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Epidermal Growth Factor-Induced Tumor Cell Invasion and Metastasis Initiated by Dephosphorylation and Downregulation of Focal Adhesion Kinase

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Upregulated epidermal growth factor (EGF) receptor (EGFR) expression and EGFR-induced signaling have been correlated with progression to invasion and metastasis in a wide variety of carcinomas, but the mechanism behind this is not well understood. We show here that, in various human carcinoma cells that overexpress EGFR, EGF treatment induced rapid tyrosine dephosphorylation of focal adhesion kinase (FAK) associated with downregulation of its kinase activity. The downregulation of FAK activity was both required and sufficient for EGF-induced refractile morphological changes, detachment of cells from the extracellular matrix, and increased tumor cell motility, invasion, and metastasis. Tumor cells with downregulated FAK activity became less adherent to the extracellular matrix. However, once cells started reattaching, FAK activity was restored by activated integrin signaling. Moreover, this process of readhesion and spreading could not be abrogated by further EGF stimulation. Interruption of transforming growth factor alpha-EGFR autocrine regulation with an EGFR tyrosine kinase inhibitor led to a substantial increase in FAK tyrosine phosphorylation and inhibition of tumor cell invasion in vitro. Consistent with this, FAK tyrosine phosphorylation was reduced in cells from tumors growing in transplanted, athymic, nude mice, which have an intact autocrine regulation of the EGFR. We suggest that the dynamic regulation of FAK activity, initiated by EGF-induced downregulation of FAK leading to cell detachment and increased motility and invasion, followed by integrin-dependent reactivation during readhesion, plays a role in EGF-associated tumor invasion and metastasis.

The invasive and metastatic stage of cancer progression correlates with poor clinical prognosis and represents the most formidable barrier to successful treatment. Cell motility and invasiveness are defining characteristics of tumors, which enable tumor cells to migrate into adjacent tissues or through limiting basement membranes and extracellular matrices. Invasive tumor cells are characterized by dysregulated cell motility in response to extracellular signals from growth factors and cytokines. In addition to roles in organ morphogenesis, maintenance, and repair, epidermal growth factor (EGF)-induced signaling has often been associated with tumor invasion and metastasis (75). EGF receptor (EGFR) overexpression has been found in many human tumors, including lung, colon, breast, prostate, brain, head and neck, thyroid, ovarian, and bladder, gliomas, and renal carcinoma (4, 20, 39, 40, 63, 71), and has been correlated with an advanced tumor stage and a poor clinical prognosis. In addition, EGFR overexpression in tumor cells is often accompanied by production of transforming growth factor alpha (TGF-α) or other EGF family ligands (73), and autocrine regulation through EGFR by such ligands has also been implicated in tumor progression. It has been reported that EGF promotes tumor cell motility and invasion (58, 62, 66). However, the basis for initiation and maintenance of the aberrant motility, which seems to be the key to understanding invasion and metastasis of tumors which overexpress EGFR, is still not known.

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Cell migration is a highly coordinated process involving precise regulation of cell adhesion and deadhesion to extracellular matrix (ECM) proteins (38). Functional regulation of the molecules involved in cell adhesion signaling should therefore be a key process in EGF-induced cell motility. Focal adhesion kinase (FAK) is a nonreceptor protein tyrosine kinase that localizes to focal adhesions, specific regions of cells that make close contacts with the ECM through transmembrane integrin molecules. FAK is associated with integrin within focal adhesions, and integrin activation by extracellular matrix ligands is associated with increased tyrosine phosphorylation and kinase activity of FAK (6, 18, 36). The activation of FAK plays an important role in integrin-mediated cell adhesion and spreading. FAK-deficient cells show decreased migration, and FAK overexpression enhances CHO cell migration (8, 28). FAK-associated or regulated proteins, such as p130^{cas}, Crk, extracellular-regulated kinase (ERK), and phosphatidylinositol (PI)-3 kinase, have been shown to function as positive regulators of adhesion receptor-mediated cell migration (9, 33, 34, 81). Despite intense investigation of the role of FAK in adhesion receptor-mediated cell attachment, spreading, and motility, very little is known about the involvement of FAK in growth factor-induced cell motility.

In this report, we demonstrate that FAK becomes dephosphorylated and inactivated upon EGF stimulation in a variety of tumor cells as well as in NIH 3T3 cells overexpressing EGFR and that downregulation of FAK activity is responsible for EGF-induced cell refractile morphological changes, detachment from the ECM, and increased tumor cell motility, invasion, and metastasis.

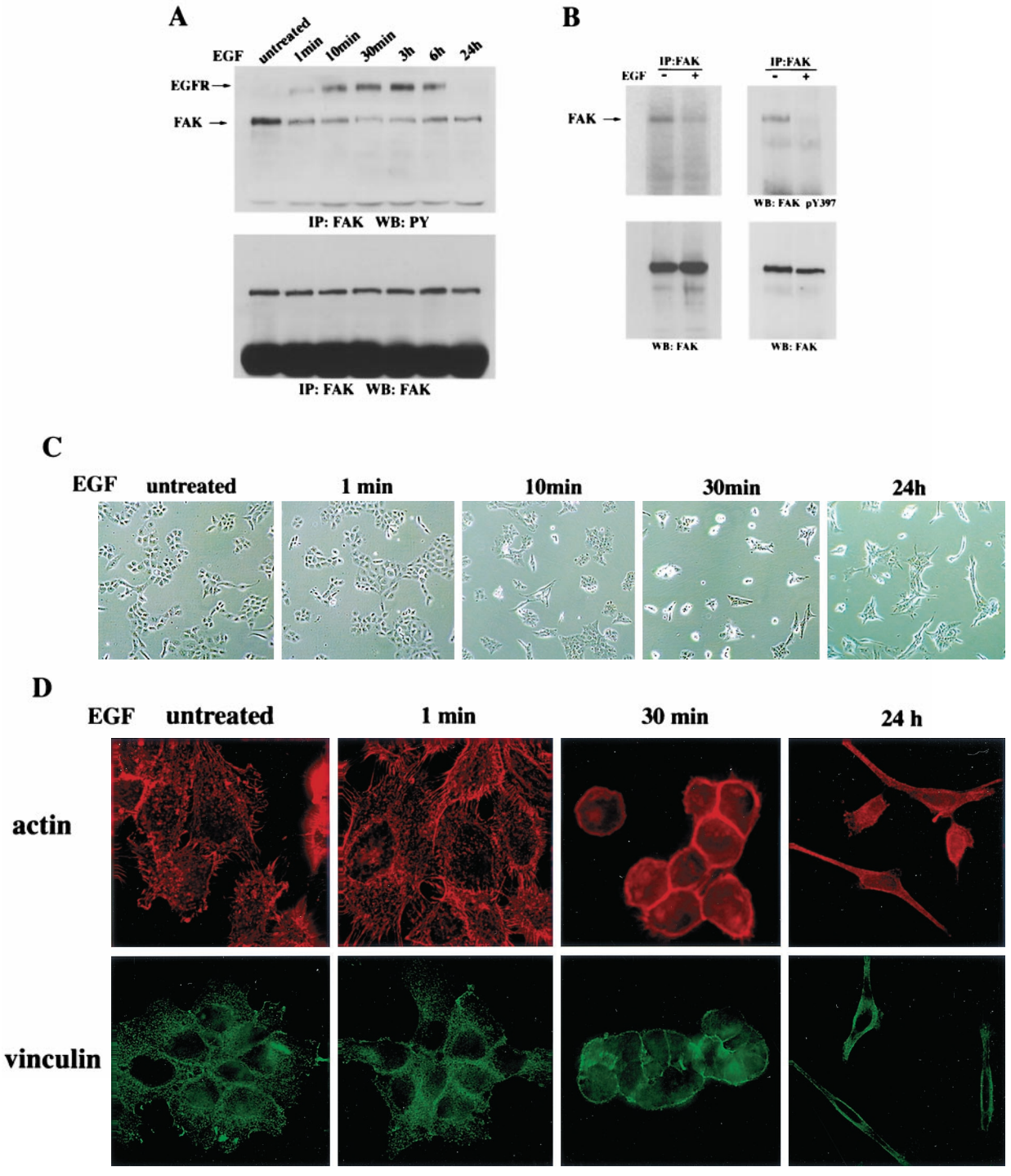

FIG. 1. EGF induces FAK dephosphorylation and inactivation prior to cell morphology changes and detachment. (A) A431 cells were treated with EGF (100 ng/ml) for the indicated times. FAK was immunoprecipitated (IP) with polyclonal FAK antiserum followed by Western blot (WB) analysis with antiphosphotyrosine (PY) antibody (upper panel) and then reprobing with polyclonal FAK antiserum (lower panel). (B) A431 cells were treated with EGF (100 ng/ml) for 30 min. FAK was immunoprecipitated with polyclonal FAK antiserum and then analyzed by in vitro kinase assay as described in Materials and Methods (upper left panel). FAK levels were assessed by immunoblotting analysis using polyclonal FAK antiserum (lower left panel). Similarly, immunoprecipitated FAK was immunoblotted with anti-phospho-FAK Tyr-397 antibodies (upper right panel), and then reprobed with polyclonal FAK antiserum (lower right panel). (C) A431 cells treated with EGF (100 ng/ml) for the indicated times were examined using a digital camera mounted on a microscope with 100× magnification or stained for actin with TRITC-labeled phalloidin or for focal adhesion with antivinculin antibody (D).

