Specific Cross-talk between Epidermal Growth Factor Receptor and Integrin $\alpha_v\beta_5$ Promotes Carcinoma Cell Invasion and Metastasis

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Abstract

Tyrosine kinase receptors and integrins play essential roles in tumor cell invasion and metastasis. Previously, we showed that epidermal growth factor (EGF) stimulation of pancreatic carcinoma cells led to invasion and metastasis that was blocked by antagonists of integrin $\alpha_v\beta_5$. Here, we show that EGF stimulates metastasis of carcinoma cells via a Src-dependent phosphorylation of p130 CAS leading to activation of Rap1, a small GTPase involved in integrin activation. Specifically, EGF receptor (EGFR)–induced Src activity leads to phosphorylation of a region within the CAS substrate domain, which is essential for Rap1 and $\alpha_v\beta_5$ activation. This pathway induces $\alpha_v\beta_5$-mediated invasion and metastasis *in vivo* yet does not influence primary tumor growth or activation of other integrins on these cells. These findings show cross-talk between a tyrosine kinase receptor and an integrin involved in carcinoma cell invasion and metastasis and may explain in part how inhibitors of EGFR affect malignant disease. [Cancer Res 2009;69(4):1383–91]

Introduction

Epidermal growth factor receptor (EGFR) signaling is important in normal epithelial developmental biology as well as for tumor cell proliferation, motility, survival, and metastasis (1). Dysregulation of EGFR signaling, including receptor overexpression and/or activation, is a significant factor in the progression of human cancers, including neoplasms of the brain, lung, breast, ovary, prostate, and pancreas (2). Function blocking antibodies and tyrosine kinase inhibitors targeting EGFRs have proven somewhat effective in various cancers (3). Although EGFR has been linked to increased tumor growth and invasion, its direct influence on the growth and malignant properties of tumors remains poorly understood.

EGFR stimulation activates Src family kinases (SFK), which mediate a variety of intracellular signaling pathways and are overexpressed or hyperactivated in some cancers (4). Activated Src kinase is involved in the rearrangement of the actin cytoskeleton, cell-matrix interactions, and cell-cell adhesions, processes that promote cell invasion implicating Src activity in tumor progression. The role of SFKs in fibronectin-dependent cellular motility has been well established in fibroblasts (5); however, their role in carcinoma cell migration has not been well defined. Pharmacologic SFK inhibitors decrease pancreatic carcinoma invasiveness *in vitro* (6) and show significant antiproliferative and antimetastatic activity in human xenograft models *in vivo* (7). Dasatinib (Sprycel) has recently received regulatory approval for the treatment of imatinib-resistant chronic myelogenous leukemia and is currently being evaluated in clinical trials for solid tumors (8, 9).

Two major substrates of activated Src kinase that influence cell migration are focal adhesion kinase (FAK) and Crk-associated substrate (p130CAS or CAS). FAK promotes the assembly of multiprotein complexes required for the turnover of focal contacts facilitating integrin-mediated cell migration and invasion (10, 11). On phosphorylation, CAS recruits Crk and DOCK180, which coordinate small GTPase activity required for cell migration and invasion (12). Src-dependent phosphorylation of CAS also confers invasive growth potential to transformed cells (13). Phosphorylation of the substrate domain (SD) of CAS is important for activation of the small GTPase Rap1 (14, 15) and invasive behavior *in vivo* but not for tumor growth (16).

Cell adhesion to the extracellular matrix (ECM) promotes an integrin-dependent association between Src and FAK (17). In addition to playing an adhesive role, integrins mediate cytosolic signaling events that affect cell proliferation, survival, and motility (18). Activation of integrins has been implicated in many pathologic processes, including tumor initiation and growth, angiogenesis, and metastasis (19). Furthermore, integrin-mediated adhesion can enhance signaling pathways by direct phosphorylation of growth factor receptors (20).

In this study, we have identified a signaling pathway leading to the spontaneous metastasis of human pancreatic carcinoma that does not affect primary tumor growth. We provide evidence for two distinct pathways of tumor cell migration that differ based on their dependence on EGF-mediated Src kinase activity and activation of integrin $\alpha_v\beta_5$. On matrix proteins such as fibronectin or collagen, cell migration is mediated by β_1 integrins and does not require EGF or Src kinase. In contrast, EGF and Src activity are required to promote phosphorylation of specific tyrosines in the SD of CAS leading to activation of Rap1 during integrin β_5-mediated cell invasion and metastasis of pancreatic carcinoma cells without influencing primary tumor growth. Thus, the EGFR/Src/β_5-dependent pathway seems to contribute to the metastatic properties of pancreatic cancer.

Materials and Methods

Antibodies and inhibitors. Antibodies were purchased from Santa Cruz Biotechnology (FAK C-20 and CSK C-20), Invitrogen (FAKpY861 and rhodamine-phalloidin), Cell Signaling Technology (phospho-extracellular signal-regulated kinase 1/2, Src pY416, pCAS Y165, pCAS Y249, and pCAS Y410), Millipore (Src GD11, Rap1, and Rac1), Sigma (β-actin), and BD Transduction Labs (Yes, CAS, and Rho). LM142, P4C10, and P1F6 antibodies

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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were prepared as described (21). The Src inhibitor SKI-606 (22) was used at 500 nmol/L. The FAK inhibitor PF228 (23) was used at 1 μmol/L.

CAS mutants. cDNA was amplified from pRc/CMV-CASmyc templates (12) using primers containing *Eco*R1 and *Bam*H1 sites surrounding the region encoding the full-length CAS protein. Mutated cDNAs were subcloned into pEGFP-C1 vector with *Eco*R1 and *Bam*H1 restriction enzymes and ligated using Rapid DNA Ligation kit (Roche). All cDNAs with YXXP mutations in pEGFR-C1 were sequenced to verify final plasmid constructs.

Cell culture. *Mycoplasma*-negative FG human pancreatic carcinoma cells (24) were grown in DMEM (Life Technologies) with 10% fetal bovine serum. For some experiments, subconfluent cells were transfected with SrcA (Y527F) in pcDNA3.1 or CAS mutations in pEGFP-C1 using the Amaxa Nucleofector I (Amaxa), cells were selected, and single-cell clones were isolated, propagated, and screened. FG cells containing CAS mutations were sorted for green fluorescent protein expression, and CAS expression was verified by immunoblotting. Cells expressing recombinant adenovirus for wild-type (WT) and kinase-dead (KD) CSK were created as described (25).

