Published in final edited form as:

Cancer Res. 2009 February 15; 69(4): 1383–1391. doi:10.1158/0008-5472.CAN-08-3612.

Specific crosstalk between EGFR and integrin $\alpha\beta 5$ promotes carcinoma cell invasion and metastasis

Jill M. Ricono¹, Miller Huang¹, Leo A. Barnes¹, Steven K. Lau¹, Sara M. Weis¹, David D. Schlaepfer¹, Steven K. Hanks², and David A. Cheresch^{1,3}

¹Moores UCSD Cancer Center, University of California, San Diego, La Jolla, California 92093, USA

²Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232, USA

³Department of Pathology, University of California, San Diego, La Jolla, California 92093, USA

Abstract

Tyrosine kinase receptors and integrins play essential roles in tumor cell invasion and metastasis. Previously, we demonstrated that EGF stimulation of pancreatic carcinoma cells leads to invasion and metastasis that was blocked by antagonists of integrin $\alpha\beta 5$. Here we show 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, EGFR induced Src activity leads to phosphorylation of a region within the CAS substrate domain which is essential for Rap1 and $\alpha\beta 5$ activation. This pathway induces $\alpha\beta 5$ -mediated invasion and metastasis in vivo yet does not influence primary tumor growth or activation of other integrins on these cells. These findings demonstrate crosstalk between a tyrosine kinase receptor and an integrin involved in carcinoma cell invasion and metastasis and may explain in part how inhibitors of EGFR impact malignant disease.

Keywords

EGF; integrin; Src; CAS; Rap1

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, has been shown to be 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). While 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. Pharmacological 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 which influence cell migration are focal adhesion kinase (FAK) and Crk-associated substrate (p130CAS or CAS). FAK promotes the assembly of multi-protein complexes required for the turnover of focal contacts facilitating integrin-mediated cell migration and invasion (10,11). Upon 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 impact cell proliferation, survival, and motility (18). Activation of integrins has been implicated in many pathological 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 upon EGF-mediated Src kinase activity and activation of integrin $\alpha\beta 5$. On matrix proteins such as fibronectin or collagen, cell migration is mediated by $\beta 1$ 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 substrate domain of CAS leading to activation of Rap1 during integrin $\beta 5$ -mediated cell invasion and metastasis of pancreatic carcinoma cells without influencing primary tumor growth. Thus, the EGFR/Src/ $\beta 5$ -dependent pathway appears to contribute to the metastatic properties of pancreatic cancer.

Methods

Antibodies and inhibitors

Antibodies were purchased from Santa Cruz Biotechnology (FAK C-20, CSK C-20), Invitrogen (FAKpY861, rhodamine-phalloidin), Cell Signaling Technology (phospho-Erk1/2, Src pY416, pCAS Y165, pCAS Y249, pCAS Y410), Millipore (Src GD11, Rap1, Rac1), Sigma (β -actin), and BD Transduction Labs (Yes, CAS, Rho). LM142, P4C10, P1F6 antibodies were prepared as described (21). The Src inhibitor SKI-606 (22) was used at 500nM. The FAK inhibitor PF-228 (23) was used at 1 μ M.

CAS mutants

cDNA was amplified from pRc/CMV-CASmyc templates (12) using primers containing EcoR1 and BamH1 sites surrounding the region encoding the full length CAS protein. Mutated cDNAs were subcloned into pEGFP-C1 vector with EcoR1 and BamH1 restriction enzymes, and ligated using Rapid DNA Ligation kit (Roche). All cDNA 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 (GIBCO BRL) with 10% FBS. 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, single cell clones were isolated, propagated and screened. FG cells containing CAS mutations were sorted for GFP expression, and CAS expression was verified by immunoblotting. Cells expressing recombinant adenovirus for wildtype (WT) and kinase-dead (KD) CSK were created as described (25).

shRNA knockdown

Integrin $\beta 5$ and non-silencing lentiviral shRNAmir in pLKO.1 expressing system were from Open Biosystems. Lentiviruses were produced in 293FT cells using Fugene transfection. Cells were selected 48hrs after infection with 1 μ g/ml Puromycin, and low-expressing cells were further selected by flow cytometry. For Rap1 knockdown, FG cells were transfected with a pool of 4 Rap1b siRNA (Qiagen) for 24hrs, serum-starved overnight, and migrations assays performed at 48hrs post-transfection.

