CrkI and CrkII Function as Key Signaling Integrators for Migration and Invasion of Cancer Cells

Sonia P. Rodrigues,1 Kelly E. Fathers,1 Gabriel Chan,2 Dongmei Zuo,3 Fawaz Halwani,1 Sarkis Meterissian,2 and Morag Park1,3,5

Abstract
Crk adaptor proteins play an important role during cellular signaling by mediating the formation of protein complexes. Increased levels of Crk proteins are observed in several human cancers and overexpression of Crk in epithelial cell cultures promotes enhanced cell dispersal and invasion, implicating Crk as a regulator of invasive responses. To determine the requirement of Crk for invasive signals, we targeted the CRKII gene by RNA interference. Consistent knockdown of CRKII was observed with two small interfering RNA targeting sequences in all human cancer cell lines tested. CRKII knockdown resulted in a significant decrease in migration and invasion of multiple malignant breast and other human cancer cell lines (MDA-231, MDA-435s, H1299, KB, and HeLa). Moreover, CRKII knockdown decreased cell spreading on extracellular matrix and led to a decrease in actin stress fibers and the formation of mature focal adhesions. Using immunohistochemistry, we show elevated CRKI/II protein levels in patients with breast adenocarcinoma. Together, these studies identify Crk adaptor proteins as critical integrators of upstream signals for cell invasion and migration in human cancer cell lines and support a role for Crk in metastatic spread.


Introduction
The progression of cancer to metastasis is dependent, in part, on the deregulation of signaling pathways involved in migration and invasion. In response to the extracellular environment, epithelial sheets or individual cells respond to different migratory signaling pathways to invade surrounding tissue (1). These transitions are also prominent in normal physiologic processes such as embryonic development, wound healing, and organogenesis. The dissection of these signaling pathways will be an important step towards developing targeted therapeutics for metastases.

Hepatocyte growth factor (HGF) and its receptor tyrosine kinase, Met, are potent modulators of epithelial dispersal and invasion, in vitro and in vivo (2). Although many signaling pathways have been identified to coordinate this process (3), we have identified a role for Crk adapter proteins downstream from the HGF/Met receptor tyrosine kinase, in both the dispersal of epithelial colonies and in epithelial morphogenesis induced by HGF (4-7).

Crk was originally isolated as the oncogene fusion product of the CT10 chicken retrovirus (v-Crk; ref. 8). Cellular homologues of v-Crk include the c-Crk gene, which encodes two alternatively spliced mRNAs that give rise to two proteins (c-CrkI and c-CrkII), and a second gene, c-CRKL. Crk proteins are composed of one Src homology 2 (SH2) and, one or two Src homology 3 (SH3) domains (9). They are adaptor proteins that direct the assembly of multimeric signaling complexes. Crk adaptor proteins and their effectors are highly conserved throughout evolution. The Crk SH2 domain binds a specific phosphorylated tyrosine motif found in proteins involved in cell spreading, actin reorganization, and cell migration. These Crk-SH2 binding proteins include the focal adhesion components, p130Cas and paxillin (10), growth factor receptor tyrosine kinases, and a docking protein Gab1, involved in epithelial dispersal and morphogenesis (5, 11, 12). The NH2-terminal SH3 domain of CrkII interacts constitutively with proline-rich motifs present within several proteins, including C3G, a nucleotide exchange factor for Rap1 (13), DOCK180, an exchange factor for Rac1 (14, 15), as well as the Ab1 tyrosine kinase (16), tyrosine phosphatase (protein tyrosine phosphatase 1B; ref. 17), the p85 subunit of phosphatidylinositol 3-kinase (18), and the c-Jun-NH2-kinase (1). Binding proteins for the COOH-terminal SH3 domain are still poorly understood.

Genetic studies in C. elegans have shown a role for CrkII and DOCK180 in phagocytosis and polarized cell migration (19, 20). Similarly, the overexpression of CrkII or CrkL in mammalian cells in vitro enhances migration when assayed as single cells (21-25) and promotes the dispersal of epithelial cell colonies (6). CrkII has been identified as a mediator of cell migration through its association with p130Cas and paxillin (23) as well as the Rac exchange
factor DOCK180 (14). Through these interactions, a role for Crk proteins has been proposed in the regulation of cell migration, morphogenesis, invasion, phagocytosis, and survival (10).