Short hairpin RNA knockdown. Integrin β_5 and nonsilencing lentiviral shRNAmir in pLKO.1 expressing system were from Open Biosystems. Lentiviruses were produced in 293FT cells using Fugene transfection. Cells were selected 48 h after infection with 1 μg/mL puromycin, and low-expressing cells were further selected by flow cytometry. For Rap1 knockdown, FG cells were transfected with a pool of four Rap1b small interfering RNAs (Qiagen) for 24 h and serum starved overnight, and migration assays were performed at 48 h after transfection.

Protein analysis. Cells were serum starved for 24 h, pretreated with inhibitors, and stimulated with EGF (50 ng/mL). Immunoprecipitation, immunoblotting, and immunofluorescence were performed as previously described (24, 26). Images were captured using a TE200E Nikon C1Si spectral confocal microscope. Cells were analyzed with a FACScan II flow cytometer (Becton Dickinson) and analysis was gated on forward and size scatter intensities, with results presented as single-variable histograms.

Cell migration. Migration assays were performed as described (24).

Immunofluorescence and microscopy. Cells were fixed in 2% paraformaldehyde, permeabilized, and incubated with 2 μg/mL rhodamine-phalloidin. Images were captured using a TE200E Nikon C1S spectral confocal microscope.

Chick embryo metastasis. The chick embryo metastasis assay was performed as described (27). Pulmonary metastasis was quantified by quantitative detection of the human *Alu* sequence present in chick lung DNA extracts normalized to chick glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using real-time quantitative PCR as described previously (28) with modifications: *Alu* probe-AGACCAGCCTGGGCAACATAGTGAAA, 5′BHQ1a-5TET; GAPDH probe-AGATGCTCTGCGGGAAAGCAGTGAAT, 5′BHQ1a-6FAM3′. A standard curve was generated through quantitative amplification of genomic DNA extracted from chick lung homogenates containing a serial dilution of FG cells and relative changes in metastasis were reported.

Small GTPase activation. For Fig. 1, FG cells were serum starved, trypsinized, plated on dishes coated with 10 μg/mL P1F6 or P4C10 antibody, and allowed to adhere for 15 min. For Fig. 5, cells were grown for 3 d and serum starved overnight before stimulation with 50 ng/mL EGF for 1 min. Rac1-GTP, Rho-GTP, and Rap1-GTP pull-down assays were performed according to the manufacturer's instructions (Millipore).

Statistics. Unless stated otherwise, bar graphs represent mean ± SD of triplicate samples. All data presented are representative of at least two experiments. *P* values were generated by two-tailed *t* test (equal variance).

Results

EGF stimulation leads to activation of Rap1 and integrin $\alpha_v\beta_5$-mediated metastasis. EGF stimulation induces $\alpha_v\beta_5$-mediated carcinoma cell migration on vitronectin, whereas cells migrate robustly on integrin β_1-mediated substrates, such as fibronectin or collagen, in an EGF-independent manner (Supplementary Fig. S1*A*

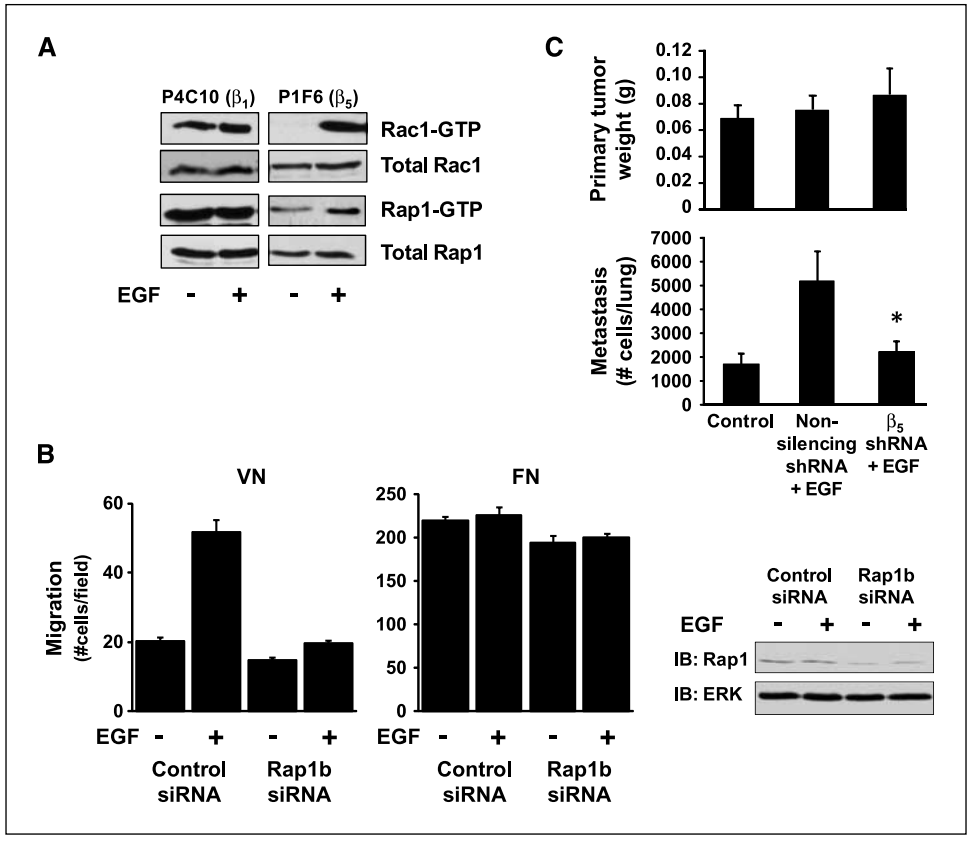

Figure 1. EGF induces $\alpha_v\beta_5$-mediated Rap1 activation and cell metastasis. *A,* EGF treatment increased activity of Rac1 and Rap1 in FG cells plated on anti-β_5, but not anti-β_1, integrin antibodies. *B,* Rap1 knockdown blocked EGF-induced cell migration on vitronectin but not fibronectin. *C,* knockdown of β_5 expression blocked EGF-induced pulmonary metastasis, but not primary tumor weight, in the chick CAM model. *Columns,* mean ($n \geq 6$); *bars,* SE. *, $P \leq 0.05$.