Protein analysis

Cells were serum-starved for 24hrs, pretreated with inhibitors, and stimulated with EGF (50ng/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 parameter histograms.

Cell migration

Migration assays were performed as described (24).

Immunofluorescence and microscopy

Cells were fixed in 2% paraformaldehyde, permeabilized, and incubated with 2 μ g/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 GAPDH using real-time qPCR as described previously (28) with modifications: *alu* probe-AGACCAGCCTGGGCAACATAGTGAAA, 5'BHQ1a-5TET; GAPDH probe-AGATGCTCTGCGGAAAGCAGTGAAT, 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 μ g/ml P1F6 or P4C10 antibody, and allowed to adhere for 15 minutes. For Fig. 5, cells were grown for 3 days and serum-starved overnight prior to stimulation with 50ng/ml EGF for 1min. Rac1-GTP, Rho-GTP and Rap1-GTP pull-down assays were performed according to manufacturer instructions (Millipore).

Statistics

Unless stated otherwise, bar graphs represent mean $\pm$ SD of triplicate samples. All data presented is 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 $\alpha\beta 5$ -mediated metastasis

EGF stimulation induces $\alpha\beta 5$ -mediated carcinoma cell migration on vitronectin, whereas cells migrate robustly on integrin $\beta 1$ -mediated substrates such as fibronectin or collagen in an EGF-independent manner (29) (Supplemental Fig. S1 A&B). Since small GTPases regulate cytoskeletal rearrangements and cell migration (30-32), their activity was measured for FG cells attached to immobilized anti-integrin $\alpha\beta 5$ or $\beta 1$. Adhesion to anti- $\beta 1$ led to Rac1 and Rap1 activity independent of EGF stimulation, whereas cells attached to anti- $\alpha\beta 5$ showed robust EGF-dependent activation of Rac1 or Rap1 (Fig. 1A). Knockdown of Rap1b expression in FG cells selectively blocked EGF-induced migration on vitronectin (Fig. 1B), supporting a role for Rap1 in the $\beta 5$ /EGF-mediated cell migratory response.

Previous studies have implicated $\beta 1$ integrins in cancer since they regulate cell adhesion and migration/invasion on tumor stroma proteins such as fibronectin, laminin and collagen (33). Since $\alpha\beta 5$ requires activation to promote cell migration, we considered whether EGF and integrin $\alpha\beta 5$ 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. While EGF stimulation had no effect on primary tumor growth, it increased pulmonary metastasis 3-fold, which was abolished by shRNA-mediated knockdown of integrin $\beta 5$ (Fig. 1C). Importantly, knockdown of $\beta 5$ did not influence $\beta 1$ integrin expression (Supplemental Fig. S1C) or primary tumor growth (Fig. 1C), indicating that EGF and $\alpha\beta 5$ coordinately and specifically regulate the spontaneous metastasis of FG cells in this model.

Src activation downstream of EGFR is required for $\alpha\beta 5$ -mediated carcinoma cell invasion and metastasis

EGF receptor 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) prior to EGF stimulation. SKI-606 suppressed EGF-induced Src phosphorylation in FG cells (Supplemental Fig. S2A) and blocked EGF-mediated migration on vitronectin yet had no effect on EGF-independent migration on collagen (Fig. 2A). To confirm a role for endogenous Src in EGF-induced migration, FG cells were transduced to express C-terminal Src kinase (CSK), an inactivator of Src family kinases (34). As expected, expression of CSK (but not kinase-dead CSK) suppressed EGF-induced Src activation (Fig. 2B) and cell migration on vitronectin (Fig. 2B). Importantly, migration on collagen was not influenced by EGF treatment or CSK expression (Fig. 2B). 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. While 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. 2C). These findings suggest that spontaneous metastasis and $\alpha\beta 5$ -mediated migration can be activated by EGF and suppressed by Src inhibition.

