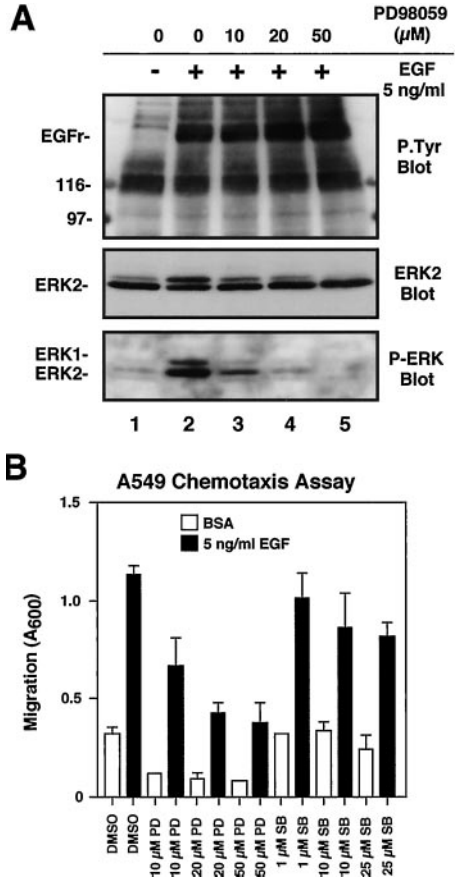

Fig. 7. ERK activity is required for EGF-initiated A549 cell motility. In *A*, serum-starved A549 cells were pretreated (30 min) with DMSO vehicle or with the indicated concentrations of the PD98059 MEK-1 inhibitor prior to EGF stimulation (5 ng/ml, 10 min). Equal total protein (~100 μg) lysates were analyzed by anti-P.Tyr (*top panel*), anti-ERK2 (*middle panel*), and phospho-specific anti-ERK (*bottom panel*) antibody blotting. IN *B*, serum-starved A549 cells were pretreated (30 min) with the indicated concentrations of the PD98059 MEK-1 inhibitor or the SB203580 p38 inhibitor and were used in modified Boyden chamber chemotaxis assays with 5 ng/ml EGF (*closed bars*) or BSA (*open bars*) as stimuli. DMSO or pharmacological inhibitors at the indicated concentrations were also present at the indicated concentrations during the motility assay. Values are means ± SD from two independent experiments.

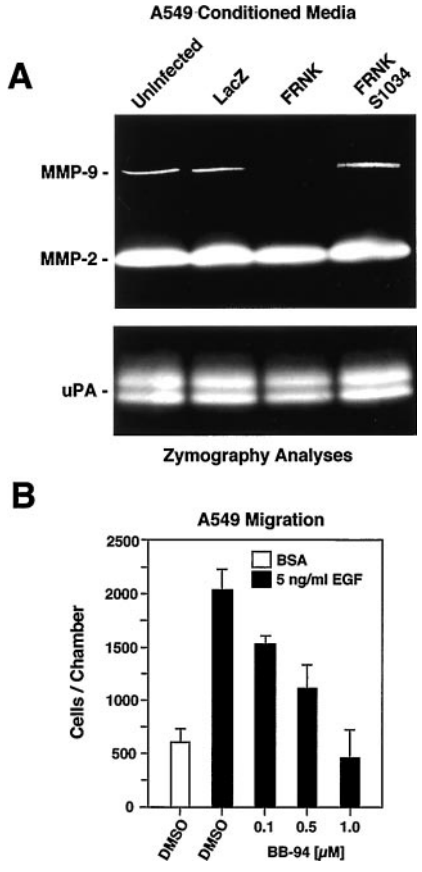

Fig. 8. FRNK expression inhibits MMP-9 secretion, and MMP activity is needed for efficient EGF-stimulated A549 cell motility. In *A*, A549 cells were infected (MOI, 5) with recombinant LacZ, FRNK, or HA-FRNK S-1034 adenoviruses or were left uninfected for 48 h and transferred to serum-free medium; after 18 h, the conditioned medium was collected and analyzed by either gelatin (*top panel*) or casein/plasminogen (*bottom panel*) zymography for MMP or uPA detection, respectively. In *B*, serum-starved A549 cells were pretreated (30 min) with either DMSO or the indicated concentrations of the batimastat BB-94 MMP inhibitor and were used in modified Boyden chamber chemotaxis assays with 5 ng/ml EGF (*closed bars*) or BSA (*open bars*) as stimuli. DMSO or BB-94 at the indicated concentrations were also present at the indicated concentrations during the motility assay. Values are means ± SD from two independent experiments.