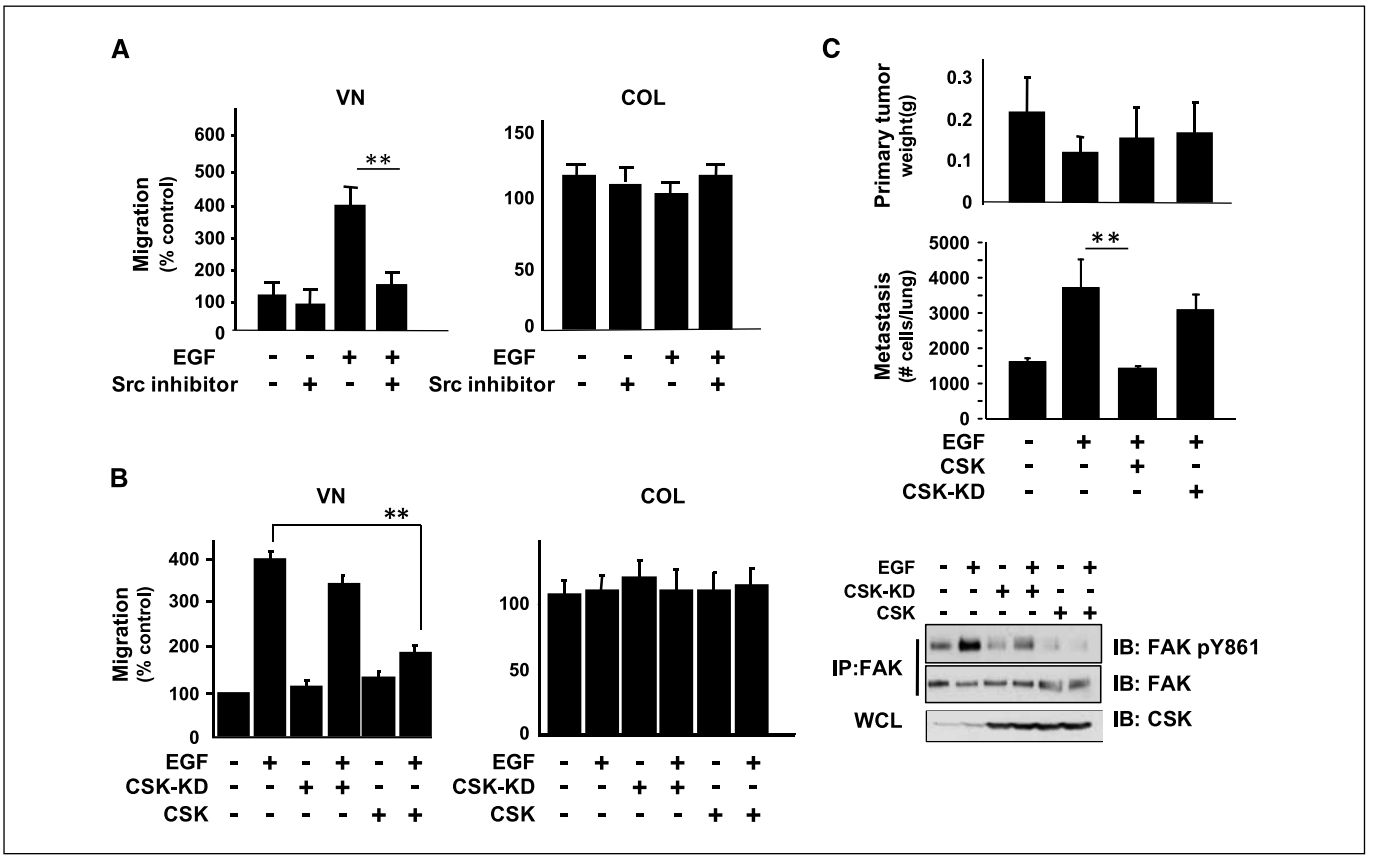

Figure 2. Src kinase is necessary for EGF-induced β_5-mediated migration and metastasis. *A,* pretreatment with a Src inhibitor (SKI-606, 500 nmol/L) blocked EGF-induced migration on vitronectin (*VN*). *B,* expression of CSK, but not CSK-KD, blocked EGF-induced migration on vitronectin. Blockade of Src kinase activity and expression of CSK and CSK-KD were confirmed by immunoblotting. **, $P \leq 0.01$. *C,* expression of CSK blocked EGF-induced pulmonary metastasis, but not primary tumor growth, in the chick CAM model. *Columns,* mean ($n \geq 6$); *bars,* SE. **, $P \leq 0.01$.

and *B*; ref. 29). Because small GTPases regulate cytoskeletal rearrangements and cell migration (30–32), their activity was measured for FG cells attached to immobilized anti-integrin $\alpha_v\beta_5$ or β_1. Adhesion to anti-β_1 led to Rac1 and Rap1 activity independent of EGF stimulation, whereas cells attached to anti-$\alpha_v\beta_5$ showed robust EGF-dependent activation of Rac1 or Rap1 (Fig. 1*A*). Knockdown of Rap1b expression in FG cells selectively blocked EGF-induced migration on vitronectin (Fig. 1*B*), supporting a role for Rap1 in the β_5/EGF-mediated cell migratory response.

Previous studies have implicated β_1 integrins in cancer because they regulate cell adhesion and migration/invasion on tumor stroma proteins, such as fibronectin, laminin, and collagen (33). Because $\alpha_v\beta_5$ requires activation to promote cell migration, we considered whether EGF and integrin $\alpha_v\beta_5$ could coordinately influence the spontaneous metastasis of FG cells *in vivo*. FG cells stimulated with EGF were implanted on the CAM of 10-day-old chick embryos. Although EGF stimulation had no effect on primary tumor growth, it increased pulmonary metastasis 3-fold, which was abolished by short hairpin RNA (shRNA)–mediated knockdown of integrin β_5 (Fig. 1*C*). Importantly, knockdown of β_5 did not influence β_1 integrin expression (Supplementary Fig. S1*C*) or primary tumor growth (Fig. 1*C*), indicating that EGF and $\alpha_v\beta_5$ coordinately and specifically regulate the spontaneous metastasis of FG cells in this model.