Src is sufficient to induce $\alpha\beta 5$ - mediated tumor cell metastasis

To determine whether activated Src is sufficient to account for $\alpha\beta 5$ -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 wild type FG cells stimulated with EGF showed robust Src activation, which was completely abolished by treatment with the Src kinase inhibitor SKI-606 (Supplemental Fig. S3). Importantly, FG cells expressing SrcA showed spontaneous migration on vitronectin, a process that was completely blocked by anti- $\alpha\beta 5$ antibody (Fig. 3A) but not with anti- $\beta 1$ integrin antibody (data not shown). Furthermore, SrcA expression did not significantly influence FG migration on collagen or fibronectin (Fig. 3A), consistent with our findings that cell migration on these $\beta 1$ -integrin substrates is independent of Src kinase activity (Fig. 2). Together these results suggest that Src activation is sufficient to trigger $\alpha\beta 5$ -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. While increased Src kinase activity did not influence primary tumor growth (Fig. 3B, upper panel), it was sufficient to induce pulmonary metastases (Fig. 3B, lower panel). To evaluate the role of $\alpha\beta 5$ in this process, tumor bearing animals were injected systemically with a function blocking (PIF6) or non-function blocking (11D1) antibody directed to integrin $\alpha\beta 5$. Blockade of integrin $\alpha\beta 5$ function completely inhibited SrcA-induced metastasis to control levels (Fig. 3B, lower panel). Importantly, increased Src activity or blockade of $\alpha\beta 5$ function did not influence primary tumor growth (Fig. 3B, upper panel). These results reveal that activation of Src kinase in combination with $\alpha\beta 5$ is necessary and sufficient for spontaneous metastasis.

Since EGFR activation of Src initiates $\alpha\beta 5$ -mediated migration of FG pancreatic carcinoma cells in vitro and in vivo, we asked whether carcinomas of distinct histological origin would migrate on vitronectin in response to activation of $\beta 5$ integrin, EGF, and Src. As observed for FG cells, MCF-7 breast carcinoma cells (Fig. 3C) as well as other tumor cell lines that express $\alpha\beta 5$ 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 (Supplemental Fig. S1B), EGF-induced migration of MCF-7 breast carcinoma cells on vitronectin selectively required $\beta 5$ integrin function (Fig. 3D). Together these results suggest a role for the Src/ $\beta 5$ signaling module in multiple carcinomas.

EGF-induced, $\alpha\beta 5$ -dependent migration in vitro and metastasis in vivo requires Src-mediated phosphorylation of a specific region within the substrate domain 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 approximately 175 kD, as well as other proteins (Fig. 4A). 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 120-140 kD (Fig. 4A, arrow). Previous studies have suggested FAK and CAS as major phospho-proteins 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. 4B). Pharmacological blockade of FAK reduced autophosphorylation of Y397 and EGF-induced phosphorylation of Y861, and disrupted EGF-induced $\alpha\beta 5$ -mediated migration on vitronectin as well as $\beta 1$ -mediated migration on fibronectin. These findings indicate that FAK plays a

generic role in cell migration on multiple substrates. Next, we considered whether EGF stimulation could lead to Src-dependent phosphorylation of p130 CAS. As measured by antibodies that specifically detect phosphotyrosines in the CAS substrate domain (SD) (36), EGF stimulation promoted phosphorylation of CAS SD, which was blocked by Src inhibition (Fig. 4C). 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. 4C). These findings reveal that Src activity is required for phosphorylation of CAS SD in FG pancreatic carcinoma cells.

Previous studies have demonstrated 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 regarding the specificity of these phosphorylation events and whether any specifically regulate 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 (Supplemental Fig. S4A).