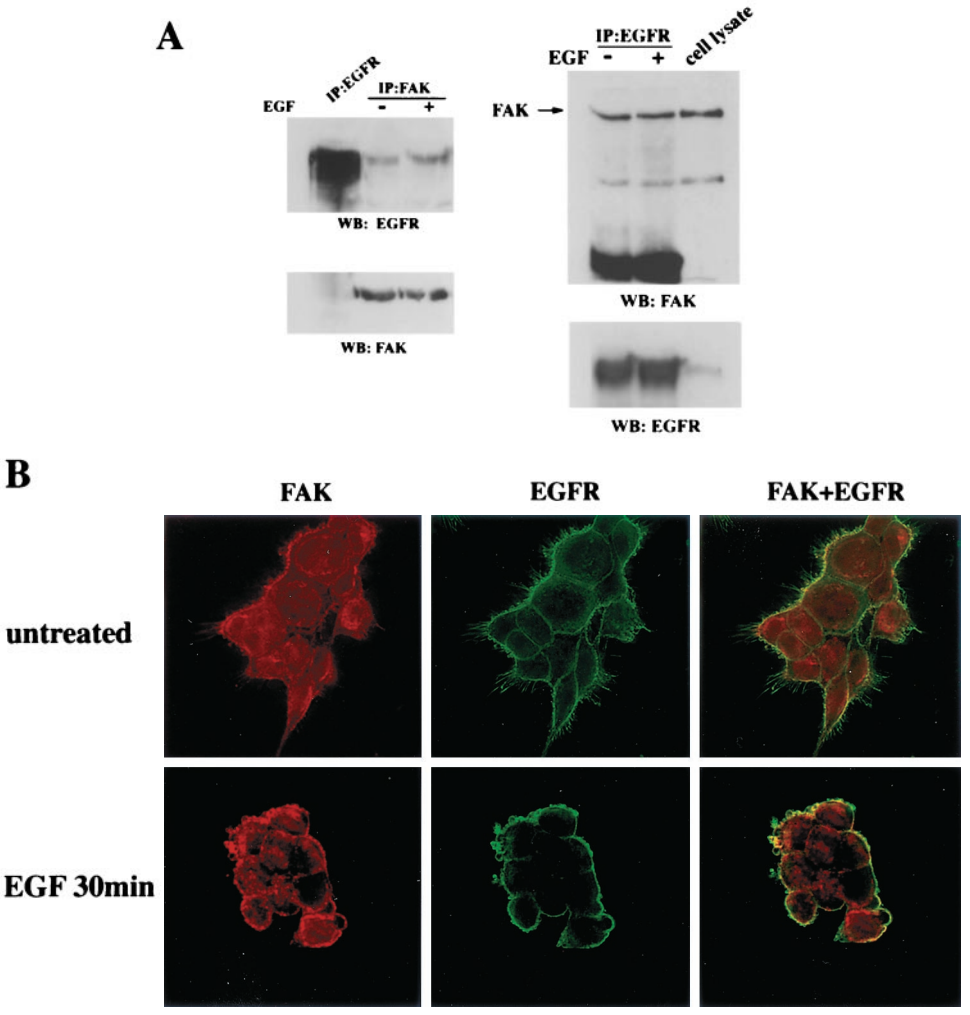

FIG. 2. FAK associates and colocalizes with EGFR. (A) A431 cells were treated with EGF (100 ng/ml) for 30 min. Immunoprecipitation (IP) was then carried out with anti-FAK antiserum, followed by immunoblotting (WB) with anti-EGFR monoclonal antibody (upper left panel). FAK protein levels were determined by immunoblot analysis using a FAK antiserum (lower left panel). Reciprocal immunoprecipitation with anti-EGFR monoclonal antibody was followed by immunoblotting with polyclonal FAK antiserum (upper right panel), and then reprobing with anti-EGFR monoclonal antibody (lower right panel). (B) Deconvolution microscopy for FAK (red), EGFR (green), and colocalized FAK and EGFR (yellow) in A431 cells that were either left untreated or treated with EGF for 30 min.

MATERIALS AND METHODS

Cells and cell culture conditions. MDA-MB-468 breast carcinoma cells and KB oral squamous carcinoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (HyClone). A431 epidermoid carcinoma cells NIH 3T3 cells, and NIH 3T3 cells overexpressing EGFR were maintained in DMEM supplemented with 10% bovine calf serum (HyClone). DU145 prostate carcinoma cells were maintained in DMEM supplemented with 1.5 g of sodium bicarbonate per liter, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10% fetal bovine serum (HyClone). Cell cultures were made quiescent by growing them to confluence and then replacing the medium with fresh medium containing 0.5% serum for 1 day.

Transfection. To generate cells expressing pp41/43^{FRNK} or S1034-FRNK, A431 cells were plated at a density of 10^5 per 100-mm-diameter dish 18 h prior to transfection with pcDNA3.1FRNK or pcDNA3.1L1034S-FRNK expression vector. Transfection was performed using Lipofectamine reagent (Gibco) according to the vendor's instructions. Transfected cultures were selected with hygromycin (200 μg/ml) for 10 to 14 days at 37°C. At that time, antibiotic-resistant colonies were picked, pooled, and expanded for further analysis under selective conditions.

Materials. AG1478 was obtained from Calbiochem. Monoclonal antibodies to the EGFR and paxillin were obtained from Transduction Laboratories, and polyclonal antibodies for FAK, p130^{cas}, ERK1, and ERK2 and monoclonal anti-phospho-ERK1 and ERK2 antibodies were from Santa Cruz Biotechnology. 4G10 monoclonal antibody for phosphotyrosine, polyclonal anti-phospho-FAK Tyr-397 antibody, and monoclonal anti-EGFR antibody were from Upstate Biotechnology. Polyclonal rabbit antisera 5591 and 5592 to the FAK C-terminal domain were produced and affinity purified as previously described (64). Human EGF, sodium orthovanadate, poly-L-lysine, fibronectin, polycolonal antivinculin antibody, and tetramethyl rhodamine isocyanate (TRITC) labeled phalloidin were purchased from Sigma. Hygromycin was from Gibco. Transwell chambers (pore size, 5 μm) containing polycarbonate membrane were from Corning Costar. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin (Ig) antibody and Texas red-conjugated anti-rabbit Ig antibody were from Southern Biotechnology Associates. A 10 mM pervanadate mixture was generated by mixing 1 ml of 10 mM sodium orthovanadate with 1 μl of 37% H_2O_2.

Immunoprecipitation and immunoblot analysis. Extraction of proteins from cultured cells was performed as previously described (42) with a modified buffer consisting of 50 mM Tris-HCl (pH 7.5), 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, leupeptin (12 μg/ml), aprotinin (20 μg/ml), 100 μM sodium vanadate, 100 μM sodium pyrophosphate, and 1 mM sodium fluoride. Cell extracts were clarified by centrifugation at 12,000 rpm, and the super-

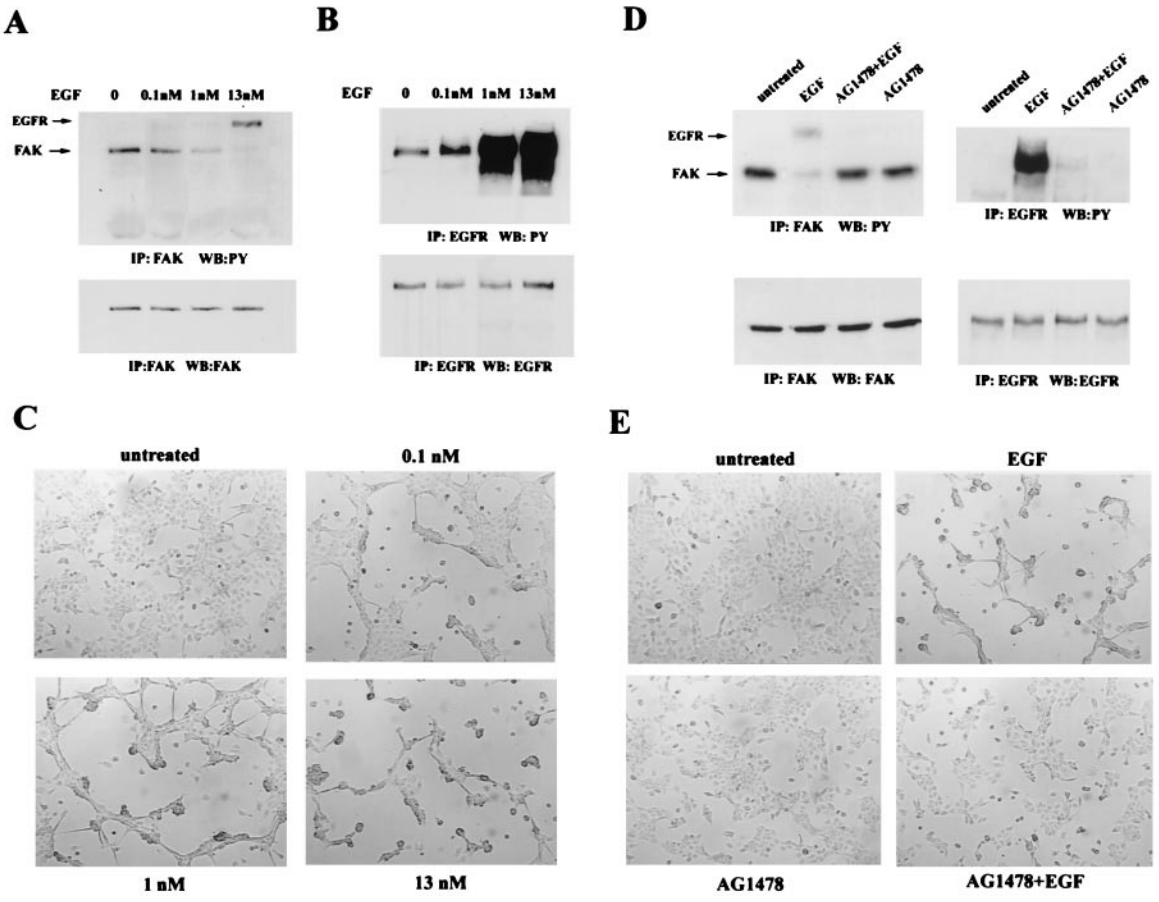

FIG. 3. FAK dephosphorylation, refractile morphological changes, and detachment of A431 cells are dependent on EGFR activation. A431 cells were treated with different doses of EGF for 30 min. The tyrosine phosphorylation levels of FAK (A) and EGFR (B) and the morphology of the cells (C) were examined as described in the legend to Fig. 1. (D) A431 cells were treated with EGF (100 ng/ml) or AG1478 (300 nM) or pretreated with AG1478 (300 nM) for 30 min before EGF treatment. The tyrosine phosphorylation levels of FAK (upper left panel) and EGFR (upper right panel) were determined by blotting with antiphosphotyrosine (PY) monoclonal antibody following immunoprecipitation with either anti-FAK or anti-EGFR antibodies. The protein levels of FAK (lower left panel) and EGFR (lower right panel) in the immunoprecipitates were determined by immunoblotting for the indicated protein. (E) A431 cells were treated with EGF (100 ng/ml) or AG1478 (300 nM) or pretreated with AG1478 (300 nM) for 30 min before EGF treatment. The morphology of A431 cells was examined as described in the legend to Fig. 1.

natants (1.5 mg of protein/ml) were subjected to immunoprecipitation with corresponding antibodies. After overnight incubation at 4°C, protein A- or G-agarose beads were added and left for an additional 3 h. Immunocomplexes were then subjected to immunoblot analysis as described previously (42).

Immunofluorescence and deconvolutional microscopy. Cells were grown on poly-L-lysine-coated glass coverslips, fixed with 4% paraformaldehyde, permeabilized in phosphate-buffered saline (PBS) containing 0.2% Triton, and blocked with 1% bovine serum albumin (BSA). Cells were incubated with TRITC-labeled phalloidin, antivinculin, or anti-FAK (Santa Cruz Biotechnology) together with anti-EGFR (Upstate Biotechnology) antibodies for 1 h at room temperature, washed, and incubated with FITC-conjugated anti-mouse Ig antibody and Texas red-conjugated anti-rabbit Ig antibody. After final washes and mounting, cells were examined using a laser scanning deconvolution microscope with a 60× oil immersion objective.

Cell migration assays. In the wound-healing assay, cells were plated at 70% confluence in 10% serum–DMEM. At 24 h after seeding, the monolayers were wounded by scoring with a sterile plastic 200-μl micropipette tip, washed, and fed with DMEM. After 48 h, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 5 min at room temperature and photographed using a low-magnification phase-contrast microscope. The extent of migration into the wound area was evaluated qualitatively. In transwell chamber (Corning-Costar) migration assays, 6×10^3 A431 cells in DMEM with 10% serum were seeded in the 6.5-mm upper chamber of transwells containing a polycarbonate membrane. DMEM with 10% serum was also added to the lower chamber. At 12 h after seeding at 37°C, EGF (100 ng/ml) was added to the upper chamber. Cells that migrated into the lower chamber were counted 72 h after addition of EGF.