expression inhibited MMP secretion as well as cell motility, A549 cells were infected with the indicated recombinant adenoviruses and conditioned cell medium was used in either gelatinase or urokinase plasminogen activator (uPA) zymography assays, respectively (Fig. 8A). Equivalent levels of MMP-9, MMP-2 and uPA activity were detected in the media from uninfected, Lac-Z, and HA-FRNK S-1034 infected A549 cells. However, FRNK expression resulted in the loss of detectable MMP-9 activity and slightly lower levels of secreted MMP2 and uPA activity from A549 cells (Fig. 8*A*). As confirmed by MMP-9 blotting (data not shown), FRNK expression resulted in a greater inhibition of MMP-9 secretion than did ISIS 15421 treatment of A549 cells (Fig. 3*C*).

Because it has been reported that either secreted or cell surface-associated MMP activity can influence the migratory as well as the invasive activity of epithelial cells (61), the batimastat BB-94 MMP inhibitor was tested for its effects on EGF-stimulated A549 cell motility in Boyden chamber assays (Fig. 8*B*). Surprisingly, BB-94 reduced EGF-stimulated A549 cell motility in a dose-dependent fashion with maximal inhibitory activity at 1 μM BB-94 (Fig. 8*B*). Because MMP-9 gene expression is regulated in part through signaling inputs downstream of both ERK/MAP and JNK/SAP kinase pathways (62, 63), our results with ISIS 15421 FAK antisense and adenoviral-mediated FRNK expression support the conclusion that FAK signaling regulates MMP-9 levels, and that MMP activity is needed for both A549 cell motility and invasion.

Studies in FAK-null Cells Show That FAK Is Required for Efficient EGF-stimulated Signaling and Cell Migration. To determine whether the results obtained after FAK antisense treatment or FRNK-mediated inhibition of FAK activity in A549 cells were similar to the complete loss of FAK function in FAK$^{-/-}$ fibroblasts, signaling and motility comparisons were made between FAK$^{-/-}$, FAK$^{+/+}$, and FAK-reconstituted (DA2) murine cells (Fig. 9). Whereas EGF-stimulated EGFr tyrosine phosphorylation was equal in FAK$^{-/-}$, FAK$^{+/+}$, and DA2 cells (Fig. 9*A*), elevated levels of EGF-stimulated