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. 5A) in a manner similar to untransfected FG cells (Supplemental Fig. S1) or vector control FG cells (data not shown). In cells expressing F1-15, EGF did not induce an α v β 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 α v β 5-dependent migration. To further identify the region within the SD that is critical for EGF-inducible α v β 5 migration, we stably expressed two additional SD mutants in these cells (F1-9 and F10-15, see Supplemental Fig. S4B). Expression of F1-9 (but not F10-15) blocked EGF-induced migration on vitronectin (Fig. 5), indicating that phosphorylation of tyrosines 1-9 is required for the EGF/Src-dependent α v β 5-mediated migration response. Importantly, expression of these CAS mutations in FG cells did not significantly influence β 1-mediated cell migration on collagen or fibronectin (data not shown). These findings suggest that one or more of the first nine tyrosines in the CAS substrate domain play a specific role in α v β 5-mediated migration.

CAS phosphorylation is generally associated with actin cytoskeleton organization and membrane ruffling (38), processes which are essential during cell invasion. In FG cells, EGF stimulation caused actin reorganization from stress fibers into filopodia (Supplemental Fig. S5, *b* arrows) and this was blocked by Src inhibition (Supplemental Fig. S5, *e* and *c*). We observed a similar pattern in cells expressing F10-15 (Supplemental Fig. S5, *d-f*), whereas cells expressing F1-9 showed little or no EGF-induced actin reorganization (Supplemental 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. 5C). 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 1-9 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. While 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. 5D). 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 $\alpha\beta 5$ -dependent manner without influencing the growth of the primary tumor. The specificity of this pathway is underscored by the finding that $\beta 1$ -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 regulate 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 $\beta 4$ 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 $\beta 1$ integrins in a transgenic mouse model of mammary tumorigenesis demonstrates that $\beta 1$ 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 $\beta 1$ - and $\beta 5$ -dependent manner, demonstrating that FAK is required for general integrin function (Fig. 4). In contrast, blockade or depletion of $\beta 5$ integrin does not inhibit initiation or growth of the primary tumor, but does reduce EGF- and Src-induced metastasis in vivo (Fig. 1 and Fig. 3).

Our results suggest 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 $\alpha\beta 5$ integrin. In the absence of EGF, $\alpha\beta 5$ ligation is not sufficient to activate the small GTPases Rac1 and Rap1. In contrast, ligation of $\beta 1$ integrin leads to activation of Rac1 and Rap1 in a manner that is independent of EGF or Src kinase activity. While $\beta 1$ integrin ligation to proteins such as collagen, fibronectin and laminin is critical for metastasis of some tumors, it appears that EGF and perhaps other cytokines can activate integrin $\alpha\beta 5$ (29) and thereby significantly enhance the metastatic capacity of various cancers (27). We do not believe that EGF exerts transcriptional regulation over $\beta 5$ integrin, since EGF treatment does not change the expression of $\beta 5$ or $\beta 1$ integrin on the surface of FG cells (Suppl. Fig. S6A). Instead, we have evidence that the EGFR and $\beta 5$ integrin form a molecular complex. Using an immunoprecipitation and immunoblotting approach, we detected EGF-induced association between $\beta 5$ and EGFR (Suppl. Fig. S6B). We believe that EGF stimulates Src to activate $\beta 5$ integrin, thus forming an EGFR/Src/ $\beta 5$ signaling module that drives migration and metastasis.

Our findings demonstrating a role for Src and $\alpha\beta 5$ in metastasis are supported by previous studies showing Src co-distributes with αv but not $\beta 1$ 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 $\alpha\text{v}\beta 5$ -mediated migration is Src-dependent in FG pancreatic carcinoma cells and in carcinomas of distinct histological origin derived from breast, ovary, or colon which use $\alpha\text{v}\beta 5$ as their primary vitronectin receptor (Table 1).

Association and trans-phosphorylation between EGFR and Src occurs 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 $\beta 1$ -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 $\alpha\text{v}\beta 5$ -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 $\alpha\text{v}\beta 5$ -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 which is regulated by Src kinases (47), binds to the E-cadherin cytoplasmic domain and is activated upon 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 $\alpha\text{v}\beta 5$ -mediated migration and metastasis.