Src activation downstream of EGFR is required for $\alpha_v\beta_5$-mediated carcinoma cell invasion and metastasis. EGFR ligation and Src activation have been linked to the growth and malignant properties of many tumors (2, 4). To assess whether EGF-mediated migration was Src dependent, FG cells were treated with a Src kinase inhibitor (SKI-606) before EGF stimulation. SKI-606 suppressed EGF-induced Src phosphorylation in FG cells (Supplementary Fig. S2*A*) and blocked EGF-mediated migration on vitronectin yet had no effect on EGF-independent migration on collagen (Fig. 2*A*). To confirm a role for endogenous Src in EGF-induced migration, FG cells were transduced to express COOH-terminal Src kinase (CSK), an inactivator of SFKs (34). As expected, expression of CSK (but not KD CSK) suppressed EGF-induced Src activation (Fig. 2*B*) and cell migration on vitronectin (Fig. 2*B*). Importantly, migration on collagen was not influenced by EGF treatment or CSK expression (Fig. 2*B*). These findings suggest that FG cells migrate via two distinct mechanisms, which differ by their requirement for EGF/Src stimulation.

To determine whether disruption of endogenous Src kinase activity by CSK can influence metastasis, FG cells expressing CSK or CSK-KD were allowed to form primary tumors and metastasize to the lungs in chick embryos. Although EGF stimulation had no effect on primary tumor growth, it enhanced the spontaneous pulmonary metastasis of these cells by >2-fold, although this was completely disrupted in cells expressing CSK (Fig. 2*C*). These findings suggest that spontaneous metastasis and $\alpha_v\beta_5$-mediated migration can be activated by EGF and suppressed by Src inhibition.

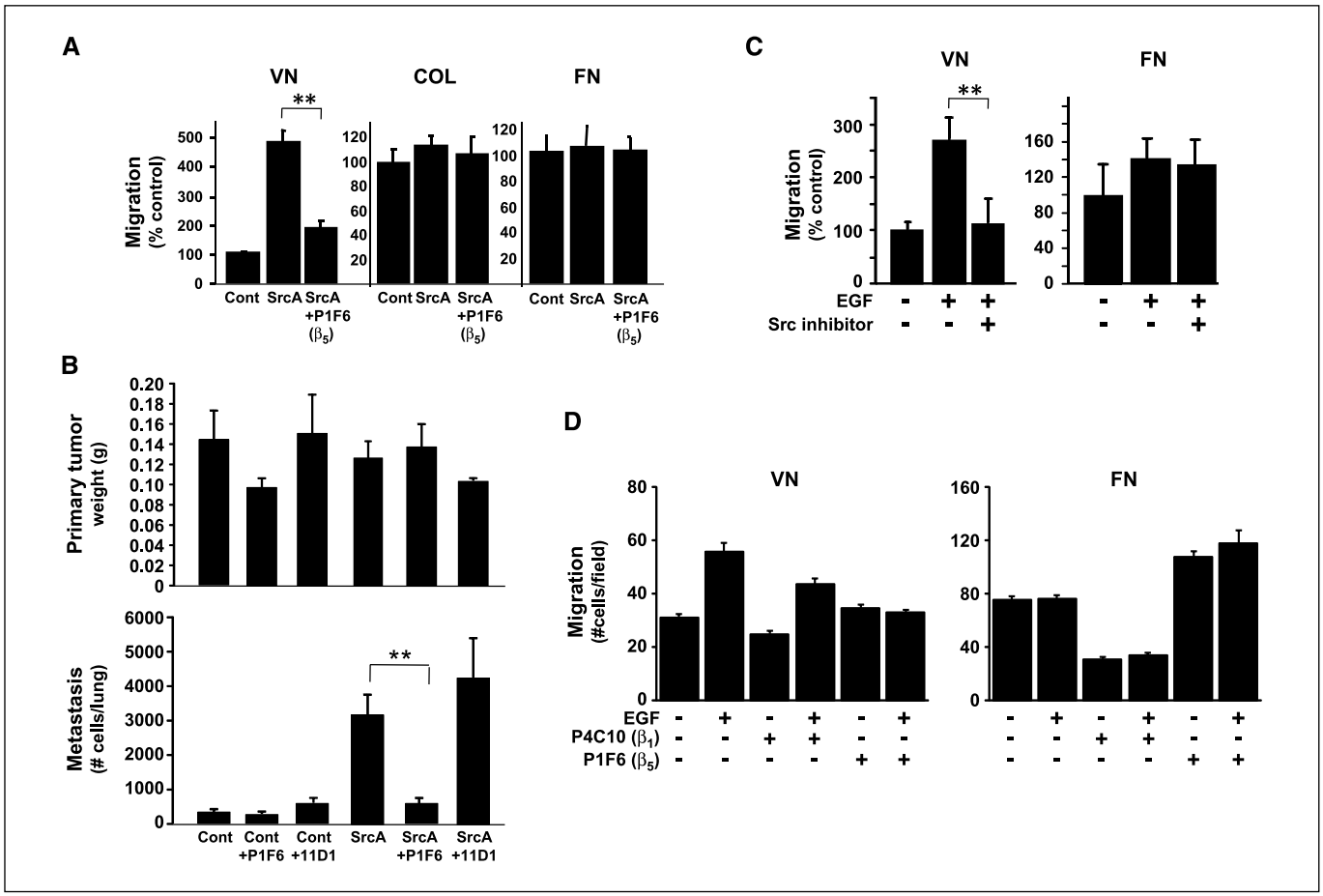

Figure 3. Src kinase is sufficient for β_5-mediated migration and metastasis. *A,* expression of active Src (*SrcA*) selectively induced migration on vitronectin, which could be blocked with anti-$\alpha_v\beta_5$. *FN,* fibronectin. *B,* i.v. injection with 100 μg of a function-blocking antibody to β_5 (P1F6), but not a nonfunction-blocking β_5 antibody (11D1), blocked pulmonary metastasis but not primary tumor growth in the chick CAM model. *Columns,* mean ($n \geq 6$); *bars,* SE. **, $P \leq 0.01$. *C,* pretreatment with a Src inhibitor (SKI-606, 500 nmol/L) blocked EGF-induced migration of MCF-7 breast carcinoma cells on vitronectin. *D,* EGF-induced migration of MCF-7 cells on vitronectin was blocked by treatment with an anti-β_5 function-blocking antibody.