In vitro invasion assay. Polymerized gels (1.0 mg/ml, final concentration) were prepared by neutralization of the collagen solution (Vitrogen 100; Collagen Corp.) with 1/6 volume of 7× DMEM concentrate. The mixed solution was diluted to a final 1× DMEM solution containing 10% serum with or without EGF (100 ng/ml) (chemokinesis) and incubated at 37°C for 24 h. Cells in DMEM with 10% serum were plated on top of the collagen gel in the presence or absence of EGF (100 ng/ml) (chemotaxis). Photographs were taken 2 days later to visualize cells that protruded into the gel surface or 5 days later to capture cells that had invaded below the gel surface. Pictures were taken using a digital camera mounted on a microscope with 100× magnification. The total number of invading cells in 10 photographic fields from two separate experiments was counted, and data were displayed in graphic format.

Adhesion assay. Serum-starved A431 cells were harvested as previously described (68). Cells were held in suspension for 40 min in DMEM containing 0.1% BSA and then plated onto either fibronectin (10 μg/ml)- or poly-L-lysine (100 μg/ml)-coated plates for different times before being photographed, trypsinized and counted, or lysed.

In vitro kinase assays. FAK was immunoprecipitated with polyclonal FAK antibodies as described above except that the lysis buffer contained no SDS. The kinase reactions were done in kinase assay buffer containing 10 μCi of [γ-^{32}P]ATP, 10 mM Tris-HCl (pH 7.4), 5 mM $MnCl_2$, 1 mM dithiothreitol, and 20 μM ATP for 20 min at 30°C. Reactions were stopped by adding an equal

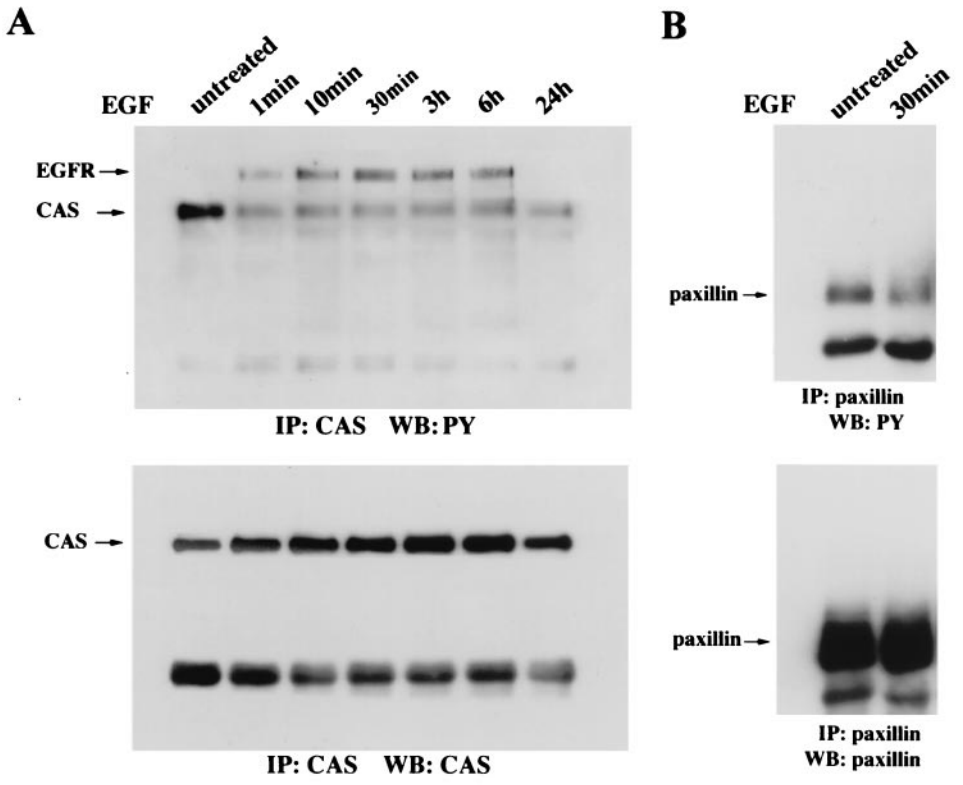

FIG. 4. p130^{cas} and paxillin are also dephosphorylated upon EGF treatment. (A) A431 cells were treated with EGF (100 ng/ml) for the indicated times. p130^{cas} was immunoprecipitated (IP) with polyclonal p130^{cas} antiserum, followed by immunoblot (WB) analysis with antiphosphotyrosine antibody (upper panel). Immunoblots were then reprobed with p130^{cas} antiserum (lower panel). (B) A431 cells were treated with EGF (100 ng/ml) for 30 min. Immunoprecipitation was carried out with antipaxillin monoclonal antibody, followed by immunoblotting with antiphosphotyrosine antibody (upper panel). Immunoblots were then reprobed with antipaxillin monoclonal antibody.

volume of 2× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and boiling for 5 min. Samples were then separated by SDS–6% PAGE, and dried gels were exposed to X-ray film.

Tumor formation in athymic nude mice. A431 cells were resuspended at a density of 1.7×10^6 cells per 150 μl in DMEM and injected subcutaneously into the right flank region of 4-week-old athymic nude mice (Harlan, Indianapolis, In). Tumors were isolated 2 weeks after injection and homogenized in lysis buffer with Rotor-Stator homogenizer. Cell extracts containing equal amount of EGFR (determined by Western blot with anti-EGFR antibody) were used for immunoprecipitation with corresponding antibodies.

DNA extraction and human Alu sequence PCR amplification. The frozen organ tissue was crushed in lysis buffer with sterile 5-ml pipettes, and genomic DNA was analyzed as described previously (35). Specific primers for human Alu sequences were Alu-sense (5′ ACG CCT GTA ATC CCA GCA CTT 3′) and Alu-antisense (5′ TCG CCC AGG CTG GAG TGC A 3′), and PCRs were performed as described previously (32).

RESULTS

EGF induces FAK dephosphorylation and inactivation prior to cell morphology changes and detachment. The human epidermoid carcinoma cell line A431, which highly overexpresses the EGFR, exhibits refractile morphological changes and detachment from the ECM upon EGF treatment (3, 12). To investigate the mechanism underlying this effect, we examined the effect of EGF treatment on the tyrosine phosphorylation state and activity of FAK and correlated these with morphological changes. Upon EGF treatment, FAK was rapidly dephosphorylated, as determined by blotting with antiphosphotyrosine antibodies. This occurred within 1 min, and FAK remained hypophosphorylated for over 24 h (Fig. 1A). Since the kinase activity of FAK is regulated by its level of tyrosine phosphorylation (7, 18), EGF-induced dephosphorylation of FAK might be expected to reduce its kinase activity. To test this, an in vitro kinase assay was carried out after immunoprecipitation with a FAK-specific antiserum. As shown in Fig. 1B, FAK immunoprecipitated from cells treated with EGF for 30 min had reduced autophosphorylation activity compared with FAK from untreated cells. Consistent with a reduction in activity, a decreased phosphorylation of Tyr-397, which is the major FAK autophosphorylation site, was observed upon EGF treatment (Fig. 1B). While it has been reported that c-Src transiently associates with FAK during integrin activation (65), we did not detect any association between c-Src and FAK either before or after EGF treatment (data not shown). It is therefore unlikely that the results of the kinase assay were severely affected by associated c-Src. Taken together, these results indicate that EGF treatment induces dephosphorylation and inactivation of FAK.

Although the EGF-induced FAK dephosphorylation occurred within 1 min after EGF treatment (Fig. 1A), EGF-induced refractile morphological changes and cell detachment were observed after 10 min of treatment with EGF and became more dramatic at 30 min of EGF stimulation (Fig. 1C). Interestingly, while some cells still had a rounded shape after being

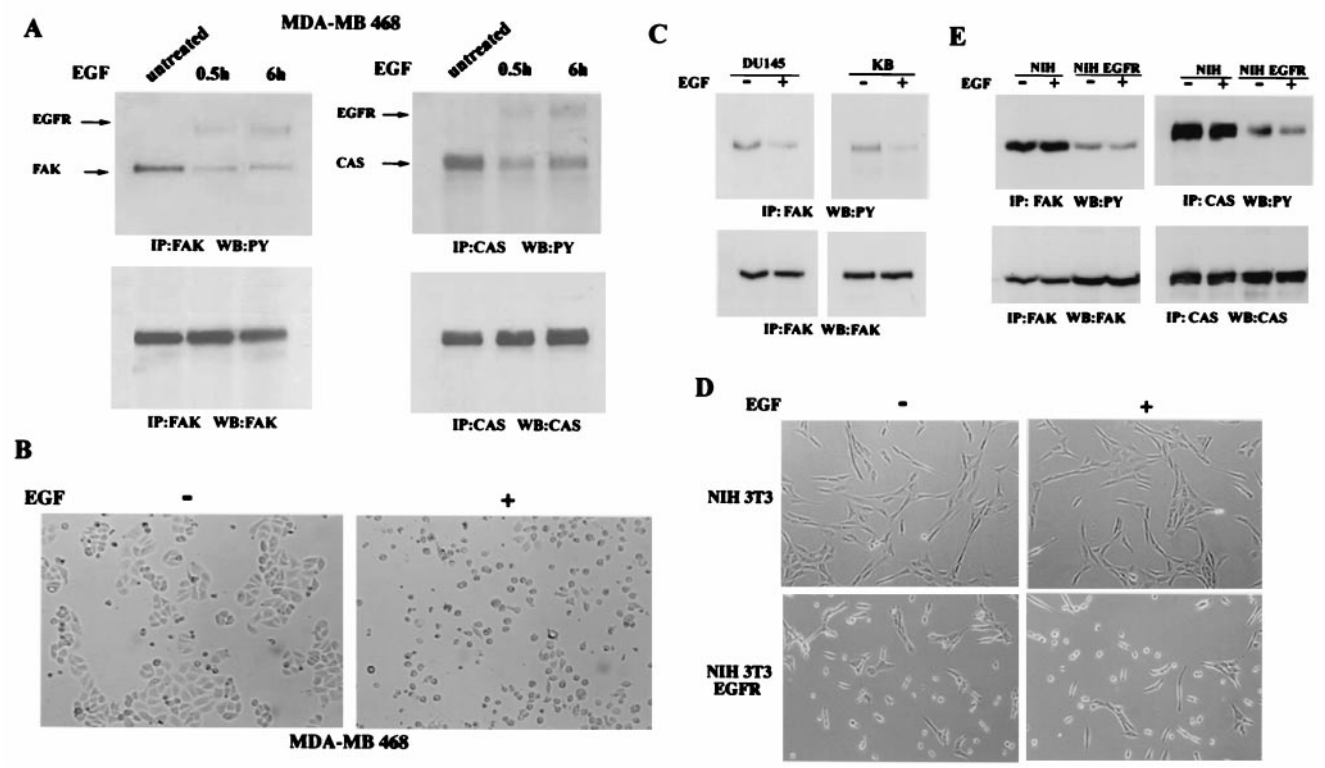

FIG. 5. EGF-induced inactivation of FAK and refractile morphological changes are general phenotypes of human tumor cells that overexpress EGFR. (A) MDA-MB468 breast cancer cells were treated with EGF (100 ng/ml) for 30 min or 6 h. The tyrosine phosphorylation levels of FAK and p130^{cas} as well as the morphology of the cells, which were either left untreated or treated with EGF for 6 h (B), were examined as described in the legend to Fig. 1. DU145 prostate cancer cells and KB oral carcinoma cells (C) and NIH 3T3 cells and NIH 3T3 EGFR cells (D and E) were either left untreated or treated with EGF (100 ng/ml) for 30 min. The morphology of the cells and tyrosine phosphorylation levels of FAK and p130^{cas} were examined as described in the legend to Fig. 1.

treated for 24 h, others became elongated and spindle shaped, resembling mesenchymal cells. Consistent with the cellular morphological changes, a dramatic actin reorganization and redistribution of the focal adhesion protein vinculin were observed after 30 min of EGF treatment, while such changes were not detectable after treatment with EGF for 1 min (Fig. 1D). These results suggest that FAK dephosphorylation and inactivation might be causally involved in the EGF-induced cell morphological changes and detachment.