Actin reorganization is required for the motility and invasiveness of cells. Src-mediated phosphorylation of tyrosines within the CAS substrate domain 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, since 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 upon selective phosphorylation of one or more of the first nine tyrosine residues in the CAS SD in a Src-dependent manner (Fig. 5 and Supplemental Fig. S5). Therefore, our results provide new insight into the role that EGFR and integrin $\alpha\text{v}\beta 5$ plays 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 1-9 within the CAS SD are specifically required for EGF-induced Rap1 activation and $\alpha\text{v}\beta 5$ -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 $\alpha\text{v}\beta 5$. Tyrosine kinase inhibitors such as Tarceva or Dasatinib, which target EGFR and Src respectively, appear 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Drs. J. Lindquist, S. Anand and D. Shields for their helpful review and critical discussion of the manuscript.

This work was supported by NIH NRSA T32 HL07195-26 (JMR), NIH RO1 DA102310 (DDS), NIH GM49882 (SKH), and NIH R01 CA45726-17 (DAC).

References

1. Izumi Y, Xu L, di Tomaso E, Fukumura D, Jain RK. Tumour biology: herceptin acts as an anti-angiogenic cocktail. *Nature* 2002;416(6878):279–80. [PubMed: 11907566]
2. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* 1995;19(3):183–232. [PubMed: 7612182]
3. Marshall J. Clinical implications of the mechanism of epidermal growth factor receptor inhibitors. *Cancer* 2006;107(6):1207–18. [PubMed: 16909423]
4. Summy JM, Gallick GE. Src family kinases in tumor progression and metastasis. *Cancer Metastasis Rev* 2003;22(4):337–58. [PubMed: 12884910]
5. Klinghoffer RA, Sachsenmaier C, Cooper JA, Soriano P. Src family kinases are required for integrin but not PDGFR signal transduction. *Embo J* 1999;18(9):2459–71. [PubMed: 10228160]
6. Ito H, Gardner-Thorpe J, Zinner MJ, Ashley SW, Whang EE. Inhibition of tyrosine kinase Src suppresses pancreatic cancer invasiveness. *Surgery* 2003;134(2):221–6. [PubMed: 12947321]
7. Trevino JG, Summy JM, Lesslie DP, et al. Inhibition of SRC expression and activity inhibits tumor progression and metastasis of human pancreatic adenocarcinoma cells in an orthotopic nude mouse model. *Am J Pathol* 2006;168(3):962–72. [PubMed: 16507911]
8. Luo FR, Barrett YC, Yang Z, et al. Identification and validation of phospho-SRC, a novel and potential pharmacodynamic biomarker for dasatinib (SPRYCELtrade mark), a multi-targeted kinase inhibitor. *Cancer chemotherapy and pharmacology*. 2008
9. Lombardo LJ, Lee FY, Chen P, et al. Discovery of N-(2-chloro-6-methyl- phenyl)-2-(6-(4-(2-hydroxyethyl)- piperazin-1-yl)-2-methylpyrimidin-4- ylamino)thiazole-5-carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays. *Journal of medicinal chemistry* 2004;47(27):6658–61. [PubMed: 15615512]
10. Avizienyte E, Wyke AW, Jones RJ, et al. Src-induced de-regulation of E-cadherin in colon cancer cells requires integrin signalling. *Nat Cell Biol* 2002;4(8):632–8. [PubMed: 12134161]
11. Shi Q, Boettiger D. A novel mode for integrin-mediated signaling: tethering is required for phosphorylation of FAK Y397. *Molecular biology of the cell* 2003;14(10):4306–15. [PubMed: 12960434]
12. Shin NY, Dise RS, Schneider-Mergener J, Ritchie MD, Kilkenny DM, Hanks SK. Subsets of the major tyrosine phosphorylation sites in Crk-associated substrate (CAS) are sufficient to promote cell migration. *The Journal of biological chemistry* 2004;279(37):38331–7. [PubMed: 15247284]
13. Brabek J, Constancio SS, Shin NY, Pozzi A, Weaver AM, Hanks SK. CAS promotes invasiveness of Src-transformed cells. *Oncogene* 2004;23(44):7406–15. [PubMed: 15273716]
14. Sawada Y, Tamada M, Dubin-Thaler BJ, et al. Force sensing by mechanical extension of the Src family kinase substrate p130Cas. *Cell* 2006;127(5):1015–26. [PubMed: 17129785]
15. Sakakibara A, Ohba Y, Kurokawa K, Matsuda M, Hattori S. Novel function of Chat in controlling cell adhesion via Cas-Crk-C3G-pathway-mediated Rap1 activation. *Journal of cell science* 2002;115 (Pt 24):4915–24. [PubMed: 12432078]
16. Brabek J, Constancio SS, Siesser PF, Shin NY, Pozzi A, Hanks SK. Crk-associated substrate tyrosine phosphorylation sites are critical for invasion and metastasis of SRC-transformed cells. *Mol Cancer Res* 2005;3(6):307–15. [PubMed: 15972849]