Src is sufficient to induce $\alpha_v\beta_5$-mediated tumor cell metastasis. To determine whether activated Src is sufficient to account for $\alpha_v\beta_5$-mediated invasion, FG cells stably expressing mutationally active Src Y527F (SrcA) were evaluated for invasion *in vitro* and *in vivo*. FG cells expressing SrcA in the absence of EGF, or WT FG cells stimulated with EGF, showed robust Src activation, which was completely abolished by treatment with the Src kinase inhibitor SKI-606 (Supplementary Fig. S3). Importantly, FG cells

Table 1. Carcinoma cells of distinct histologic origin were analyzed for the role of Src in $\alpha_v\beta_5$-mediated migration

Carcinoma cell lines (origin)	Fluorescence-activated cell sorting analysis of integrin β subunits			Requirement of Src kinase for $\alpha_v\beta_5$-mediated migration (vitronectin)	Requirement of Src kinase for β_1-mediated migration (fibronectin)
	β_1	β_3	β_5		
FG (pancreatic)	+++++	−	++	+++	−
Mia PaCa-2 (pancreatic)	+++++	−/+	++	+++	−
2008 (ovarian)	+++++	−	++	+++	−
MCF-7 (breast)	+++++	−	+++	+++	−
HT-29 (colon)	+++++	−	+++	+++	−
Panc-1 (pancreatic)	+++++	+++	+++	+	−
MDA-MB-435 (breast)	+++++	+++	++	−	−

NOTE: Fluorescence-activated cell sorting analysis was used to determine $\alpha_v\beta_5$, β_3, or β_1 integrin expression on the cell surface. Requirement of Src kinase in cell migration was determined by stimulating cells with or without EGF (50 ng/mL) and/or pretreated with Src inhibitor (SKI-606, 500 nmol/L). Cells were allowed to migrate toward vitronectin or fibronectin.

expressing SrcA showed spontaneous migration on vitronectin, a process that was completely blocked by anti-$\alpha_v\beta_5$ antibody (Fig. 3*A*) but not with anti-β_1 integrin antibody (data not shown). Furthermore, SrcA expression did not significantly influence FG migration on collagen or fibronectin (Fig. 3*A*), consistent with our findings that cell migration on these β_1-integrin substrates is independent of Src kinase activity (Fig. 2). Together, these results suggest that Src activation is sufficient to trigger $\alpha_v\beta_5$-dependent cell migration.

We next tested whether activated Src was sufficient to induce spontaneous metastasis of FG cells. FG cells or those expressing SrcA were implanted onto the CAM of 10-day-old chick embryos and allowed to form primary tumors and spontaneous pulmonary metastases. Although increased Src kinase activity did not influence primary tumor growth (Fig. 3*B, top*), it was sufficient to induce pulmonary metastases (Fig. 3*B, bottom*). To evaluate the role of $\alpha_v\beta_5$ in this process, tumor-bearing animals were injected systemically with a function-blocking (P1F6) or nonfunction-blocking (11D1) antibody directed to integrin $\alpha_v\beta_5$. Blockade of integrin $\alpha_v\beta_5$ function completely inhibited SrcA-induced metastasis to control levels (Fig. 3*B, bottom*). Importantly, increased Src activity or blockade of $\alpha_v\beta_5$ function did not influence primary tumor growth (Fig. 3*B, top*). These results reveal that activation of Src kinase in combination with $\alpha_v\beta_5$ is necessary and sufficient for spontaneous metastasis.

Because EGFR activation of Src initiates $\alpha_v\beta_5$-mediated migration of FG pancreatic carcinoma cells *in vitro* and *in vivo*, we asked whether carcinomas of distinct histologic origin would migrate on vitronectin in response to activation of β_5 integrin, EGF, and Src. As observed for FG cells, MCF-7 breast carcinoma cells (Fig. 3*C*) as well as other tumor cell lines that express $\alpha_v\beta_5$ as their primary vitronectin receptor (Table 1) showed an EGF-inducible, Src-mediated cell migration response selectively on vitronectin. Furthermore, as observed for FG cells (Supplementary Fig. S1*B*), EGF-induced migration of MCF-7 breast carcinoma cells on vitronectin selectively required β_5 integrin function (Fig. 3*D*). Together, these results suggest a role for the Src/β_5 signaling module in multiple carcinomas.

EGF-induced, $\alpha_v\beta_5$-dependent migration *in vitro* and metastasis *in vivo* require Src-mediated phosphorylation of a specific region within the SD of CAS. To investigate the mechanism by which EGF-mediated Src activity leads to increased metastasis, lysates from FG cells stimulated with EGF in the presence or absence of a Src inhibitor were probed with anti-phosphotyrosine to identify relevant Src substrates. As expected, EGF stimulation led to phosphorylation of EGFR at ~175 kDa, as well as other proteins (Fig. 4*A*). Following Src inhibition, a number of these proteins showed a marked decrease in tyrosine phosphorylation, most prominently a cluster of phosphorylated bands in the range of 120 to 140 kDa (Fig. 4*A, arrow*). Previous studies have suggested FAK and CAS as major phosphoproteins in this size range for EGF-stimulated FG cells (24).

Src activity contributes to phosphorylation of FAK at Y576, Y577, Y861, and Y925, whereas Y397 is a FAK autophosphorylation site (35). In FG cells stimulated with EGF, phosphorylation of FAK Y397 was not increased above background levels (Fig. 4*B*). Pharmacologic blockade of FAK reduced autophosphorylation of Y397 and EGF-induced phosphorylation of Y861, and disrupted EGF-induced $\alpha_v\beta_5$-mediated migration on vitronectin as well as β_1-mediated migration on fibronectin. These findings indicate that FAK plays a generic role in cell migration on multiple substrates. Next, we