FAK associates and colocalizes with EGFR. FAK immunoprecipitated from cells treated with EGF was associated with a 175-kDa phosphotyrosine-containing protein, which is the size of EGFR (Fig. 1A). Immunoblotting of FAK immunoprecipitates with EGFR antibodies showed that EGFR was associated with FAK before EGF stimulation and that EGF induced a limited increase in association (Fig. 2A). Conversely, FAK was detected in EGFR immunoprecipitates both from untreated cells and following EGF treatment (Fig. 2A). These results indicate that there is a constitutive association between FAK and EGFR in A431 cells. Consistent with this, EGFR colocalized with FAK in focal contacts in both untreated and EGF-treated cells (Fig. 2B).

FAK dephosphorylation, cellular morphological changes, and detachment of A431 cells are dependent on EGFR activation but independent of cell proliferation. It has been reported that there is dual dosage effect of EGF on A431 cell growth, in which a low dose of EGF (3 to 100 pM) stimulates cell growth, while a high dose (>1 nM) inhibits cell growth (3, 12, 30). To investigate whether cell detachment and the downregulation of FAK activity are cell proliferation-related events, A431 cells were treated with different doses of EGF. As shown in Fig. 3A, FAK was dephosphorylated even with low dose of EGF, which reportedly stimulates cell growth, and became further dephosphorylated with higher doses. The kinetics of FAK dephosphorylation correlated with the kinetics of EGFR activation (Fig. 3B), which implies that FAK dephosphorylation is EGFR activation dependent but not related to cell proliferation. Correlating with the kinetics of FAK dephosphorylation, cell morphology and attachment started to change at low levels of EGF stimulation, and this change became more dramatic at high levels (Fig. 3C). These observations suggest a connection between downregulation of FAK activity and cell detachment; both of these processes are dependent on EGFR activation but unrelated to cell proliferation.

To confirm that FAK dephosphorylation, cellular morphological changes, and detachment are EGFR activation dependent, the potent EGFR kinase inhibitor AG1478 was used. As shown in Fig. 3D, both EGF-induced FAK dephosphorylation and EGFR phosphorylation were blocked by AG1478 pretreatment for 30 min, whereas short-term treatment with AG1478 alone did not affect the basal level of FAK and EGFR tyrosine phosphorylation. The refractile morphological

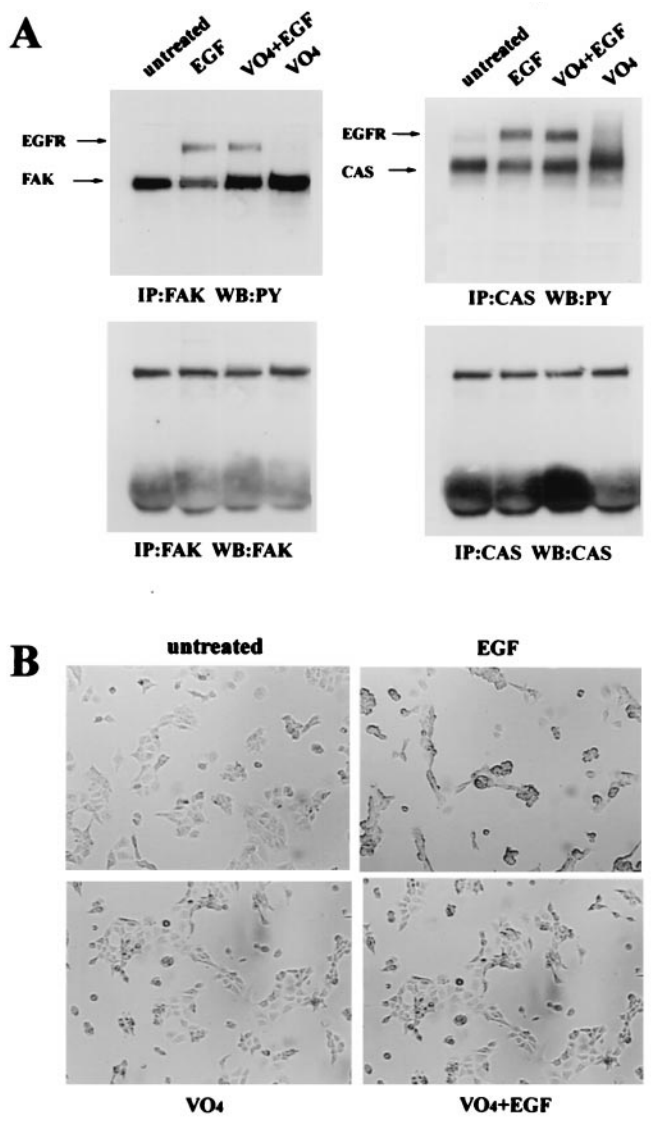

FIG. 6. EGF-induced phenotype changes of A431 cells is PTP dependent. A431 cells were treated with EGF (100 ng/ml) or pervanadate (50 μM) or pretreated with pervanadate (50 μM) for 30 min before EGF treatment. The tyrosine phosphorylation levels of FAK (A) and cell morphology (B) were examined as described in the legend to Fig. 1.

changes and cell detachment following EGF stimulation were also completely blocked by pretreatment with AG1478, while short-term treatment with AG1478 by itself had no effect on the morphology of A431 cells (Fig. 3E). These data indicate that EGFR kinase activation is required for EGF-induced FAK dephosphorylation, refractile morphological changes, and cell detachment.

p130^{cas} and paxillin are also dephosphorylated upon EGF treatment. p130^{cas} and paxillin, which associate with and are phosphorylated by FAK, are both important components of focal adhesions (57, 65, 69). To investigate whether p130^{cas} and paxillin are also regulated by EGF stimulation, p130^{cas} and paxillin were immunoprecipitated from cells treated with EGF for different periods of time and then immunoblotted with an antiphosphotyrosine antibody. Both p130^{cas} and paxillin were dephosphorylated in response to EGF, and the kinetics of p130^{cas} dephosphorylation correlated with the kinetics of FAK dephosphorylation upon EGF stimulation (Fig. 4A and B). These data indicate that EGFR activation negatively regulates the kinase activity of FAK and associated downstream signaling molecules.

EGF-induced FAK inactivation and refractile morphological changes are general phenotypes of human tumor cells that overexpress EGFR. In many human tumor cells, EGFR overexpression correlates with aggressive invasion and high metastasis rates, suggesting that there might be a general mechanism for invasion and metastasis dependent on EGFR activation. To exclude that the EGF-induced phenotypic changes demonstrated above are cell line specific, the MDA-MB468 breast carcinoma cell line, which also overexpresses EGFR, was treated with EGF for different times. As shown in Fig. 5A, both FAK and p130^{cas} were dephosphorylated upon EGF stimulation, and cells exhibited a rounded morphology (Fig. 5B). Similarly, dephosphorylation of FAK was observed in DU145 prostate carcinoma cells and KB oral squamous carcinoma cells, both of which also overexpress EGFR (Fig. 5C). Interestingly, NIH 3T3 EGFR cells, which overexpress human EGFR, were highly refractile even before EGF treatment (Fig. 5D). Consistent with these morphological changes, the tyrosine phosphorylation levels of FAK and p130^{cas} were significantly reduced even in unstimulated NIH 3T3 EGFR cells compared to the high tyrosine phosphorylation level of FAK and p130^{cas} in parental NIH 3T3 cells (Fig. 5E). Upon EGF treatment, additional changes in cell morphology and a further small reduction in the tyrosine phosphorylation levels of FAK and p130^{cas} were observed in NIH 3T3 EGFR cells, but not in NIH 3T3 cells (Fig. 5D and E). These data indicate that EGFR activation-induced downregulation of FAK activity and its downstream signaling, as well as refractile morphological changes and cell detachment, are general phenotypes of human tumor cells overexpressing EGFR. Such phenotypic changes might play an important role in tumor cell motility.

EGF-induced phenotypic changes of A431 cells are PTP dependent. At least two mechanisms might explain the EGF-induced downregulation of FAK activity: one is through inhibition of an upstream signaling molecule that can activate FAK, and the other is through involvement of a protein-tyrosine phosphatase (PTP). To examine whether the FAK dephosphorylation is PTP dependent, pervanadate, a general PTP inhibitor, was applied before EGF treatment. As shown in Fig. 6A, pretreatment with pervanadate blocked EGF-induced dephosphorylation of FAK and p130^{cas}. Moreover, pretreatment with pervanadate also partially blocked EGF-induced refractile morphological changes and cell detachment (Fig. 6B). Similar results were obtained with cells treated with phenylarsine oxide (data not shown), another PTP inhibitor, which was previously reported to cause selective inhibition of FAK dephosphorylation (44, 48, 59). These data indicate that PTP-mediated downregulation of FAK activity might be at least partly responsible for EGF-induced cell detachment and morphological changes.