17. Mitra SK, Schlaepfer DD. Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr Opin Cell Biol* 2006;18(5):516–23. [PubMed: 16919435]
18. Aplin AE, Howe A, Alahari SK, Juliano RL. Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. *Pharmacol Rev* 1998;50(2):197–263. [PubMed: 9647866]
19. Hehlhans S, Haase M, Cordes N. Signalling via integrins: implications for cell survival and anticancer strategies. *Biochimica et biophysica acta* 2007;1775(1):163–80. [PubMed: 17084981]
20. Moro L, Dolce L, Cabodi S, et al. Integrin-induced epidermal growth factor (EGF) receptor activation requires c-Src and p130Cas and leads to phosphorylation of specific EGF receptor tyrosines. *The Journal of biological chemistry* 2002;277(11):9405–14. [PubMed: 11756413]
21. Cheresch DA, Smith JW, Cooper HM, Quaranta V. A novel vitronectin receptor integrin (alpha v beta x) is responsible for distinct adhesive properties of carcinoma cells. *Cell* 1989;57(1):59–69. [PubMed: 2467745]
22. Golas JM, Arndt K, Etienne C, et al. SKI-606, a 4-anilino-3-quinolinecarbonitrile dual inhibitor of Src and Abl kinases, is a potent antiproliferative agent against chronic myelogenous leukemia cells in culture and causes regression of K562 xenografts in nude mice. *Cancer Res* 2003;63(2):375–81. [PubMed: 12543790]
23. Slack-Davis JK, Martin KH, Tilghman RW, et al. Cellular characterization of a novel focal adhesion kinase inhibitor. *The Journal of biological chemistry* 2007;282(20):14845–52. [PubMed: 17395594]
24. Klemke RL, Leng J, Molander R, Brooks PC, Vuori K, Cheresch DA. CAS/Crk coupling serves as a “molecular switch” for induction of cell migration. *J Cell Biol* 1998;140(4):961–72. [PubMed: 9472046]
25. Takayama Y, Tanaka S, Nagai K, Okada M. Adenovirus-mediated overexpression of C-terminal Src kinase (Csk) in type I astrocytes interferes with cell spreading and attachment to fibronectin. Correlation with tyrosine phosphorylations of paxillin and FAK. *The Journal of biological chemistry* 1999;274(4):2291–7. [PubMed: 9890993]
26. Klemke RL, Cai S, Giannini AL, Gallagher PJ, de Lanerolle P, Cheresch DA. Regulation of cell motility by mitogen-activated protein kinase. *J Cell Biol* 1997;137(2):481–92. [PubMed: 9128257]
27. Brooks PC, Klemke RL, Schon S, Lewis JM, Schwartz MA, Cheresch DA. Insulin-like growth factor receptor cooperates with integrin alpha v beta 5 to promote tumor cell dissemination in vivo. *J Clin Invest* 1997;99(6):1390–8. [PubMed: 9077549]
28. Zijlstra A, Mellor R, Panzarella G, et al. A quantitative analysis of rate-limiting steps in the metastatic cascade using human-specific real-time polymerase chain reaction. *Cancer Res* 2002;62(23):7083–92. [PubMed: 12460930]
29. Klemke RL, Yebra M, Bayna EM, Cheresch DA. Receptor tyrosine kinase signaling required for integrin alpha v beta 5-directed cell motility but not adhesion on vitronectin. *J Cell Biol* 1994;127(3):859–66. [PubMed: 7525598]
30. Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* 1992;70(3):401–10. [PubMed: 1643658]
31. Han J, Lim CJ, Watanabe N, et al. Reconstructing and deconstructing agonist-induced activation of integrin alphaIIb beta3. *Curr Biol* 2006;16(18):1796–806. [PubMed: 16979556]
32. Retta SF, Balzac F, Avolio M. Rap1: a turnabout for the crosstalk between cadherins and integrins. *European journal of cell biology* 2006;85(34):283–93. [PubMed: 16546572]
33. van der Flier A, Sonnenberg A. Function and interactions of integrins. *Cell and tissue research* 2001;305(3):285–98. [PubMed: 11572082]
34. Nada S, Yagi T, Takeda H, et al. Constitutive activation of Src family kinases in mouse embryos that lack Csk. *Cell* 1993;73(6):1125–35. [PubMed: 8513497]
35. Hanks SK, Ryzhova L, Shin NY, Brabek J. Focal adhesion kinase signaling activities and their implications in the control of cell survival and motility. *Front Biosci* 2003;8:d982–96. [PubMed: 12700132]
36. Fonseca PM, Shin NY, Brabek J, Ryzhova L, Wu J, Hanks SK. Regulation and localization of CAS substrate domain tyrosine phosphorylation. *Cell Signal* 2004;16(5):621–9. [PubMed: 14751547]