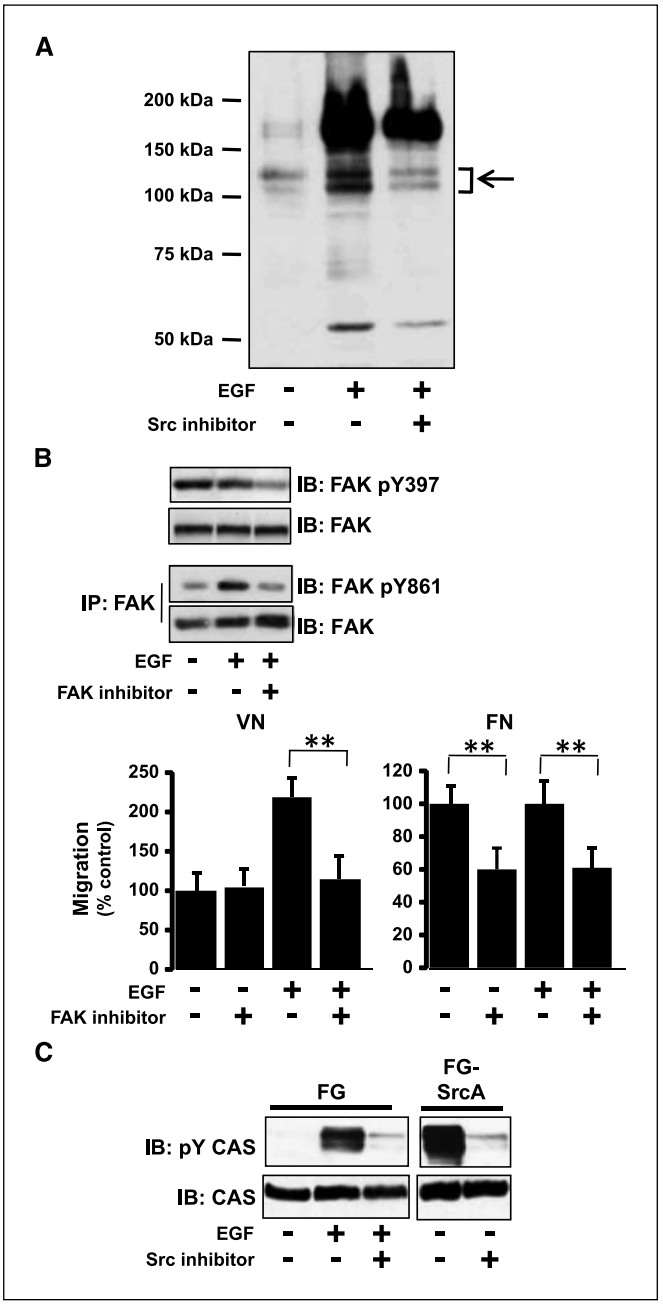

Figure 4. FAK and CAS are substrates of Src in FG pancreatic carcinoma cells. *A, arrow,* Src inhibition (SKI-606, 500 nmol/L) blocked EGF-induced tyrosine phosphorylation of 120- to 140-kDa proteins. *B,* treatment with a FAK inhibitor (PF228, 1 mmol/L) blocked FAK autophosphorylation on Y397, EGF-induced FAK pY861, and EGF-induced migration on vitronectin and fibronectin. **, $P \leq 0.01$. *C,* the Src inhibitor (SKI-606, 500 nmol/L) blocked EGF-induced CAS phosphorylation in FG cells, and constitutive CAS activity in FG cells expressing active Src.

considered whether EGF stimulation could lead to Src-dependent phosphorylation of p130 CAS. As measured by antibodies that specifically detect phosphotyrosines in the CAS SD (36), EGF stimulation promoted phosphorylation of CAS SD, which was blocked by Src inhibition (Fig. 4*C*). In the absence of growth factor stimulation, FG cells expressing constitutively active SrcA exhibited increased CAS SD phosphorylation, which was abolished by Src inhibition (Fig. 4*C*). These findings reveal that Src activity is

required for phosphorylation of CAS SD in FG pancreatic carcinoma cells.

Previous studies have shown that complete deletion of the CAS SD inhibits the assembly of docking proteins leading to loss of cell migration on various matrix proteins (24). However, little is known about the specificity of these phosphorylation events and whether any specifically regulates EGF-mediated cell invasion or metastasis. There are 15 tyrosine-containing motifs (YXXP) in the SD of CAS that represent putative Src phosphorylation sites (12, 37). To assess whether these sites were responsive to Src activity, we stably expressed WT CAS or CAS containing Y/F mutations of the 15 YXXP phosphorylation SD sites (F1-15) in FG cells. EGF stimulation resulted in Src-dependent phosphorylation of WT CAS, whereas cells expressing the F1-15 mutant CAS showed no phosphorylation (Supplementary Fig. S4*A*).

To evaluate the role of phosphorylation of the CAS SD in EGF-induced migration, FG cells expressing the WT or F1-15 CAS constructs were allowed to migrate on vitronectin or fibronectin in the presence or absence of EGF. On vitronectin, FG cells expressing WT CAS responded to EGF stimulation (Fig. 5*A*) in a manner similar to untransfected FG cells (Supplementary Fig. S1) or vector control FG cells (data not shown). In cells expressing F1-15, EGF did not induce an $\alpha_v\beta_5$-mediated migration response on vitronectin, although migration on fibronectin was normal (data not shown), suggesting that one or more sites within the CAS SD are critically involved in EGF-mediated $\alpha_v\beta_5$-dependent migration. To further identify the region within the SD that is critical for EGF-inducible $\alpha_v\beta_5$ migration, we stably expressed two additional SD mutants in these cells (F1-9 and F10-15; see Supplementary Fig. S4*B*). Expression of F1-9 (but not F10-15) blocked EGF-induced migration on vitronectin (Fig. 5), indicating that phosphorylation of tyrosines 1 to 9 is required for the EGF/Src-dependent, $\alpha_v\beta_5$-mediated migration response. Importantly, expression of these CAS mutations in FG cells did not significantly influence β_1-mediated