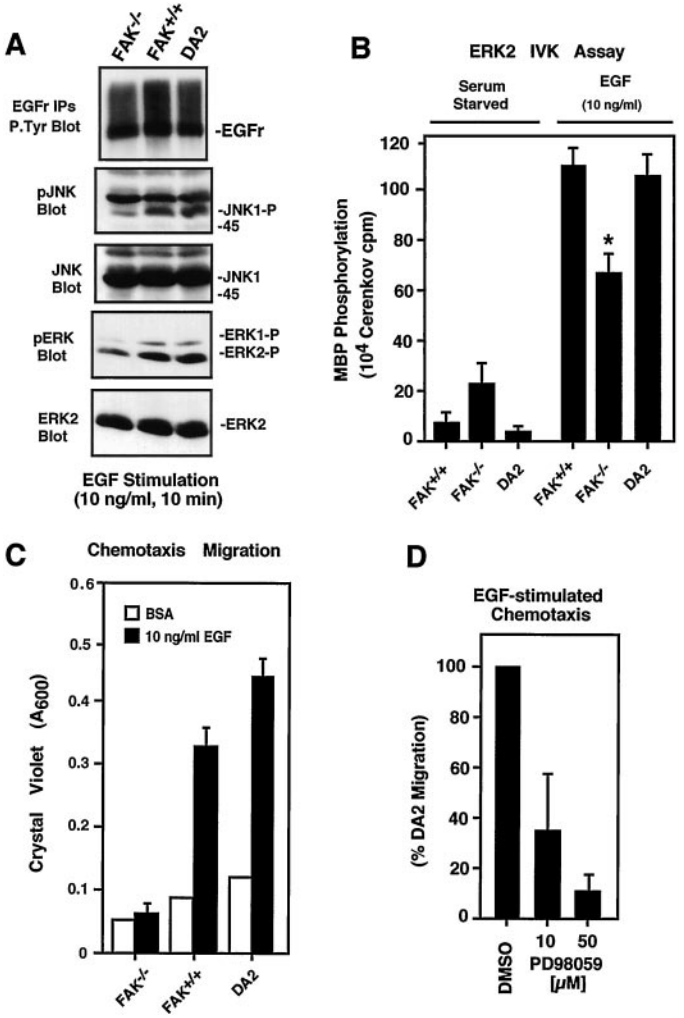

Fig. 9. FAK promotes EGF-stimulated JNK-1 and ERK2 activation, which is required for fibroblast cell motility. In *A*, EGFr IPs made from lysates of EGF-stimulated primary FAK-null (*FAK*$^{-/-}$), normal (*FAK*$^{+/+}$), and FAK-reconstituted (*DA2*) fibroblasts were analyzed by anti-P.Tyr blotting. Equal total protein (~100 μg) lysates made from EGF-stimulated FAK$^{-/-}$, FAK$^{+/+}$, and DA2 fibroblasts were analyzed by phospho-specific anti-JNK (*pJNK-1, top panel*) or phospho-specific anti-ERK (*pERK, lower middle panel*) antibody blotting, which was sequentially followed by anti-JNK1 (*upper middle panel*) or anti-ERK2 (*bottom panel*) blotting of the same membranes, respectively. *B*, ERK2 IPs were made from either serum-starved or EGF-stimulated FAK$^{+/+}$, FAK$^{-/-}$, or DA2 cells, and ERK2-associated IVK activity was measured by phosphorylation of MBP and quantified by Cerenkov counting. Values are means ± SD from three determinations. ERK2 activity in EGF-stimulated FAK$^{-/-}$ cells was significantly different from ERK2 activity in FAK$^{+/+}$ and DA2 cells ($P < 0.025$). In *C*, serum-starved FAK$^{-/-}$, FAK$^{+/+}$, or DA2 cells were used in Boyden chamber chemotaxis assays using either BSA or EGF (10 ng/ml) as stimuli for 6 h. Values are means ± SD from three independent experiments. In *D*, serum-starved DA2 cells were pretreated (30 min) with either DMSO or the indicated concentrations of PD98059 MEK-1 inhibitor and were used in Boyden chamber chemotaxis assays with 10 ng/ml EGF as a stimulus for 6 h. Values are means ± SD from two independent experiments. DMSO or PD98059 at the indicated concentrations was also present at the indicated concentrations during the motility assay.

JNK-1 and ERK2 activation were detected in FAK-containing cells by phospho-ERK and phospho-JNK blotting (Fig. 9*A*). To quantify the differences in EGF-stimulated ERK activation, direct measurements of endogenous ERK2 IVK activity toward MBP were performed (Fig. 9B). Whereas a higher basal ERK2 activity level was present in lysates from serum-starved $FAK^{-/-}$ cells, EGF-stimulated ERK2 activity was significantly higher in lysates of $FAK^{+/+}$ and DA2 cells compared with $FAK^{-/-}$ cells (Fig. 9*B*). Similar findings of enhanced EGF-stimulated endogenous JNK-1 IVK activity toward GST-c-Jun were measured in $FAK^{+/+}$ and DA2 compared with $FAK^{-/-}$ cells (data not shown). Although these results show that FAK is not absolutely required for EGF-stimulated ERK2 and JNK-1 activation, our combined A549 and 293T cell signaling studies unequivocally demonstrate that FAK positively contributes to EGF-stimulated JNK-1 and ERK2 activation.