Activation of FAK by integrin engagement blocks EGF-induced FAK dephosphorylation and also prevents morphological changes and cell detachment from the ECM. If downregula-

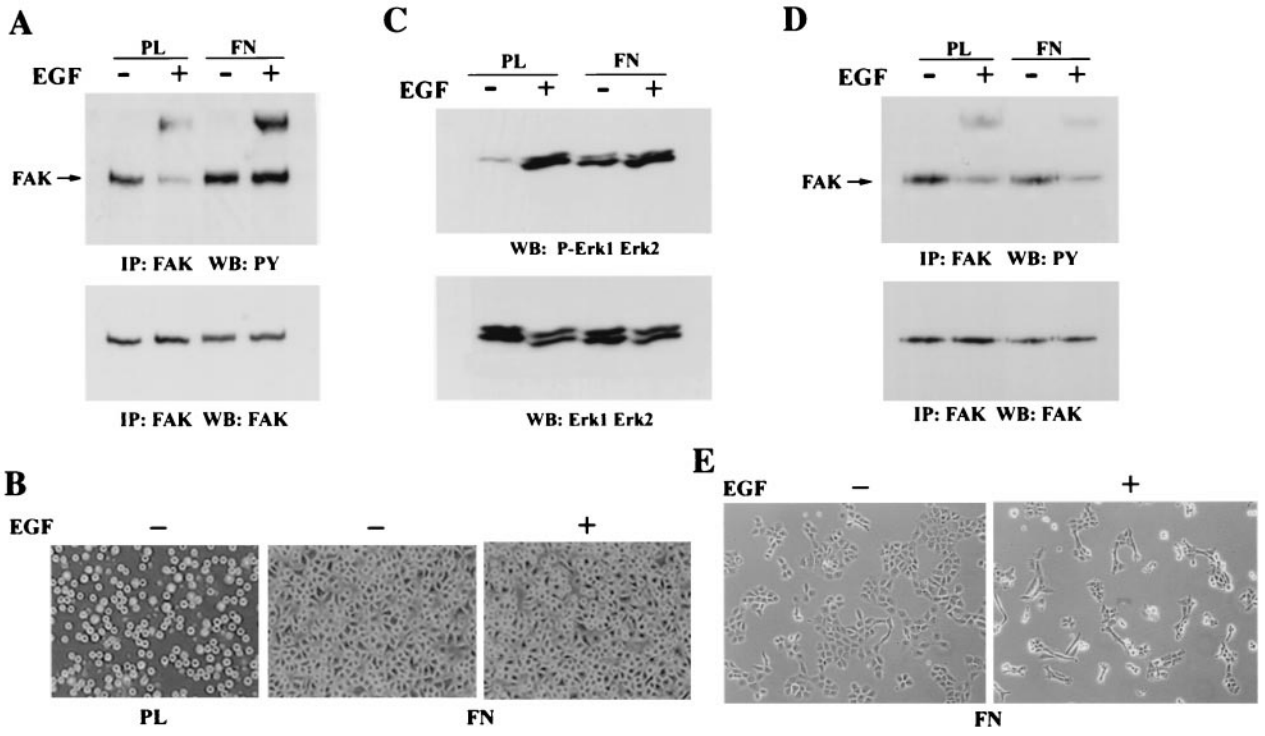

FIG. 7. Activation of FAK by integrin engagement blocks EGF-induced FAK dephosphorylation and also prevents morphological changes and cell detachment from the ECM. A431 cells were trypsinized and kept suspended in DMEM with 0.1% BSA for 40 min before plating onto either fibronectin (FN)- or poly-L-lysine (PL)-coated plates for 40 min (A and B) or 12 h (D and E) prior to 20 min of EGF (100 ng/ml) treatment. The tyrosine phosphorylation levels of FAK and cell morphology were examined as described in the legend to Fig. 1. (C) The activation of ERK1 and ERK2 was determined by Western blot analysis using anti-phospho-ERK1 and -ERK2 monoclonal antibodies (upper panel). The blot was reprobed with anti-ERK1 and -ERK2 antisera (lower panel).

tion of FAK activity resulting from EGF-induced dephosphorylation is responsible for refractile morphological changes and cell detachment from the ECM, FAK in an active state should prevent these EGF-induced phenotypic changes. It is known that integrin receptor engagement through binding of extracellular ligands such as fibronectin results in the phosphorylation and activation of FAK. Therefore, integrin-induced FAK activation might be able to counteract the effect of EGFR activation on focal adhesion. To test this hypothesis, A431 cells in suspension were seeded on plates coated with either fibronectin or poly-L-lysine. Forty minutes after being seeded, cells on fibronectin-coated plates showed spreading processes, while the cells plated on poly-L-lysine still exhibited a rounded morphology (Fig. 7B). This indicates that fibronectin binding to integrins activates FAK and promotes cell spreading, while poly-L-lysine, to which integrins do not bind, does not increase FAK activity or promote cell spreading. After treatment with EGF for 20 min, FAK was dephosphorylated in cells plated on poly-L-lysine but not in cells plated on fibronectin (Fig. 7A). Moreover, integrin signaling prevented the EGF-induced refractile morphology change and cell detachment from the ECM (Fig. 7B). The dominant effect of integrin signaling only occurred during the process of adhesion and spreading, since EGF was still able to induce dephosphorylation of FAK and morphological changes in cells plated on fibronectin for 12 h (Fig. 7D and E). To test whether integrin signaling completely interrupts the signal transduction induced by EGFR activation, EGF-stimulated ERK activation was examined. As shown in Fig. 7C, EGF treatment of cells plated on either fibronectin or poly-L-lysine for 40 min led to activation of ERK1 and ERK2, although plating cells on fibronectin alone stimulated ERK1 and ERK2 activity significantly. These data indicate that downregulation of FAK is required for EGF-induced rounding, refractile morphological changes, and cell detachment from the ECM. However, during the process of cell adhesion, integrin signaling exerts a dominant effect and prevents EGF-induced dephosphorylation of FAK, morphological changes, and cell detachment from the ECM, even though EGF can induce other signaling events, such as activation of ERK.

Inhibition of FAK by expression of pp41/43^{FRNK} results in refractile morphological changes and cell detachment. The C-terminal domain of FAK contains binding sites for a number of molecules, including the adaptor proteins p130^{cas} (22, 56, 57) and Grb2 (64), the cytoskeletal proteins paxillin and talin (11, 25), PI-3 kinase (10, 19), and the GTPase-activating protein GRAF (26). The C-terminal domain of FAK also contains a focal adhesion targeting sequence that is necessary and sufficient for recruiting FAK to focal adhesions. In some cells, the C-terminal domain of FAK is also expressed as a separate protein called pp41/43^{FRNK} (FRNK for FAK-related nonkinase). Overexpression of pp41/43^{FRNK} inhibits integrin-stimulated tyrosine phosphorylation of FAK, paxillin, and tensin and delays the formation of focal adhesions and chicken embryo

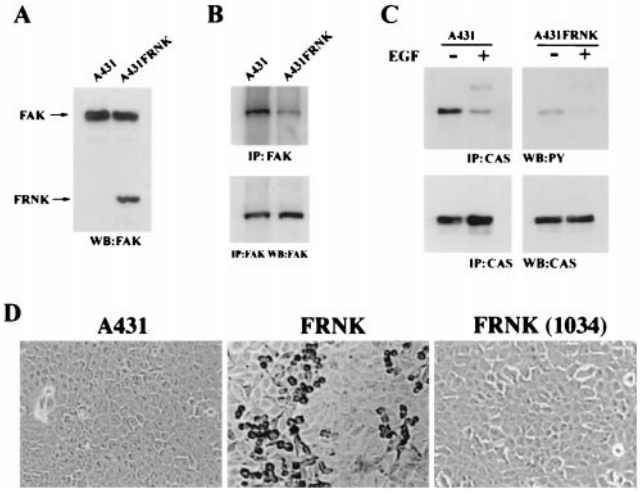

FIG. 8. Inhibition of FAK by expression of pp41/43^{FRNK} results in refractile morphological changes and cell detachment. (A) The level of pp41/43^{FRNK} overexpression in A431 cells was determined by immunoblotting (WB) with anti-FAK polyclonal antiserum recognizing the C terminus of FAK. (B) FAK was immunoprecipitated (IP) with polyclonal FAK antiserum and used to perform the in vitro kinase assay as described in Materials and Methods. (C) The tyrosine phosphorylation levels of p130^{cas} without or with EGF (100 ng/ml) treatment and the morphology of A431 cells, A431 cells expressing FRNK and A431 cells expressing the point mutant L1034S-FRNK (D) were examined as described in the legend to Fig. 1.

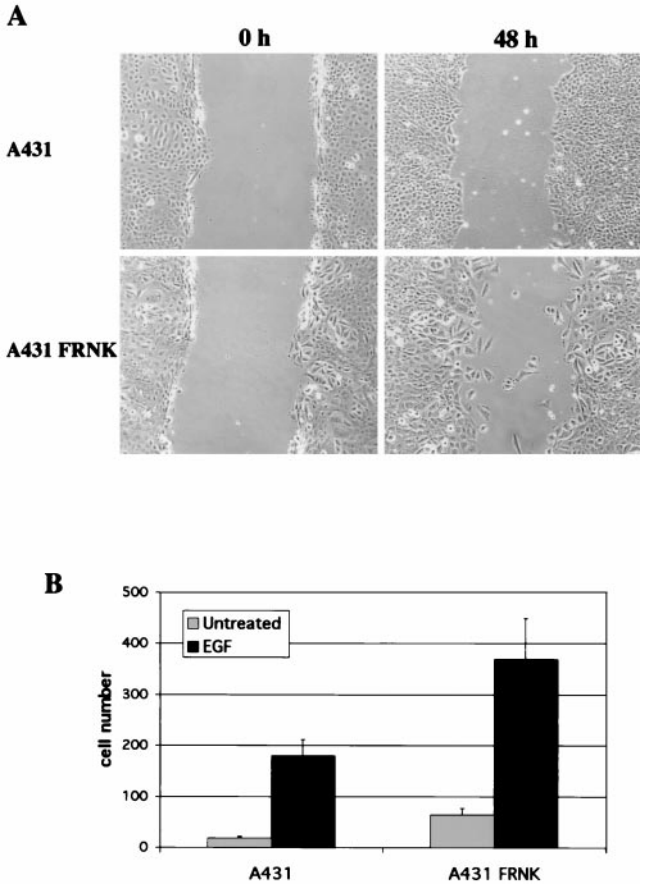

FIG. 9. EGF-induced downregulation of FAK activity promotes tumor cell motility and invasion. (A) A431 cells or A431 cells expressing FRNK were plated at 70% confluence in DMEM with 10% serum. At 24 h after seeding, the cell monolayers were wounded by scraping with a 200-μl plastic micropipette tip, washed, and then refed with complete DMEM. After 48 h, cells were fixed with 4% paraformaldehyde and photographed as described in the legend to Fig. 1. (B) Six thousand A431 cells or A431 cells expressing pp41/43^{FRNK} in DMEM with 10% serum were seeded in the 6.5-mm upper chamber of transwells containing a polycarbonate membrane. At 12 h after seeding, EGF (100 ng/ml) was added to the upper chamber. The cells that migrated into the lower chamber were counted 72 h after addition of EGF. Data represent the mean ± standard deviation of two independent experiments. (C) Cells in the presence or absence of EGF (100 ng/ml) (chemokinesis) were plated on the top of collagen gel with or without admixed EGF (100 ng/ml) (chemotaxis). Five days after plating, cells were photographed at a focal plane beneath the surface to visualize cells that have penetrated the gel. The number of invading cells in 10 photographic fields from two separate experiments was counted (D).

cell spreading on fibronectin (60, 61). Thus, pp41/43^{FRNK} functions as an inhibitor of FAK and adhesion signaling.

To examine whether pp41/43^{FRNK} expression causes a phenotype that mimics EGF-induced rounding, refractile morphology changes, and cell detachment from the ECM, pp41/43^{FRNK} was stably expressed in A431 cells at a level comparable to endogenous FAK (Fig. 8A). Expression of pp41/43^{FRNK} in A431 cells reduced FAK autophosphorylation activity relative to that in parental A431 cells (Fig. 8B). It also reduced the basal level of p130^{cas} tyrosine phosphorylation (Fig. 8C). Moreover, expression of pp41/43^{FRNK} resulted in cells piling up and exhibiting a refractile morphology in the absence of EGF treatment, whereas expression of L1034S-FRNK, a mutant that does not localize to focal contacts (67), did not affect cell morphology (Fig. 8D, and data not shown). Treatment of pp41/43^{FRNK}-expressing cells with EGF resulted in further dephosphorylation of p130^{cas} (Fig. 8C). Therefore, downregulation of FAK either by EGF-induced dephosphorylation or by inhibition as a result of expression of pp41/43^{FRNK} results in the same phenotypic changes. This indicates that the functional downregulation of FAK is sufficient and required for EGF-induced refractile morphological changes and cell detachment.