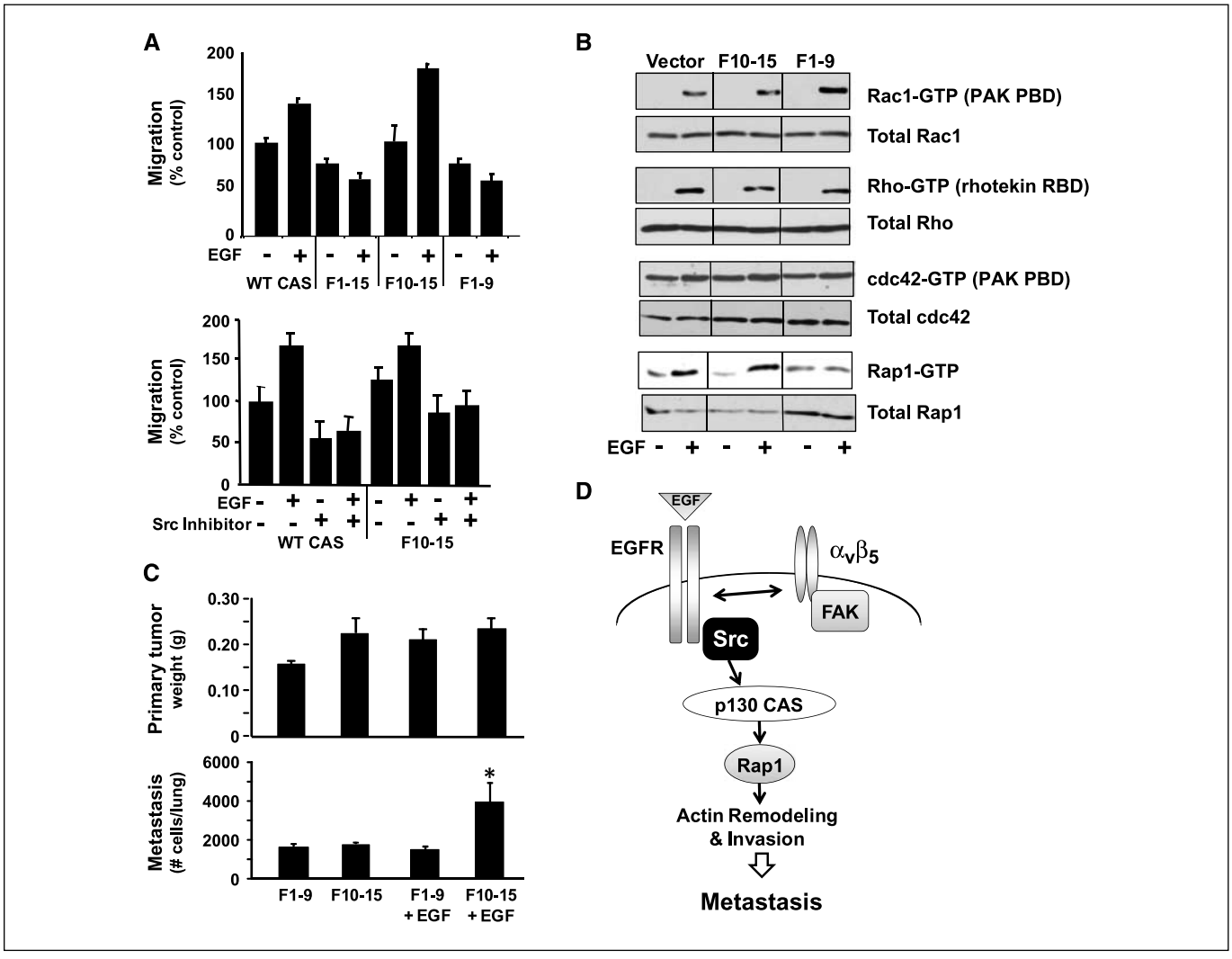

Figure 5. The first nine YXXP tyrosine residues in the CAS SD are required for $\alpha_v\beta_5$-mediated migration and metastasis. *A,* expression of the F1-15 or F1-9 CAS mutants blocked EGF-induced migration toward vitronectin. The Src inhibitor (SKI-606, 500 nmol/L) blocked EGF-induced migration of FG cells expressing WT CAS or the F10-15 CAS mutant. *B,* expression of the F1-9 (but not F10-15) CAS mutant blocked EGF-induced Rap1 activity but did not affect Rac, Rho, or cdc42. Vertical lines separate samples from a single gel. *C,* expression of the F1-9 CAS mutant blocked EGF-induced pulmonary metastasis but not primary tumor growth in the chick CAM model. *Columns,* mean ($n = 7$); *bars,* SE. *, $P \leq 0.05$. *D,* EGF stimulates EGFR to recruit and activate Src kinase and β_5 integrin, leading to activation of CAS and Rap1, which facilitate actin remodeling to enable cell invasion and metastasis.

cell migration on collagen or fibronectin (data not shown). These findings suggest that one or more of the first nine tyrosines in the CAS SD play a specific role in $\alpha_v\beta_5$-mediated migration.

CAS phosphorylation is generally associated with actin cytoskeleton organization and membrane ruffling (38), processes that are essential during cell invasion. In FG cells, EGF stimulation caused actin reorganization from stress fibers into filopodia (Supplementary Fig. S5, *b, arrows*) and this was blocked by Src inhibition (Supplementary Fig. S5, *e* and *c*). We observed a similar pattern in cells expressing F10-15 (Supplementary Fig. S5, *d–f*), whereas cells expressing F1-9 showed little or no EGF-induced actin reorganization (Supplementary Fig. S5, *h*).

To determine a role for small GTPases in CAS-regulated actin cytoskeleton organization, we investigated the activation state of four small GTPases (Rac1, Rho, cdc42, and Rap1) in FG cells expressing F10-15 or F1-9 mutations. EGF stimulation activated Rac1 and Rho (but not cdc42) in FG cells expressing vector control, F10-15, or F1-9 mutations (Fig. 5*C*). In contrast, EGF-dependent activation of Rap1, a positive regulator of integrin activation (32), occurred only in cells expressing the vector control or F10-15, but not the F1-9 mutant. Furthermore, Src inhibition abolished EGF-induced Rap1 activation in F10-15 cells (data not shown). These findings suggest that tyrosine residues 1 to 9 of the CAS SD are responsible for EGF-mediated, Src-dependent activation of Rap1 that promotes actin reorganization and cell metastasis.

To extend these results, FG cells expressing CAS mutations were tested for spontaneous metastasis *in vivo*. Whereas EGF stimulated metastasis of FG cells expressing F10-15, cells expressing the F1-9 mutant were not responsive to EGF and thus failed to metastasize in this model (Fig. 5*D*). However, primary tumor growth was identical among both mutant lines in the presence or absence of EGF. Similarly, FG cells expressing WT CAS metastasized in response to EGF, whereas F1-15 did not (data not shown). Together, these results provide evidence that EGF initiates a pathway of cell migration that depends on Src kinase activation and the specific phosphorylation of CAS leading to Rap1 activation. These events lead to the spontaneous metastasis of pancreatic carcinoma cells in an integrin $\alpha_v\beta_5$-dependent manner without influencing the growth of the primary tumor. The specificity of this pathway is underscored by the finding that β_1-mediated migration on matrix proteins such as fibronectin or collagen does not require EGF, Src, or CAS. Thus, we have identified two distinct pathways of tumor cell migration that differ based on Src dependency.

Discussion

Epithelial cancer cells metastasize in a series of linked, sequential steps that lead to remodeling and invasion of the extracellular matrix and ultimately tumor cell mobilization. Identification of contributors that activate the migration machinery is critical to understand tumor cell dissemination to secondary sites. In this study, we have identified signaling events coordinated by EGF and a specific integrin that regulates the invasive behavior of carcinoma cells.