Aggressive and invasive tumors can aberrantly activate intracellular pathways required for cell migration and invasion. Elevated levels of CrkI and CrkII proteins and mRNA are found in various human tumors (26), including glioblastomas (27) and lung cancers (26, 28). Moreover, c-Crk/II mRNA expression was predominantly increased in lung tumors that were at more advanced stages as well as those associated with poor survival (26). Together, these studies support a role for Crk in tumor progression.

Much of our understanding about the role of Crk in mammalian cells derives from overexpression studies. Although RNA interference of CrkII has been shown to limit membrane ruffling of aortic endothelial cells (29) and CrkII (30) and CrkL knockout mice (31) have been established, limited analysis of cells derived from these mice has been published to date. To investigate a requirement for Crk in migration and invasion in human tumors, we have examined the association of Crk protein levels in human breast cancer and have used RNA interference directed against CrkII in human cancer cell lines. We show elevated levels of CrkII proteins in breast adenocarcinoma and show a key role for Crk adaptor proteins in the enhanced migration and invasion signaling programs of multiple human cancer cell lines.

Results
CrkII Proteins in Human Breast Cancer

The molecular mechanisms important to the progression of breast cancer invasion and metastasis are poorly understood. The most important prognostic indicator in breast cancer is the lymph node status. We examined the level of proteins from the c-Crk gene, CrkI and CrkII, by immunohistochemistry in primary breast tumors using two cohorts of patients with breast adenocarcinoma, including 8 node-negative stage I cancer patients in group 1, and 12 node-positive stage III cancer patients in the other. The patient characteristics are summarized in Table 1. Immunohistochemical analysis of paraffin sections, using sera that recognized CrkI and CrkII, revealed positive staining for CrkI or CrkII in adjacent normal ductal epithelial cells as well as in adenocarcinoma cells. Using a semiquantitative analysis, 60% of all the tumors displayed elevated immunostaining for CrkII in comparison with adjacent normal ducts (Fig. 1A). By cohort, the elevated CrkII immunostaining was evident in 63% of stage I and 58% of stage III samples. There was no statistical difference in proportion of tumors displaying elevated CrkII levels between stages ($\chi^2$ test, $P = 0.853$).

CrkII Protein Levels Are Decreased in Multiple Human Cancer Cell Lines following Small Interfering RNA Treatment

To investigate the function of Crk adaptor proteins in migration and invasion of human cancer cell lines, we used the previously published CrkII small interfering RNA (siRNA) duplex targeted to the human CrkI and CrkII mRNA, siCrk1, with the sequence 5'-AAUAGGAGAUCAGAGUUUGA-3' and 5'-UCAAACUCUCUCUCUAUUiG-3' (29) along with a novel, secondary siRNA, termed siCrk2, with the sequence 5'-AGGAGAUCAGAGAGUACdTdT-3' and 5'-GAUUCUCAAGAUGUCUCUdTdT-3' (Fig. 2A). The ability of siCrk to knock down CrkII protein levels was tested by cotransfecting the human cervical carcinoma cells HeLa with cDNA expressing human CrkII. In the absence of siRNA, CrkII was robustly expressed following transient transfection with a CrkII cDNA expression vector for 24 hours and this was reduced in the presence of siCrk1 (Fig. 2B). It should be noted that this reduction was not to endogenous levels, suggesting that the siCrk1 may not be sufficient to completely ablate the high levels of expression from the CrkII cDNA plasmid. In HeLa cells, endogenous CrkII protein is readily detectable whereas CrkI is only expressed at low to nondetectable levels. To examine the ability of siRNAs to knock down endogenous Crk protein levels, HeLa cells were transfected with increasing concentrations of siCrk1, with a threshold of 100 pmol/L (Fig. 2C). CrkII protein levels, 24 hours posttransfection, were efficiently suppressed in a dose-dependent manner (Fig. 2C).