EGF-induced downregulation of FAK activity promotes tumor cell motility and invasion. To investigate whether EGF-induced cell morphological changes and detachment from the ECM might be causally involved in the invasive and metastatic behavior of EGFR-overexpressing carcinomas, we investigated the effect of downregulating FAK activity on the motility and invasion of tumor cells. In a monolayer wound-healing assay, A431 cells expressing pp41/43^{FRNK} were able to migrate into the wound at a rate greater than the front of cells moved in by proliferation, whereas parental A431 cells or A431 cells expressing the localization-defective S1034-FRNK (data not shown) repaired the wound by cell proliferation-mediated front movement only (Fig. 9A). EGF treatment caused A431 cells, especially A431 cells expressing pp41/43^{FRNK}, to float into the medium and to reattach, which makes the wound-healing assay a less accurate way to assess EGF-associated migration. A more quantitative assay, the chamber mobility assay, was therefore carried out. A431 cells and A431 cells expressing pp41/43^{FRNK} were plated on transwell plates for 12 h and then treated with EGF. EGF treatment significantly promoted migration of cells through a porous membrane, and

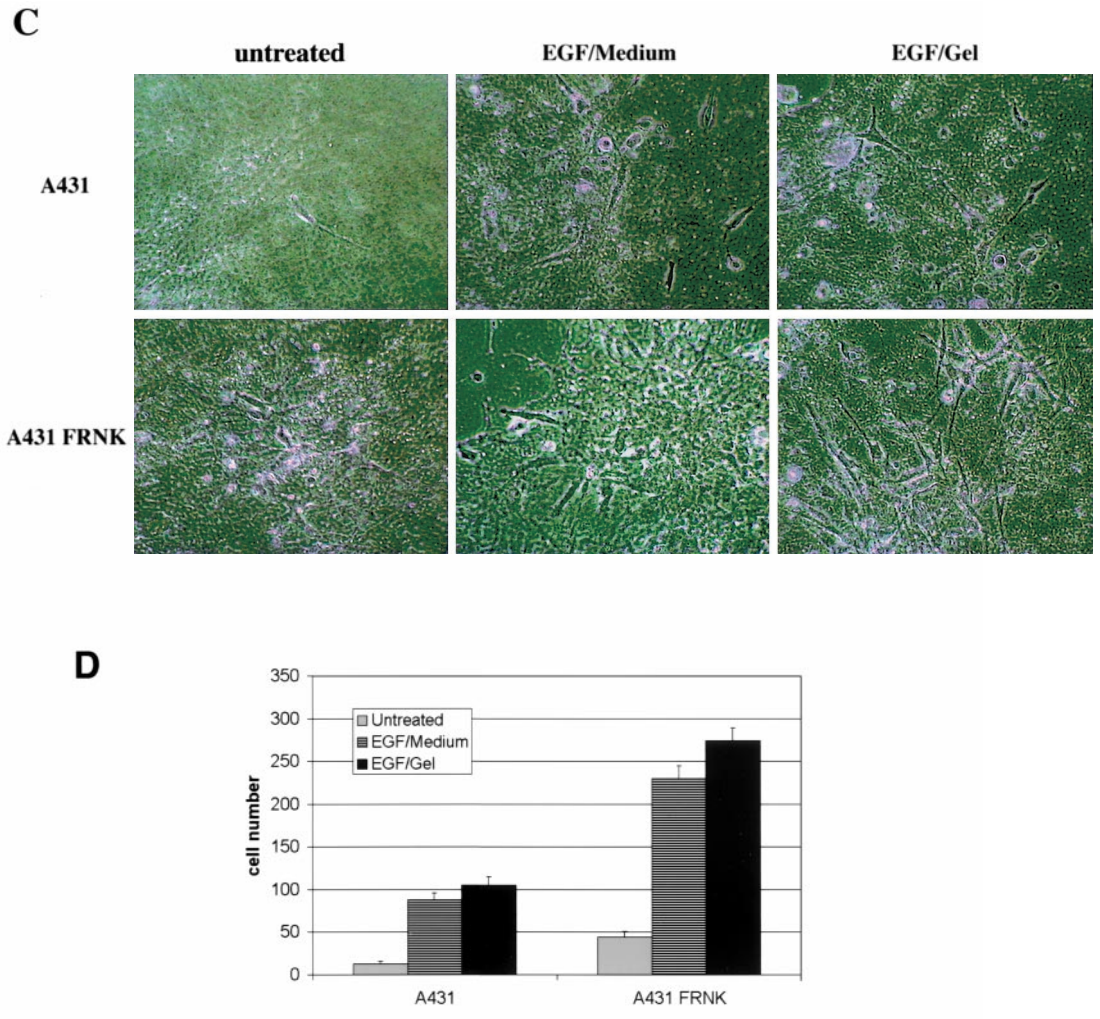

FIG. 9—*Continued.*

expression of pp41/43^{FRNK} also led to increased migration (Fig. 9B). Moreover, EGF treatment further enhanced cell migration of A431 cells overexpressing pp41/43^{FRNK}. Finally, EGF-induced chemokinesis and chemotaxis of cells were examined by adding EGF to the cell culture medium or into a collagen gel, respectively. In either case, EGF promoted the invasion of A431 cells with characteristic spiky and dendrite-like morphology that penetrated and migrated below the surface of the collagen gel. A431 cells expressing pp41/43^{FRNK} showed enhanced invasion in both the absence and presence of EGF compared to parental A431 cells. (Fig. 9C and D). These data indicate that EGF-induced downregulation of FAK activity promotes the motility and invasion of tumor cells.

EGF-treated A431 cells with inhibited FAK activity are still able to form new adhesions on fibronectin which restores FAK activity. After tumor cells detach from a tumor mass or the ECM in vivo, they must be able to migrate and adhere to a new ECM to form metastatic deposits. The previous data demonstrate that EGF-induced morphological changes, cell detachment, and increased cell motility result from inactivation of FAK. Next, we investigated the ability of EGF-treated A431 cells with downregulated FAK activity to readhere. A431 cells, trypsinized and kept in suspension for 40 min, were treated for 10 min with EGF before being plated on poly-L-lysine- or fibronectin-coated plates. In contrast to untreated cells, A431 cells treated with EGF exhibited greatly delayed attachment and spreading on fibronectin-coated plates but not on poly-L-lysine-coated plates (Fig. 10A and B). While cells treated with EGF and plated on poly-L-lysine-coated plates still exhibited dephosphorylated FAK, cells treated with EGF and plated on fibronectin-coated plates for 40 min had restored FAK tyrosine phosphorylation (Fig. 10C). These data indicate that the cells with inactivated FAK become less adherent to certain ECM. However, once the processes of adhesion and spreading have started, integrin-activated signaling can rescue the function of FAK, which in turn can promote the process of adhesion and spreading. More importantly, this process cannot be blocked by EGF stimulation. The ability of EGF-treated A431 cells to adhere and spread in spite of being delayed by downregulated FAK function may help tumor cells in vivo establish metastatic deposits.

Effect of EGF autocrine regulation on FAK phosphorylation and tumor cell invasion. EGFR overexpression is often accompanied by tumor cell production of TGF-α or other EGF

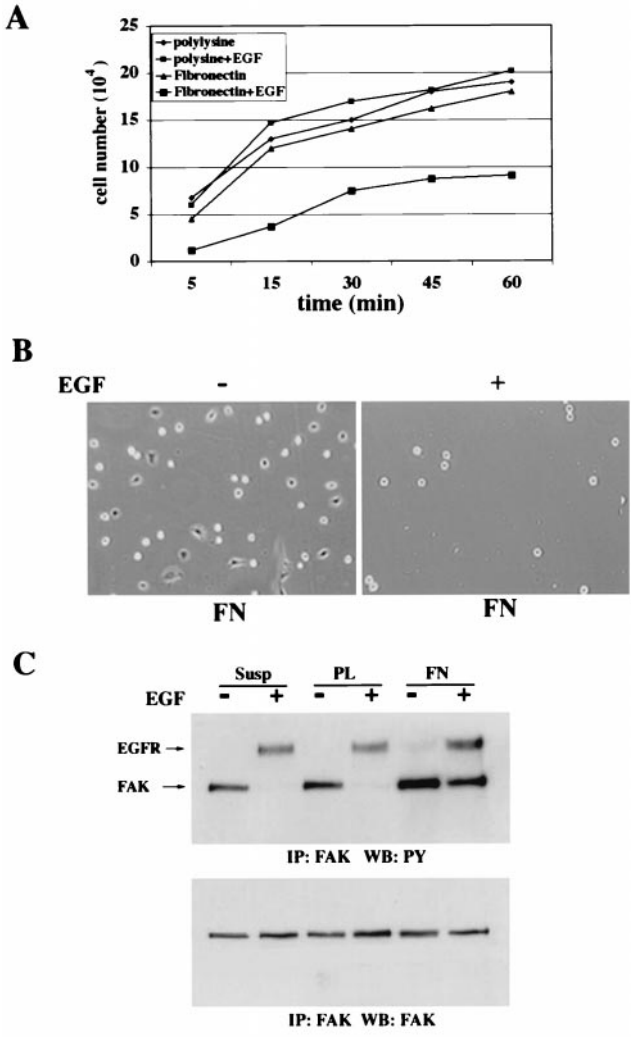

FIG. 10. EGF-treated A431 cells with inhibited FAK activity are still able to form new adhesions on fibronectin. After being trypsinized and kept in suspension (Susp) for 40 min in DMEM containing 0.1% BSA, 2.5×10^5 A431 cells were treated with EGF (100 ng/ml) or not treated for 10 min, followed by plating onto either fibronectin (FN)- or poly-L-lysine (PL)-coated plates for the indicated times. The attached cells were collected and counted (A), photographed 40 min after seeding (B), or lysed for examination of FAK tyrosine phosphorylation levels (C) as described in the legend to Fig. 1. Data represent the mean ± standard deviation of two independent experiments.

family ligands, and autocrine regulation via EGF family ligands has been implicated in tumor progression. A431 cells, breast and prostate epithelial cells, and tumor cell lines have autocrine EGFR-stimulating loops (14, 37, 43, 71). To test the effect of autocrine regulation on FAK phosphorylation, A431 cells were grown for 10 days in the absence or presence of EGF (100 ng/ml) or AG1478 (300 nM), which interrupts the autocrine regulation loop by inhibition of EGFR activation. As shown in Fig. 11A, A431 cells treated with AG1478 showed enhanced tyrosine phosphorylation of both FAK and $p130^{cas}$ in comparison to untreated cells. In the presence of AG1478, A431 cells became flatter and larger and grew in a monolayer, recapitulating the phenotypes of nontransformed cells despite the fact that their proliferation rate was not significantly changed (data not shown) (Fig. 11B). Cells treated with EGF for 10 days maintained both FAK and $p130^{cas}$ tyrosine phosphorylation at reduced levels, lost cell-cell contact inhibition, grew on top of each other, and became elongated and spindle-shaped, resembling mesenchymal cells (Fig. 11A and B). Moreover, AG1478 treatment, which leads to increased FAK tyrosine phosphorylation, blocked the invasion of A431 cells into a collagen gel, as well as the increased invasion induced by added EGF (Fig. 11C and D).