37. Goldberg GS, Alexander DB, Pellicena P, Zhang ZY, Tsuda H, Miller WT. Src phosphorylates Cas on tyrosine 253 to promote migration of transformed cells. *The Journal of biological chemistry* 2003;278(47):46533–40. [PubMed: 12972425]
38. Cheresch DA, Leng J, Klemke RL. Regulation of cell contraction and membrane ruffling by distinct signals in migratory cells. *J Cell Biol* 1999;146(5):1107–16. [PubMed: 10477763]
39. Eble JA, Haier J. Integrins in cancer treatment. *Current cancer drug targets* 2006;6(2):89–105. [PubMed: 16529540]
40. Falcioni R, Antonini A, Nistico P, et al. Alpha 6 beta 4 and alpha 6 beta 1 integrins associate with ErbB-2 in human carcinoma cell lines. *Experimental cell research* 1997;236(1):76–85. [PubMed: 9344587]
41. Guo W, Pylayeva Y, Pepe A, et al. Beta 4 integrin amplifies ErbB2 signaling to promote mammary tumorigenesis. *Cell* 2006;126(3):489–502. [PubMed: 16901783]
42. White DE, Kurpios NA, Zuo D, et al. Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. *Cancer Cell* 2004;6(2):159–70. [PubMed: 15324699]
43. Felsenfeld DP, Schwartzberg PL, Venegas A, Tse R, Sheetz MP. Selective regulation of integrin--cytoskeleton interactions by the tyrosine kinase Src. *Nat Cell Biol* 1999;1(4):200–6. [PubMed: 10559917]
44. Haskell MD, Slack JK, Parsons JT, Parsons SJ. c-Src tyrosine phosphorylation of epidermal growth factor receptor, P190 RhoGAP, and focal adhesion kinase regulates diverse cellular processes. *Chem Rev* 2001;101(8):2425–40. [PubMed: 11749381]
45. Behrens J, Vakaet L, Friis R, et al. Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/beta-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. *J Cell Biol* 1993;120(3):757–66. [PubMed: 8425900]
46. Takeda H, Nagafuchi A, Yonemura S, Tsukita S, Behrens J, Birchmeier W. V-src kinase shifts the cadherin-based cell adhesion from the strong to the weak state and beta catenin is not required for the shift. *J Cell Biol* 1995;131(6 Pt 2):1839–47. [PubMed: 8557750]
47. Fukuyama T, Ogita H, Kawakatsu T, Inagaki M, Takai Y. Activation of Rac by cadherin through the c-Src-Rap1-phosphatidylinositol 3-kinase-Vav2 pathway. *Oncogene* 2006;25(1):8–19. [PubMed: 16170364]
48. Balzac F, Avolio M, Degani S, et al. E-cadherin endocytosis regulates the activity of Rap1: a traffic light GTPase at the crossroads between cadherin and integrin function. *Journal of cell science* 2005;118(Pt 20):4765–83. [PubMed: 16219685]
49. Hogan C, Serpente N, Cogram P, et al. Rap1 regulates the formation of E-cadherin-based cell-cell contacts. *Molecular and cellular biology* 2004;24(15):6690–700. [PubMed: 15254236]
50. Radha V, Rajanna A, Mitra A, Rangaraj N, Swarup G. C3G is required for c-Abl-induced filopodia and its overexpression promotes filopodia formation. *Experimental cell research* 2007;313(11):2476–92. [PubMed: 17475248]