Under all experimental conditions, levels of growth factor receptor binding protein 2 (Grb2) in cell lysates remained similar, indicating that overall protein levels were not decreased by siCrk1 treatment (Fig. 2C). Transfection with a control nontargeting siRNA did not reduce CrkII protein levels (Fig. 2D). The decrease in CrkII protein levels was observed by Western blot analysis to last up to 5 days (120 hours) posttransfection (Fig. 2E). Optimal conditions for transfection with siRNA were established using HeLa cells and were subsequently applied to multiple human cancer cell lines including MDA-231, MCF-7, and MDA-435s breast cancer cell lines, KB oral epithelial cancer cells, and H1299 lung cancer cells. All cell lines examined express CrkI and CrkII and although the levels of CrkI and CrkII vary in response to siCrk1, suppression of Crk was observed in all cancer cell types examined (Fig. 2F).

We examined the specificity of siCrk1 and siCrk2 in MDA-231 cells as both siRNAs are targeted to different regions of the CRK gene (Fig 2A). Endogenous CrkI and CrkII are readily detected by Western blot analysis of total proteins in MDA-231 cells, and protein levels for both were decreased following transient transfection with either siCrk1

| Table 1. Patient Cohorts Used to Examine CrkI/II Protein Expression Levels by Immunohistochemistry |
|---------------------------------|-----------------|-----------------|
| No. patients | Stage I | Stage III |
| No. ductal adenocarcinoma | 8 | 12 |
| No. lobular adenocarcinoma | 8 | 8 |
| % 5-y survival | 87.5 | 50 |
| Median overall survival (d) | 3,360 | 1,733 |
| No. recurrences | 2 | 5 |
| Median disease-free survival (d) | 3,360 | 905 |

*$\chi^2$ test, $P = 0.853.$
or siCrk2 (Fig. 3A), however, complete ablation of detectable CrkI or CrkII protein was not observed. To assess the effect of transfection efficiency and the cellular uptake of siRNA, Cy3-labeled siRNAs were transiently transfected into MDA-231 breast cancer cells. As shown in Fig. 3B, nearly 100% of MDA-231 cells are positive following transient transfection with Cy3-labeled siRNAs. To assess whether suppression of Crk protein levels had an effect on apoptosis or impaired cell proliferation during the time course of our biological assays, cell proliferation rates were analyzed over a 72-hour period in MDA-231 mock cells and siCrk-transfected cells, during which time CrkI/II protein levels remain suppressed. During that period, no difference in cell number was observed between the two cell types (Fig. 3C). The induction of early apoptotic events was assayed by Annexin V staining in both HeLa and MDA-231 cells. Forty-eight hours posttransfection, control cells transfected with lipid alone and siCrk1-transfected cells were incubated for 15 minutes with 100 nL of Annexin V-fluorescein. As a positive control for apoptosis, cells were exposed to 50 J/m² UV light and incubated at 37°C overnight, and assayed in parallel with siRNA-transfected cells. Annexin V staining confirmed that both HeLa and MDA-231 cells underwent activation of apoptotic events when exposed to 50 J/m² UV light. However, no significant activation of early apoptotic events was observed in either mock transfected cells or siCrk-transfected cells (Fig. 3D). These results show that the decrease in Crk protein levels observed following transfection with siCrk does not promote apoptosis or alter proliferation of MDA-231 cells maintained in serum.

Migration of Multiple Cancer Cell Lines Is Decreased following CrkI/II Small Interfering RNA Treatment