Autocrine regulation plays a role in FAK phosphorylation not only in vitro, but also in vivo. To examine this, 1.7×10^6 A431 cells were injected subcutaneously into athymic nude mice, and tumors were isolated 2 weeks later. Compared with A431 cells treated with AG1478 (300 nM) for 10 days in culture, tumor cells from the mice showed reduced FAK tyrosine phosphorylation (Fig. 11E). This result implies that EGF family ligands secreted by the tumor cells or surrounding stromal cells cause the dephosphorylation of FAK.

Inhibition of FAK by expression of pp41/43^{FRNK} increases tumor metastasis rates in vivo. Inhibition of FAK by pp41/43^{FRNK} expression in A431 cells increases cell motility and invasion into a collagen gel. To test whether tumor cells with downregulated FAK function can potentiate tumor metastasis in vivo, 1.7×10^6 A431 cells or A431 cells expressing pp41/43^{FRNK} were injected subcutaneously into athymic mice. Two weeks after injection, three of six mice injected with A431 cells expressing pp41/43^{FRNK} had died, whereas all six mice injected with A431 cells survived. To detect any possible A431 cell-derived metastases, the liver, pancreas, spleen, kidneys, lungs, brain, and femurs were isolated from each mouse, and genomic DNA was extracted from each tissue. PCR amplification was carried out with primers positioned in the most conserved areas of human Alu sequences, which represent about 5% of the human genomic DNA sequence (32). Five metastatic organs were detected from two mice injected with A431 cells expressing pp41/43^{FRNK}; both mice died within 2 weeks after injection, whereas only one metastatic organ was found in the group of mice injected with parental A431 cells (Fig. 12). Immunoblotting of EGFR immunoprecipitates with anti-human EGFR antibodies also detected variable levels of human EGFR from the metastatic organ lysates (data not shown). These data provided additional evidence supporting the notion that downregulation of FAK activity promotes tumor cell invasion and metastasis.

DISCUSSION

The majority of deaths from cancers are due not to primary tumors, but to tumor invasion and metastasis. One reason for the poor prognosis for cancer patients with tumors overexpressing EGFR is an associated invasive or metastatic phenotype (63, 76). In this study, we demonstrated that EGF induced refractile morphology changes, detachment from the ECM, and a mesenchymal phenotype in A431 human epidermoid carcinoma cells which overexpress EGFR. The appearance of the morphological changes and cell detachment correlated with tyrosine dephosphorylation and reduced kinase activity of FAK. $p130^{cas}$ and paxillin, which are also components of focal adhesions and substrates of FAK, showed a similar kinetic profile of dephosphorylation upon EGF treatment. This is consistent with FAK's having a role in the tyrosine phosphoryla-

tion of p130^{cas} and paxillin (56, 65, 69) and indicates that EGF induces a rapid downregulation of focal adhesion signaling as well as destruction of the focal adhesion complex. EGF-induced dephosphorylation of FAK was also observed in other tumor cells having aberrantly high EGFR expression. Interestingly, NIH 3T3 EGFR cells, which are transformed upon EGF treatment and become able to grow in soft agar (data not shown), exhibit greatly reduced levels of tyrosine-phosphorylated FAK and p130^{cas} even in the absence of EGF treatment, compared to normal NIH 3T3 cells. Inhibition of focal contact formation and reduced tyrosine phosphorylation level of p130^{cas} and paxillin are also observed in NIH 3T3 cells transformed by p185^{neu} (41). Therefore, an initial downregulation of FAK activity by EGFR activation is a general phenomenon existing in human tumor cells and rodent cells that have aberrant overexpression of EGFR or EGFR family members.

The activation of FAK that resulted from the activation of integrin signaling prevented EGF-induced dephosphorylation of FAK, refractile morphological changes, and cell detachment from the ECM. This indicates that the effect of integrin-activated signaling during the process of cell adhesion is dominant over the effect induced by EGF on focal adhesion and demonstrates that functional downregulation of FAK is required for EGF to induce these phenotypic changes. Consistent with this conclusion, expression of pp41/43^{FRNK}, which inhibits FAK and FAK downstream signaling, resulted in refractile morphological changes and cell detachment, indicating that downregulation of FAK activity by itself is sufficient for these changes. Both EGF treatment and overexpression of pp41/43^{FRNK} promoted the migration and invasion of A431 cells, and a synergistic effect on cell motility and invasion was observed when EGF treatment was applied with expression of pp41/43^{FRNK}. Moreover, inhibition of FAK by expression of pp41/43^{FRNK} led to increased tumor metastasis in athymic mice. This indicates that EGF-induced downregulation of FAK activity, which results in morphological changes and cell detachment, is an initial and essential event for tumor cell motility and invasion. It has been shown in a previous report that expression of pp41/43^{FRNK} has an inhibitory effect on EGF-induced migration of FAK$^{-/-}$DA2 clonal fibroblasts which have been transfected to reexpress FAK (67). This difference from our results can most likely be ascribed to cell type-specific differences in signaling between mouse fibroblasts and epidermoid tumor cells expressing abnormally high levels of EGFR. Other differences between our findings and previous reports are also likely due to cell type-specific responses. For instance, it has been reported that stable expression of pp41/43^{FRNK} did not affect the morphology of vascular smooth muscle cells and that it inhibited platelet-derived growth factor-induced ERK2 activation (24). In contrast, we observed significant effects on the morphology of the cells induced by FAK inhibition, but no inhibitory effect on EGF-induced ERK activation (Fig. 1, 7, and 8, and data not shown).

Prevention of FAK dephosphorylation by treatment with the PTP inhibitors pervanadate and phenylarsine oxide partially blocked EGF-induced refractile morphological changes and cell detachment, suggesting that the functional downregulation of FAK by a PTP is important for EGF to induce these phenotypic changes. The PTPs SHP-2, PTEN, PTP-1B, and PTP-PEST were previously shown to be directly or indirectly responsible for FAK and p130^{cas} dephosphorylation (16, 23, 44, 70). In 293 cells, FAK was dephosphorylated upon transient overexpression of either SHP-2 or PTP-PEST. However, we were unable to detect a dramatic effect on EGF-induced FAK dephosphorylation in A431 cells stably expressing either SHP-2 or PTP-PEST (data not shown). This might be due to relatively low levels of stably expressed SHP-2 or PTP-PEST or to the involvement of another PTP. PTP-mediated FAK dephosphorylation has also been observed when other types of receptor protein tyrosine kinases are activated by ligands, including the insulin-like growth factor 1 (IGF-1) receptor and EphA2 (21, 48). The activation of IGF-1 receptor led to the migration and invasion of MCF-7 human breast cancer cells, which correlated with the tyrosine dephosphorylation of FAK, p130^{cas}, and paxillin (21, 49), further implying that FAK dephosphorylation induced by growth factor stimulation is an important event during growth factor-induced tumor cell migration and invasion.

The inhibitory effect of the EGFR inhibitor AG1478 and the graded response of cells to different doses of EGF show that the magnitude of morphological changes and cell detachment is dependent on the extent of FAK dephosphorylation, which in turn depends on the magnitude of EGFR activation. Our finding that EGFR was associated with FAK is consistent with a recent report (67). In that study it was further shown that the isolated FAK N-terminal domain associates with EGFR in 293T cells, suggesting that the FAK N-terminal domain is important for mediating interactions with EGFR (67). The authors also found that FAK association with EGFR was only detected upon EGFR activation. While we believe that the preassociation of unstimulated EGFR with FAK that we observed in EGFR-overexpressing cells is constitutive, we cannot exclude the possibility that it is due to a low level of constitutive kinase activity caused by the abnormally high level of expression of EGFR. Importantly, we do not know whether FAK association with EGFR is needed for EGF-induced FAK dephosphorylation. Growth factor stimulation of some cell types or attenuation of FAK function can elicit cell detachment and apoptosis (3, 30, 79). Consistent with this, we also observed that A431 cells had a slower growth rate in the presence of high concentrations than of low concentrations of EGF (data not shown). However, a high concentration of EGF did not have any inhibitory effect on A431 tumors in athymic mice (17), suggesting that EGF-induced three-dimensional cell-cell or cell-ECM interactions in vivo are also important regulators of tumor cell proliferation.

Growth factor-induced cell motility represents a cellular behavior distinct from adhesion receptor-induced motility. The former mode of motility represents chemokinesis and chemotaxis, while the latter is referred to as haptokinesis and haptotaxis (75). Normal epithelial cells with functional adhesion receptors and FAK move as a coherent sheet, in which each cell keeps contact with its neighboring cells as well as the ECM (13). Integrin receptor engagement leads to FAK activation and enhanced phosphorylation of FAK Tyr-397, which provides a binding site for Src and PI-3 kinase. FAK, c-Src, and PI-3 kinase act in a coordinated manner to activate ERK2, which combines with other activated downstream signaling molecules to provide survival signals and enhance cell spreading and migration. Inhibition of FAK, either through overex-