Fig. 2. Src kinase is necessary for EGF induced β 5-mediated migration and metastasis
 (A) Pretreatment with a Src inhibitor (SKI-606, 500nM) blocked EGF-induced migration on vitronectin. (B) Expression of CSK, but not CSK kinase dead (CSK-KD), blocked EGF-induced migration on vitronectin. Blockade of Src kinase activity and expression of CSK and CSK-KD was 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. mean $\pm$ SEM, $n \geq 6$, $**p \leq 0.01$.

Fig. 3. Src kinase is sufficient for $\beta 5$ -mediated migration and metastasis

(A) Expression of active Src (SrcA) selectively induced migration on vitronectin which could be blocked with anti- $\alpha\beta 5$. (B) Intravenous injection with 100 μ g of a function-blocking antibody to $\beta 5$ (P1F6), but not a non-function blocking $\beta 5$ antibody (11D1), blocked pulmonary metastasis but not primary tumor growth in the chick CAM model. mean $\pm$ SEM, $n \geq 6$, $**p \leq 0.01$. (C) Pretreatment with a Src inhibitor (SKI-606, 500nM) 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- $\beta 5$ function-blocking antibody.

Fig. 4. FAK and CAS are substrates of Src in FG pancreatic carcinoma cells

(A) Src inhibition (SKI-606, 500nM) blocked EGF-induced tyrosine phosphorylation of 120-140kD proteins (arrow). (B) Treatment with a FAK inhibitor (PF228, 1mM) 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, 500nM) blocked EGF-induced CAS phosphorylation in FG cells, and constitutive CAS activity in FG cells expressing active Src (SrcA).

Fig. 5. The first 9 YXXP tyrosine residues in the CAS substrate domain 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, 500nM) 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 impact 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. mean $\pm$ SEM, $n=7$, $*p \leq 0.05$. (D) EGF stimulates EGFR to recruit and activate Src kinase and $\beta 5$ integrin, leading to activation of CAS and Rap1, which facilitate actin remodeling to enable cell invasion and metastasis.

Carcinoma cells of distinct histological origin were analyzed for the role of Src in $\alpha v\beta 5$ -mediated migration. FACS analysis was used to determine $\alpha v\beta 5$, $\beta 3$ or $\beta 1$ integrin expression on the cell surface. Requirement of Src kinase in cell migration was determined by stimulating cells with or without EGF (50ng/ml) and/or pretreated with Src inhibitor (SKI-606, 500nM). Cells were allowed to migrate toward vitronectin (VN) or fibronectin (FN).

Table 1

Carcinoma cell lines (origin)	FACS analysis of integrin beta subunits:				Requirement of Src kinase for $\alpha v\beta 5$ - mediated migration (VN)	Requirement of Src kinase for $\beta 1$ - mediated migration (FN)
	$\beta 1$	$\beta 3$	$\beta 5$			
FG (Pancreatic)	++++	-	++		+++	-
Mia PaCa-2 (Pancreatic)	+++++	-/+	++		+++	-
2008 (Ovarian)	+++++	-	++		+++	-
MCF-7 (Breast)	+++++	-	+++		+++	-
HT-29 (Colon)	+++++	-	+++		+++	-
Panc-1 (Pancreatic)	+++++	+++	+++		+	-
MDA-MB-435 (Breast)	+++++	+++	++		-	-