Previous studies have shown that overexpression of Crk enhances cell motility and promotes epithelial dispersal. However, the overexpression of mutant Crk proteins acting as dominant interfering proteins decreased these biological responses, implicating Crk in the transmission of integrin-dependent or growth factor–dependent signals for cell migration (4-6, 32-35). To assess whether Crk adaptor proteins act as critical integrators of signals for cell migration in human cancer cell lines, siRNA duplexes targeting CrkI/II, as described above, were transfected into HeLa, MDA-231, MDA-435s, KB, and H1299 cells and their effect on cell migration and invasion was assayed. The inherent migration capacity of these cell lines in serum was assayed 48 hours following transfection with siCrk using transwell migration assays as previously described (23, 36). The degree of "knockdown" of the CRK gene products, CrkI and CrkII, was assayed for each experiment where half of transfected cells were processed for biochemistry. For each cancer cell line tested, a significant decrease in cell migration was observed after 24 hours (Fig. 4A). For example, migration of breast cancer cell lines, MDA-231 and MDA-435s, was routinely decreased by 75% following transfection with siCrk. A similar decrease in migration of 50% was observed for the H1299 and KB cell lines, whereas the migration of HeLa cells transfected with siCrk was consistently reduced by 30% when compared with control transfected cells or HeLa cells transfected with a nontargeting siRNA (Fig. 4A). All experiments were done at least thrice per cell line, and all showed comparable results with P < 0.05. For all cell lines tested, a decrease in CrkII and CrkI protein levels was observed (Fig. 4B).

Overexpression of Crk enhances epidermal growth factor–induced cell dispersal and tubulogenesis (5), and dominant negative mutants of Crk block HGF-induced cell scatter in Madin-Darby canine kidney cells (6). To determine if Crk is required for HGF-enhanced cell migration in aggressive cancer cells, we treated MDA-231 and HeLa cells with siCrk1 or siCrk2 in the presence or absence of HGF (34 ng/mL). HGF stimulation enhanced migration of MDA-231 cells by ~50% when compared with their unstimulated counterparts (Fig. 4C). In a similar manner to unstimulated MDA-231 cells, Crk

---

**FIGURE 1.** Immunohistochemistry analysis of CrkI/II proteins in stage I and III breast cancers. Paraffin sections of tumor specimens were analyzed by immunohistochemistry using an anti-Crk monoclonal antibody that recognizes both CrkI and CrkII (black arrows) and counterstained with hematoxylin. Digital photographs were captured using a 40× objective.
siRNA pretreatment decreased HGF induced migration by 60% (Fig. 4C). Similar results were obtained with HeLa cells (data not shown). These results support a role for Crk adaptor proteins as central integrators for upstream signals involved in cell migration. These results also highlight a possible role for Crk proteins in cell invasion.

**Crk Small Interfering RNA Inhibits Invasion of Multiple Cancer Cell Lines**

To assay the effect of Crk knockdown on the ability of cancer cells to invade an extracellular matrix, the invasion of cancer cells transfected with siCrk in Fig. 4A was examined using transwells coated with 100 μg/cm² of matrigel. Forty-eight hours posttransfection, 5 × 10⁴ cells/mL were seeded on top of the matrigel coating and incubated for 24 hours. Cells that invade through the matrix are visualized by staining the lower membrane with 0.1% crystal violet in 20% methanol and quantified. The ability of MDA-231, MDA-435s, H1299, and KB cells to invade through matrigel was decreased up to 80% compared with control cells when transfected with siCrk, whereas that of HeLa cells was decreased by 60% (Fig. 5A). All experiments were done at least thrice per cell line, where all showed comparable results with \( P < 0.05 \). In all cases, transfection with the control siRNA gave no significant decrease in cell invasion nor a decrease in CrkII protein levels (Fig. 5B). Hence, depletion of CrkI/II proteins by siCrk decreased cell invasion through matrigel in a similar manner to cell migration. To further assess the selectivity of action of Crk siRNA, we examined human breast cancer T47D cells that show increased invasion following overexpression of CrkII (Fig. 5B; ref. 6). Control T47D cells show a decrease in cell invasion following transfection with siCrk1 (28%, Fig. 5C); moreover, the enhanced cell invasion of CrkII overexpressing cells was reversed following transfection with siCrk1 (Fig. 5C).

**CrkI/II Small Interfering RNA Decreases Cell Adhesion**

Cell migration requires the ability to form and break focal adhesion complexes, a process that occurs during cell adhesion and spreading. To examine the effect of siCrk1 on cell adhesion and spreading, MDA-231 cells were trypsinized and replated on fibronectin 48 hours posttransfection and subjected to videomicroscopy. Time lapse videomicroscopy of mock and siCrk-transfected MDA-231 cells revealed that mock control cells begin to spread and develop lamellipodia by 10 minutes post-plating and by 30 minutes have developed large lamellipodia (Fig. 6A). In contrast, siRNA-transfected cells display smaller lamellipodia and cells retain a round and refractile morphology for a prolonged time, when compared with control cells, consistent with decreased spreading. Moreover, when assayed for attachment and spreading at later times, cells transfected with siCrk formed less stable adhesions and detached from the substratum (Fig. 6B and C).