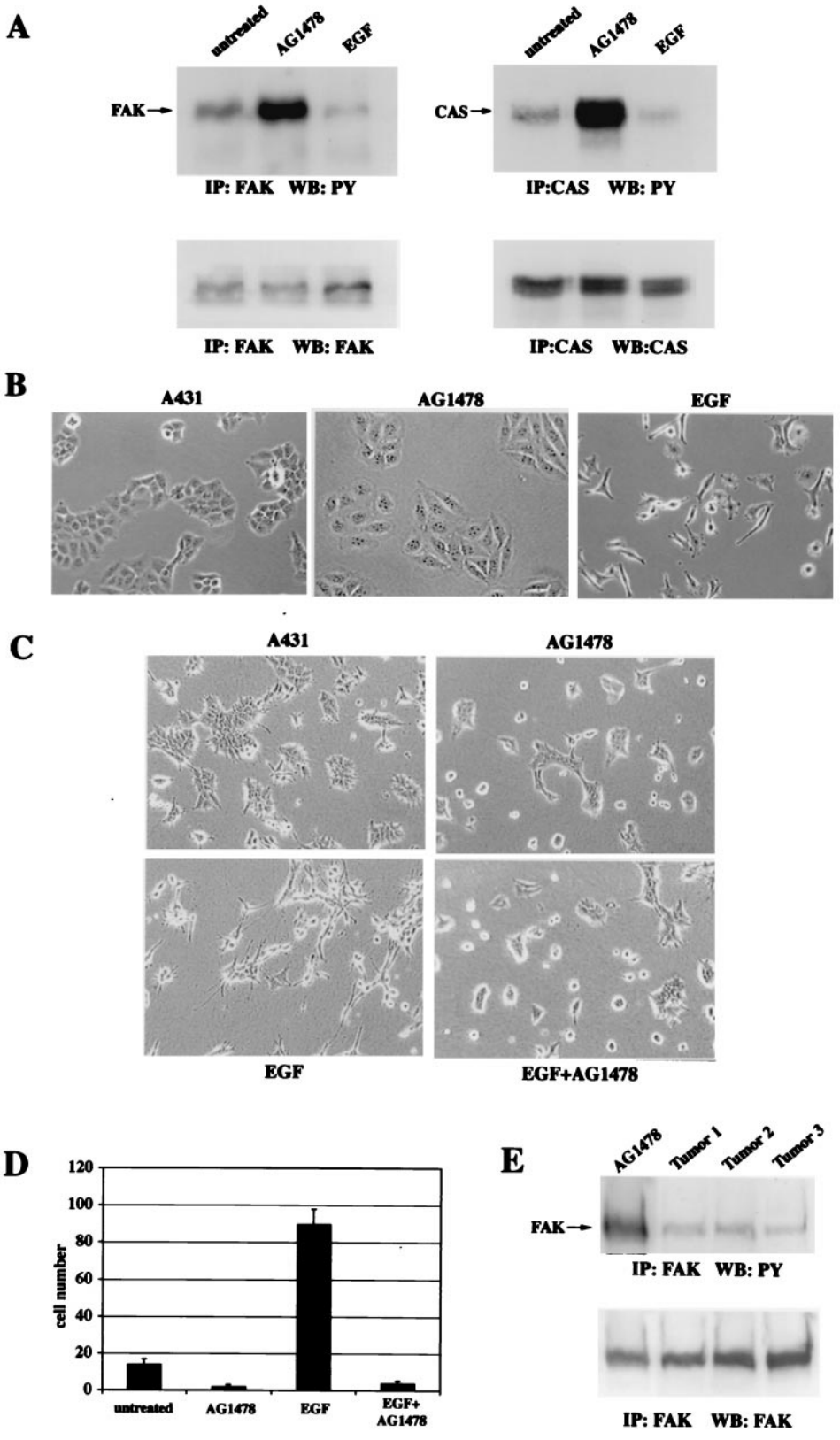

FIG. 11. Effect of EGFR autocrine regulation on FAK phosphorylation. A431 cells were grown in the absence or presence of EGF (100 ng/ml) or AG1478 (300 nM) for 10 days. The tyrosine phosphorylation levels of FAK and p130^{cas} (A) and the morphology of cells (B) were examined as described in the legend to Fig. 1. (C) A431 cells in the presence of EGF (100 ng/ml) and/or AG1478 (300 nM) were plated on the top of collagen gel. The cells were photographed 2 days later for invasion on the gel surface. Five days after plating, the number of invading cells that have penetrated the gel in 10 photographic fields from two separate experiments was counted (D). (E) A total of 1.7×10^6 A431 cells were injected

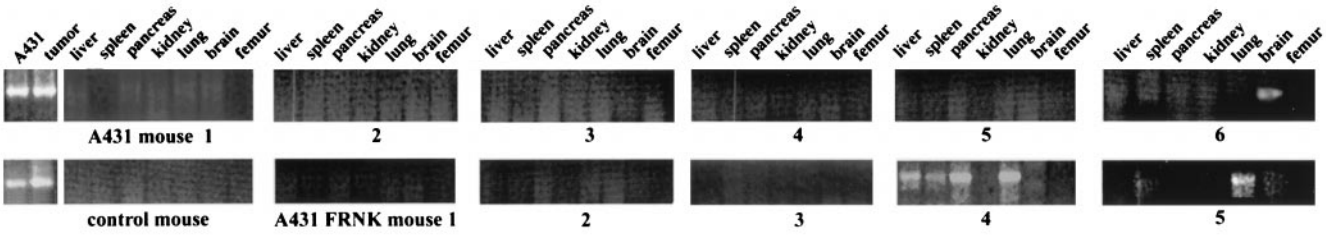

FIG. 12. Inhibition of FAK by expression of pp41/43^{FRNK} increases tumor metastasis rates in vivo. A total of 1.7×10^6 A431 cells or A431 cells expressing pp41/43^{FRNK} were injected subcutaneously into the flank region of athymic mice. Tumor, liver, pancreas, spleen, kidneys, lungs, brain, and femurs were isolated from each mouse, and genomic DNA from A431 cells (control), tumors (control), and each organ was examined. PCR amplification was carried out as described in Materials and Methods with primers positioned in the most conserved areas of human Alu sequences. The upper panel shows PCR amplification from mice injected with A431 cells, and the lower panel shows products from a control mouse or mice injected with A431 cells expressing pp41/43^{FRNK}, as indicated.

pression of FRNK, by antisense, or with antibodies, results in inhibited migration and increased apoptosis of cultured cells (52, 65, 66). The ability to move individually appears to be an exclusive attribute of carcinoma cells. We have shown here that EGF-induced inactivation of FAK results in cell detachment from the ECM, involving a disruption of cell-ECM contacts and cell-cell contacts. EGF concomitantly provides the signals for cell survival and migration through FAK activity-independent activation of Src, ERK, mitogen-activated protein kinase, and PI-3 kinase (2, 29). These events induce a disruption of the polarized cell monolayer and a morphological transition towards a mesenchymal phenotype (shown after 24 h of treatment with EGF), which is considered a marker of invasiveness (5). This subset of cells must loosen their attachments to the primary tumor mass to create a leading edge free of cell-cell constraints, recognize the surrounding stroma or matrix, and then actively migrate into and /or through that space. The process of cell migration requires proteolytic degradation, as has been shown in EGF-induced motility and invasion in human breast cancer cells, or at least minimal degradation, as has been shown in TGF-α–EGFR autocrine loop-promoted prostate carcinoma cell invasion, in which the cells may migrate along tissue planes and through pores (15, 50, 75, 78).

Other data also support the roles of focal adhesion disassembly in cell movement. EGF-enhanced cell motility correlates with focal adhesion disassembly (77). FAK$^{-/-}$ fibroblasts, which proliferate normally, surprisingly have abnormally high numbers of focal contacts, enhanced adhesion to the ECM, and reduced motility. Normal to elevated tyrosine phosphorylation of p130^{cas} and paxillin is found in FAK$^{-/-}$ cells (28, 74), and this may contribute to enhanced cell adhesion. Another possible reason for the enhanced adhesion of FAK$^{-/-}$ cells is that these cells overexpress the FAK-related tyrosine kinase Pyk2. The reexpression of FAK in FAK$^{-/-}$ cells markedly inhibits adhesion-dependent Pyk2 tyrosine phosphorylation without altering the level of Pyk2 (53), providing additional support that cells expressing high levels of Pyk2 could be compensating, at least partially, for the loss of FAK in cell adhesion. In A431 cells, Pyk2 tyrosine phosphorylation was not increased upon EGF treatment (data not shown), though FAK became dephosphorylated. A recent report showed that FAK$^{-/-}$ cells migrate slower upon EGF stimulation than FAK$^{+/+}$ fibroblasts, while stable reexpression of FAK in FAK$^{-/-}$ cells enhances EGF-induced migration (67, 68). These data also imply that a high number of focal contacts in FAK$^{-/-}$ cells might be the reason for slower migration upon EGF treatment, while enhanced migration by FAK reexpression is possibly due to a decrease in the number of focal contacts. That functional downregulation of FAK is required for initiation of migration was further supported by the fact that ectopic expression of constitutively active forms of FAK, paxillin, or α5 or β1 integrin inhibits myoblast migration (27) and a polymeric form of fibronectin inhibits tumor metastasis in vivo (55).

Contradictory roles for FAK in cell motility have been proposed in a number of reports. Enhanced cell migration has been observed in CHO cells overexpressing FAK (8, 9), and elevated FAK expression has been linked to the increased invasive potential of human tumors (54). However, PTP-PEST-null fibroblasts (1) and cells containing an N-terminally truncated form of SHP-2 PTP (80) or overexpressing a dominant-negative mutant of SHP-2 PTP (45) all exhibited elevated tyrosine phosphorylation of FAK and p130^{cas}, a larger number of focal adhesion contacts, and reduced migration abilities. One explanation for this complexity is that FAK function is dynamically regulated by dephosphorylation and phosphorylation during cellular locomotion. Our data, which support this hypothesis, indicate that EGF-induced inactivation of FAK by dephosphorylation results in loose cell attachment, mesenchymal phenotypic changes, and initiation of cell motility. Though it delayed the reattachment of cells to fibronectin-coated plates, FAK phosphorylation was rapidly restored once integrin binding to extracellular ligands occurred upon reattachment. More importantly, the process of cell readhesion, which involves integrin-activated signaling, was not interrupted by high concentrations of EGF, even though the EGFR was still competent to stimulate signaling events such as ERK activation.

A431 cells, breast and prostate epithelial cells, and tumor cell lines have autocrine EGF receptor-stimulating loops (14, 37, 43, 71). There is growing evidence that much of the induced

subcutaneously into the flank region of nude mice. Tumors were isolated and homogenized in lysis buffer 2 weeks after injection. As a control, cultured A431 cells were treated with AG1478 (300 nM) for 10 days before lysis. Cell lysates which contained equal amounts of human EGFR as determined by Western blot analysis with anti-EGFR antibody (data not shown) were used for immunoprecipitation. The tyrosine phosphorylation levels of FAK were examined as described in the legend to Fig. 1.

cell motility in tumor cells is due to autocrine signals (31, 46, 72). A431 cells growing in the presence of an EGFR inhibitor, which interrupts TGF-α–EGFR autocrine regulation by inhibition of EGFR activation, have the same phenotype as nontransformed cells. Moreover, the cells have increased focal adhesions which accompany increased FAK expression (51) and increased levels of tyrosine-phosphorylated FAK and p130^{cas}, but decreased cell invasion. Consistent with this, tumor cells grown in athymic mice exhibit reduced FAK tyrosine phosphorylation. Therefore, a mechanism for metastasis of tumor cells having high levels of EGFR might be that the autocrine growth factors functioning as chemokinetic signals stimulate dephosphorylation of FAK, which results in cell detachment from the tumor mass or ECM. The detached cells, which have reduced focal contacts and become less adherent, migrate to a new site, followed by reactivation of FAK and reattachment to the ECM, and then form metastatic deposits. This hypothesis is further supported by a recent report showing that among six related melanoma lines isolated from different metastases from the same patient, FAK expression was absent only from the line derived from peripheral blood, which grew mostly in suspension, while the cell lines derived from a few attached cells all expressed FAK (47).

In summary, we have demonstrated that FAK activity was functionally downregulated by dephosphorylation upon EGF stimulation in a variety of tumor cell lines overexpressing EGFR. Functional downregulation of FAK was essential and sufficient for EGF-induced cell morphological changes, cell detachment from the ECM, and increased cell motility, invasion, and metastasis. Tumor cells with inactivated FAK became less adherent to the ECM. However, once cells started reattaching, FAK activity was reversed by an involvement of activated integrin signaling. Therefore, downregulation of FAK activity might be essential and required for early metastatic spreading, enabling vascular circulation of tumor cells without adhesion. Once tumor cells reattach to the ECM, integrin stimulation of FAK promotes adhesion and the growth of a metastatic tumor.

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