$FAK^{-/-}$ cells are refractory to both integrin and growth factor-stimulated cues promoting directed migration (14). FAK reexpression in these cells promotes increased focal contact remodeling events (17) and EGF-stimulated motility (14). When analyzed by Boyden chamber motility assays, EGF stimulated a 3- to 4-fold enhancement of DA2 and normal $FAK^{+/+}$ primary fibroblast migration compared with the lack of a $FAK^{-/-}$ cell migration response (Fig. 9C). To determine whether elevated EGF-stimulated ERK2 activation in DA2 cells contributed to enhanced cell migration, Boyden chamber motility assays were performed in the presence of the MEK1 PD98059 inhibitor (Fig. 9*D*). Increasing concentrations of PD98059 inhibited EGF-stimulated DA2 cell motility in a dose-dependent manner (Fig. 9*D*). Although these results confirm that FAK-enhanced ERK2 activation after EGF-stimulation is important in promoting cell motility in both primary fibroblasts and A549 adenocarcinoma cells, transient transfection of $FAK^{-/-}$ cells with activated MEK1 did not rescue EGF-stimulated $FAK^{-/-}$ motility defects (data not shown). From these studies, we conclude that FAK functions as an important signaling platform to coordinate multiple signaling events needed for optimal EGF-stimulated cell motility.

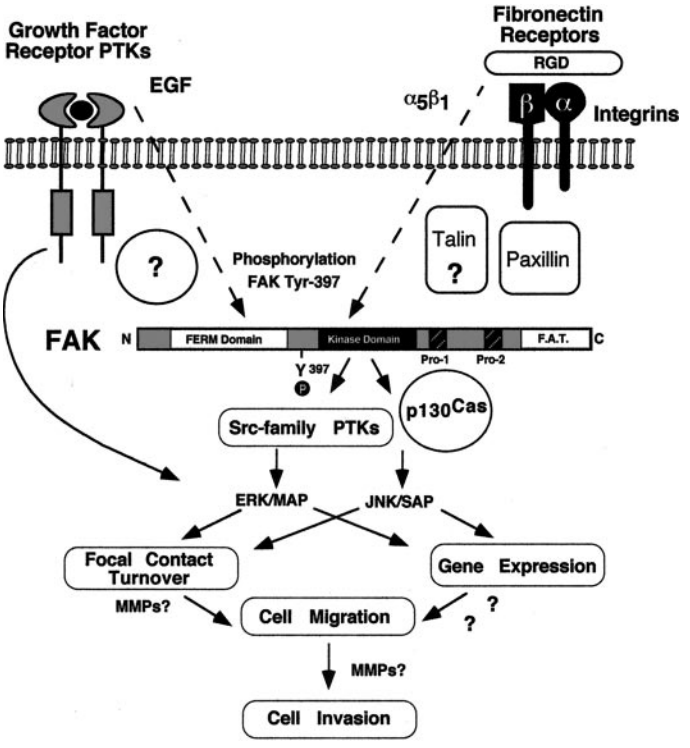

Fig. 10. Model of FAK function in linking integrins and the EGFr to cell migration. FAK localizes to fibronectin-linked $\alpha_5\beta_1$ integrin-containing focal contact sites through the binding of integrin-associated proteins such as paxillin and talin to the focal adhesion targeting (*F.A.T.*) motif within the FAK-CT domain. FAK associates with an activated EGFr complex through interactions mediated by the FERM homology region within the FAK-NT domain. These FAK-CT and FAK-NT domain connections to the respective transmembrane receptors in addition to FAK phosphorylation at Tyr-397 are required for FAK function in enhancing cell motility. Integrin-stimulated FAK autophosphorylation at Tyr-397 or EGF-stimulated *trans*-phosphorylation of FAK at Tyr-397 promote the SH2 domain-mediated recruitment of Src-family PTKs and the formation of a FAK-Src-$p130^{Cas}$ signaling complex. Multiple signaling pathways connect the FAK-Src-$p130^{Cas}$ complex to the activation of ERK2/MAP and JNK/SAP kinase cascades. This complex facilitates focal contact turnover in an undefined manner, and FAK-mediated enhancement of the extent and duration of integrin- and EGF-stimulated ERK2 and JNK activation may also affect gene expression events. It is likely that a combination of these signaling inputs regulates cell contractility and spreading during the processes of migration. FAK-mediated regulation of MMP secretion could promote both cellular focal contact remodeling events and cell motility as well as matrix degradation and increased cell invasion.