There is a growing body of literature implicating integrins in cancer. Integrin-mediated adhesion leads to intracellular signaling events that regulate cell survival, proliferation, and migration (39). For example, integrin β_4 physically interacts with ErbB2 in breast cancer cells (40) and contributes to the initiation, growth, and invasion of ErbB2-induced mammary tumors in transgenic mice (41). Moreover, ablation of β_1 integrins in a transgenic mouse model of mammary tumorigenesis shows that β_1 is important for primary tumor initiation and growth through activation of FAK (42). Accordingly, treatment of FG cells with a FAK inhibitor reduced cell migration in an integrin β_1- and β_5-dependent manner, showing that FAK is required for general integrin function (Fig. 4). In contrast, blockade or depletion of β_5 integrin does not inhibit initiation or growth of the primary tumor but does reduce EGF- and Src-induced metastasis *in vivo* (Figs. 1 and 3).

Our results suggest that there are distinct integrin-mediated pathways of tumor cell migration that differ based on their dependency on EGF, small GTPases, Src activity, and the activation state of $\alpha_v\beta_5$ integrin. In the absence of EGF, $\alpha_v\beta_5$ ligation is not sufficient to activate the small GTPases Rac1 and Rap1. In contrast, ligation of β_1 integrin leads to activation of Rac1 and Rap1 in a manner that is independent of EGF or Src kinase activity. Although β_1 integrin ligation to proteins such as collagen, fibronectin, and laminin is critical for metastasis of some tumors, it seems that EGF and perhaps other cytokines can activate integrin $\alpha_v\beta_5$ (29) and thereby significantly enhance the metastatic capacity of various cancers (27). We do not believe that EGF exerts transcriptional regulation over β_5 integrin because EGF treatment does not change the expression of β_5 or β_1 integrin on the surface of FG cells (Supplementary Fig. S6*A*). Instead, we have evidence that the EGFR and β_5 integrin form a molecular complex. Using an immunoprecipitation and immunoblotting approach, we detected EGF-induced association between β_5 and EGFR (Supplementary Fig. S6*B*). We believe that EGF stimulates Src to activate β_5 integrin, thus forming an EGFR/Src/β_5 signaling module that drives migration and metastasis.

Our findings showing a role for Src and $\alpha_v\beta_5$ in metastasis are supported by previous studies showing that Src codistributes with α_v but not β_1 integrins at the cell substrate interface and reduces adhesive bonds to vitronectin but not fibronectin (43). In our study, Src inhibition did not affect adhesion of FG pancreatic carcinoma cells (data not shown) but did inhibit migration on vitronectin and reduce metastasis (Fig. 2). Indeed, we found that $\alpha_v\beta_5$-mediated migration is Src dependent in FG pancreatic carcinoma cells and in carcinomas of distinct histologic origin derived from breast, ovary, or colon, which use $\alpha_v\beta_5$ as their primary vitronectin receptor (Table 1).

Association and transphosphorylation between EGFR and Src occur when these proteins are either highly expressed or constitutively activated (44), as they often are in cancer cells. Accordingly, inhibition of EGFRs or Src kinase completely abrogates cell migration on vitronectin but does not influence integrin β_1-mediated migration on fibronectin or collagen. In fact, Src inhibition abolished EGF-induced pulmonary metastasis without affecting primary tumor growth (Fig. 2). Together, these results suggest that Src kinase plays a pivotal role in regulating $\alpha_v\beta_5$-mediated cell migration that is critical for the spontaneous metastatic property of pancreatic carcinoma cells.

Previous studies have linked Src expression to increased invasion and metastasis (7). However, it is not clear how Src contributes to this process at the molecular level. Elevated Src kinase activity in epithelial cells has long been associated with the weakening of cell-cell adhesion (45, 46). In fact, Src-induced deregulation of E-cadherin requires integrin signaling (10). FG cells selected for their capacity to spontaneously migrate in an $\alpha_v\beta_5$-dependent manner are highly metastatic and show a loss of cell-cell contact by the reduction of cell surface E-cadherin. This loss of E-cadherin at cell-cell junctions directly correlates to increased Src activity within the

cells (data not shown). Moreover, C3G, a Rap1GEF that is regulated by Src kinases (47), binds to the E-cadherin cytoplasmic domain and is activated on cell-cell adhesion weakening (48), thereby activating Rap1. However, the role of Rap1 activation in E-cadherin–mediated adherens junctions remains controversial (48, 49). Our studies suggest that Rap1 may be activated in response to Src-induced weakening of cell-cell junctions to specifically promote $\alpha_v\beta_5$-mediated migration and metastasis.

Actin reorganization is required for the motility and invasiveness of cells. Src-mediated phosphorylation of tyrosines within the CAS SD can recruit CrkII (12), which is necessary for CAS-mediated migration and membrane ruffling (24). Filopodia formation requires a Cas-Crk-C3G signaling pathway leading to Rap1 activation (15) and overexpression of C3G promotes filopodia formation independent of the small GTPases Rho, Rac1, or cdc42 (50). This is consistent with our results because EGF induced activity of Rho and Rac1 in FG cells expressing the CAS F1-9 or F10-15 mutations. However, Rap1 activation and filopodia formation are dependent on selective phosphorylation of one or more of the first nine tyrosine residues in the CAS SD in a Src-dependent manner (Fig. 5; Supplementary Fig. S5). Therefore, our results provide new insight into the role that EGFR and integrin $\alpha_v\beta_5$ play in regulating metastatic disease. Specifically, Src signaling to CAS is able to activate a selective integrin-dependent migratory response leading to spontaneous metastasis. Interestingly, complete deletion of the CAS SD inhibits Rap1 activity (15) and generally inhibits cell migration on a wide array of matrix proteins (24). We show here that tyrosine residues 1 to 9 within the CAS SD are specifically required for EGF-induced Rap1 activation and $\alpha_v\beta_5$-mediated migration *in vitro* and spontaneous metastasis *in vivo* (Fig. 5).

These studies reveal a role for EGF and its downstream effector Src kinase in mediating carcinoma metastasis through the specific activation of integrin $\alpha_v\beta_5$. Tyrosine kinase inhibitors such as Tarceva or dasatinib, which target EGFR and Src, respectively, seem to provide clinical benefit in cancer patients. Furthermore, several integrin antagonists are also under clinical evaluation. We propose that these agents and other inhibitors that interfere with the pathway described here may serve to control the invasive and metastatic properties of a wide array of malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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