To examine the consequence of CrkI/II knockdown on the actin cytoskeleton and focal adhesions, we examined the localization of focal adhesion targeting proteins paxillin and vinculin by indirect immunofluorescence. MDA-231 cells transfected with siCrk1 were trypsinized and replated onto...
fibronectin-coated coverslips 48 hours posttransfection. When fixed 16 hours post-plating, control MDA-231 cells had developed abundant actin stress fibers, as visualized by phalloidin, whereas siCrk1-transfected cells showed reduced actin stress fibers throughout the cell. Bundled actin was still observed within lamellipodium-like structures at the cell periphery. Consistent with a decrease in actin stress fibers, siCrk1-transfected cells show reduced focal adhesions as

---

**FIGURE 3.** CrkI/II targeting siRNAs specifically knock down protein levels in a panel of tumor cell lines. A. MDA-231 cells were transfected with CrkI/II and protein lysates were analyzed by Western blot using antibodies against CrkI/II. Actin protein levels were used as a loading control. An additional construct of CrkI/II siRNA was designed and its protein knockdown efficiency was evaluated by Western blot analysis. Actin protein levels were used as a loading control. B. CrkI/II siRNAs were labeled with a CY3 marker and transfection efficiency was assessed by indirect immunofluorescence. All cells show CY3 staining, indicating that CrkI/II siRNAs were readily transfected into MDA-231 cells. Images were acquired using a Zeiss confocal microscope and a 63× objective. C. Transfection of CrkI/II siRNAs does not affect cell proliferation. In MDA-231 cells transfected with lipid alone and CrkI/II siRNAs, cell proliferation rates were analyzed. Cell counts were taken every 20 hours. No significant change in cell proliferation was observed posttransfection with CrkI/II siRNAs. D. Transfection of CrkI/II siRNA does not affect cell death. HeLa and MDA-231 cells were transfected as previously described. Positive controls were exposed to 50 J/m² of UV radiation and incubated overnight. Cells were analyzed 48 hours posttransfection for early-apoptotic stages using Annexin V staining and for necrosis using propidium iodide (data not shown). Both assays showed that the transfection with siRNAs did not induce cell death. Images were obtained using a 10× objective on a Zeiss AxioVert 135 microscope.
observed through reduced vinculin and paxillin staining when compared with control cells (Fig. 7). Together, these results support a role for Crk proteins for the establishment or maintenance of strong cell-matrix adhesions.

**Discussion**

Despite the accumulation of evidence for a function of Crk adaptor proteins in cell adhesion and cell migration, its significance in inherently motile cancer cells still remains elusive. Much of what has been proposed about CrkI/II in epithelial cells is based on overexpression studies or the use of dominant interfering mutants of CrkI/II. These approaches, although informative, are limited by the challenges inherent in overexpression systems. In this article, we report that knockdown of CrkI/II expression by RNA interference leads to a dramatic decrease in cell migration and invasion in multiple human tumor cell lines. Duplex siRNA–based
methods have the advantage of being able to nearly abrogate RNA expression and proteins of CrkI and CrkII and permit the consequences to be examined in multiple tumor cell lines (Fig. 2). Moreover, the acute and transient effects of siRNA-mediated CrkI/II down-regulation provide an alternative experimental approach to study the functions of CrkI and CrkII, avoiding the possible effect of compensatory mechanisms developed in knockout mice.

The present study establishes that transfection with CrkI/II siRNA duplexes attenuates CrkI and CrkII protein expression levels (Figs. 2 and 3), without affecting the expression of endogenous proteins, such as actin or another adaptor protein, Grb2, demonstrating the specificity of this gene targeting approach. Furthermore, the transfection with an siRNA duplex designed against a separate region of the CRKI/II gene was similarly effective at specifically knocking down CrkI/II protein expression.