DISCUSSION

The ability of tumor cells to migrate from the site of the primary tumor and to invade surrounding tissues is a prerequisite for metastasis. FAK is a PTK that can promote increased motility of multiple cell types (15). Because the expression of FAK is elevated in metastatic, compared with noninvasive, human tumor samples (28), increased FAK expression has been correlated with the genesis of an invasive phenotype. However, the exact molecular connections of FAK to increased human tumor cell invasion have not been defined. In this study, we provide evidence that FAK functions as an important integration point for the regulation of EGF- and serum-stimulated signals promoting human tumor cell motility and invasion, respectively. FAK contributed to EGF-stimulated JNK/SAP kinase activation and also to EGF-stimulated ERK2/MAP kinase activation, which was a required signaling component promoting cell migration. We also found that the inhibition of FAK expression or function resulted in decreased MMP-9 secretion and the inhibition of *in vitro* A549 cell invasion through a reconstituted basement membrane. Because we did not observe increased cell apoptosis with FAK antisense treatment of p53+/+ A549 cells (64, 65), our results support a potential therapeutic role for inhibitors of FAK expression or activity in the control of human tumor cell dissemination.

As shown in a working model of FAK function (Fig. 10), FAK indirectly connects to clustered integrins at cell substratum contact sites via interactions with integrin-associated proteins such paxillin and talin (66). In fibroblasts and smooth muscle cells, FAK can also associate with activated growth factor receptors through interactions mediated by the FERM homology region within the FAK NT domain (14, 26). Thus, through stimulated interactions with growth factor receptors and integrins, FAK is localized within a receptor-proximal position to integrate both growth factor and extracellular matrix cues that promote cell motility.

In many different cell types, FAK phosphorylation at Tyr-397 either through integrin-stimulated FAK kinase domain activation or through growth factor-stimulated *trans*-phosphorylation creates a high affinity binding motif for the SH2 domain of Src-family PTKs (Fig. 10). The transient signaling complex formed by FAK and Src-family PTKs leads to enhanced Src-family PTK activity (67), SH2 domain-mediated recruitment of Grb2 through FAK phosphorylation at Tyr-925 (15), and enhanced $p130^{Cas}$ tyrosine phosphorylation promoting Crk adaptor protein binding (68). Integrin-stimulated FAK activation and the recruitment of Grb2 and Crk into the FAK-Src complex can lead to enhanced activation of targets such as the ERK2 and JNK kinases, respectively (66, 69). Our findings that stable reexpression of FAK within $FAK^{-/-}$ fibroblasts enhanced EGF-stimulated ERK2 and JNK activation, and that inhibition of FAK function through either antisense or FRNK expression in A549 or 293T cells impairs EGF-stimulated JNK-1 and ERK2 activation, support the conclusion that FAK is a positive regulator of these signaling events.

In this context, it is interesting to note that reduction of FAK expression by antisense oligonucleotides and inhibition of FAK function by FRNK had distinct effects on either EGF-stimulated JNK or ERK2 activation in A549 cells. Whereas reduced FAK expression resulted in the inhibition of EGF-stimulated $p130^{Cas}$ phosphorylation, Src-$p130^{Cas}$ complex formation, and JNK activation, the effect on EGF-stimulated ERK2 activation was only minor. Because residual FAK in the antisense-treated cells was highly phosphorylated at Tyr-397, we speculate that this remaining pool of FAK may still support sufficient Src-family PTK recruitment and the generation of signals leading to ERK2 activation. In contrast, adenovirus-mediated expression of FRNK did not affect FAK protein levels, but instead resulted in the quantitative de-phosphorylation of FAK at Tyr-397. This negative influence of FRNK expression on FAK Tyr-397 phosphorylation has been observed in other cell types (14, 26) and may be mediated by the competitive displacement of a FAK-integrin linkage (23). Although FRNK does not detectably associate with the EGFr, FRNK expression disrupts the association of FAK with an activated EGFr signaling complex (14). Because FRNK expression inhibits both the extent and the duration of EGF-stimulated JNK and ERK2 activation, these results suggest that FAK has to be localized appropriately within the cell to become phosphorylated at Tyr-397 and to participate in EGF-stimulated signaling events.

With respect to signals promoting cell motility, studies with human glioblastoma cells have linked ERK2 activation with enhanced random cell motility whereas FAK-$p130^{Cas}$ signaling connections were connected to directionally persistent cell migration (70). Our findings that FAK antisense treatment inhibited EGF-stimulated but not random A549 cell motility are consistent with the glioblastoma cell findings and support the existence of a distinct FAK-$p130^{Cas}$-JNK motility-promoting pathway. FRNK expression inhibited both random and EGF-stimulated A549 cell motility which is consistent with the inhibition of both ERK2 and JNK signaling pathways, respectively. Although cell migration is a complex process regulated by multiple mechanisms, ERK2 can affect actin-myosin contractility (71) and the strength of integrin-matrix connections (13, 72). It is potentially through these linkages that ERK2 inhibition results in a global negative effect on cell motility.

Whereas the use of specific inhibitors of the ERK/MAP kinase pathway have elucidated its role in cell motility, it remains undetermined whether FAK-$p130^{Cas}$ signaling connections to targets such as JNK are causally involved in EGF-stimulated A549 cell migration because of the lack of specific pharmacological JNK inhibitors. Motility stimulated by enhanced $p130^{Cas}$-Crk coupling has been shown to be dependent on the small GTPase Rac, which can promote actin cytoskeletal reorganization and membrane ruffling (73). In *Drosophila*, genetic inactivation of either Rac or components of JNK/SAP kinase pathway result in similar cell migration defects during embryonic development (74). Genetic inactivation of MEKK1 of the JNK/SAP kinase cascade in murine fibroblasts results in the inhibition of growth factor-stimulated cell migration and JNK activation (54, 55). Whereas JNK can directly interact with proteins involved in actin reorganization events (75), enhanced cell migration may be mediated in part through alterations in gene transcription events. Indeed, EGF-stimulated epithelial cell migration is attenuated by inhibitors of RNA synthesis (76, 77). In many instances, the extent and duration of ERK and or JNK activation can alter early response gene expression events after growth factor stimulation of cells through the direct phosphorylation of transcription factors (78). To this end, direct comparisons of $FAK^{-/-}$, $FAK^{+/+}$, and FAK reconstituted DA2 cells showed that FAK enhanced the EGF-stimulated early response gene expression of JunB and FosB as detected using RNase protection assays.[4]

With regard to FAK and gene expression, other FAK antisense studies have provided support that FAK expression, Ras, and ERK2 activation are important components promoting integrin-stimulated *MMP-9* gene expression in ovarian carcinoma cells (51). In A549 cells, we found that both FAK antisense treatment and FRNK expression inhibited MMP-9 secretion. Whereas sustained ERK activation can enhance MMP-9 secretion from cells (63), the *MMP-9* gene promoter region contains multiple transcription factor binding sites and is regulated in part through both ERK and JNK kinase pathway activation (62). $FAK^{-/-}$ cells are defective in Concanavalin A-dependent secretion of both MMP-2 and MMP-9, which was rescued by FAK reexpression (52). Whereas genes such as *IRS-1* are differentially regulated in $FAK^{-/-}$ and FAK-reconstituted cells (79), ongoing investigations are aimed at elucidating the molecular mechanism(s) of how FAK may regulate MMP secretion in A549 as well as in FAK-reconstituted cells.

The connection of FAK to increased MMP secretion is also notable for the fact that MMP activity was required for EGF-stimulated A549 cell motility on a collagen substrate. Whereas increased MMP secretion can enhance tumor cell invasion through basement membrane barriers, the role of MMP activity in promoting cell motility is more complex. In both smooth muscle (80) and airway epithelial cells (61), MMP-9 expression at the leading edge of migrating cells functions to promote matrix proteolysis leading to the modulation of cell-associated integrin-matrix contacts. FAK-null cells exhibit an elevated number of integrin-matrix contact sites, and this has been linked to the motility defects of these cells (16). Stable reexpression of FAK promotes enhanced cell-substratum contact turnover, and this has been correlated to the transient inhibition of Rho GTPase activity (17). Whereas it is unknown whether the inhibition of Rho signaling is distinct from the mechanisms through which FAK can enhance MMP secretion, we conclude that FAK activation can potentially affect integrin-matrix contact stability through regulatory points on the inside (Rho regulation) and outside (MMP secretion) of cells. Because FAK expression is elevated in invasive humor tumors, we propose that FAK activation in an *in vivo* environment may synchronize both MMP-mediated extracellular matrix breakdown and cell motility, thereby, facilitating an invasive phenotype.

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