**FIGURE 5.** Crk siRNA inhibits invasion of cancer cells. A. Assays for cell invasion were carried out 48 hours posttransfection using 5 × 10⁴ cells. Cells were seeded onto matrigel covered boyden chambers and assayed. Cells remaining on bottom of the porous membrane were fixed in formalin phosphate, stained with 0.2% crystal violet, extensively washed with H₂O, and left to dry overnight. Using a Retiga 1300 digital camera and a Zeiss AxioVert 135 microscope, bottom layers of the transwell were imaged in five separate fields for each condition using a 10× objective in phase contrast. Image analysis of these assays was carried out using Northern Eclipse and Scion Image. A minimum of three experiments was done. Bars, SD of the three experiments. B. For each experiment, the extent of CrkI/II knockdown was assessed by Western blot analysis. Cells were trypsinized 48 hours posttransfection and either seeded into boyden chambers for invasion or lysed to estimate CrkI/II protein levels. Proteins derived from whole cell lysates were separated by PAGE, transferred to a membrane, and immunoblotted with anti-CrkI/II sera or actin. C. To confirm the specificity of siCrk1 within a biological context, we examined the invasion capacity of T47D cancer cells and those that overexpress CrkII compared with their siCrk1-transfected counterparts, using the assay described above. A minimum of three experiments was done. Bars, SD of the three experiments.
levels (siCrk2, Fig. 3A). Annexin V assays and proliferation counts confirmed that CrkI/II knockdown did not significantly enhance cell death or decrease cell proliferation throughout the duration of our assays (Fig. 3).

A major function assigned to CrkI and CrkII from overexpression studies is an enhanced signal for cell migration (6, 23, 25, 27). The effect of Crk knockdown on migration and invasion of single cells, as evaluated using Boyden chamber transwell assays, revealed a significant decrease in the migration and invasion capacity of various highly invasive human cancer cell lines as well as a decrease in HGF-enhanced migration (Figs. 4 and 5). These results are consistent with a role for Crk as a modulator of intracellular signals downstream from various growth factor receptors, such as the Met/HGF receptor (6, 37), vascular endothelial growth factor receptor (38), and ErbB2/Neu receptor (24), many of which are activated in human cancers (39). Although the signals that promote the invasive phenotype of these cell lines have not been characterized, these data show that Crk adaptor proteins play an important role in integrating signals for migration and invasion of highly migratory and invasive human cancer cell lines (Figs. 3-6).

Cell motility is a complex event that is dependent on the coordinated remodeling of the actin cytoskeleton and the regulated assembly and turnover of focal adhesions (for review see ref. 40). Crk knockdown resulted in decreased adhesion and spreading of cells as visualized by time-lapse microscopy (Fig. 6). Time-lapse microscopy of CrkI/II siRNA–transfected MDA-231 carcinoma cells further confirmed that a decrease in total levels of Crk proteins interferes with the ability of MDA-231 cells to form membrane protrusions, an event required during cell spreading, adhesion, and cell motility (Fig. 6). Such biological defects may result from the inability of these cells to sustain strong focal contacts with the extracellular matrix or to maintain actin polymerization at these sites.

**FIGURE 6.** CrkI/II targeting siRNAs decrease cell adhesion and spreading. White arrows, lamellipodia and membrane protrusions in spreading cells. A. Time-lapse video-microscopy of MDA-231 cells transfected with CrkI/II siRNAs adhering to a fibronectin substrate. Forty hours posttransfection, cells were trypsinized and plated onto fibronectin-coated 35-mm plates and time-lapse video-microscopy was done using a Zeiss AxioVert 135 microscope and Northern Eclipse software. Frames were captured every 2 minutes. Transfection efficiency was analyzed by both immunofluorescence and Western blot analysis (data not shown). B. MDA-231 cells were transfected with siRNAs as previously described. Cells were trypsinized 48 hours posttransfection and replated on coverslips coated with 20 μg/mL fibronectin. Cells were washed and fixed in 4% paraformaldehyde at indicated time points. Images were captured using a Zeiss AxioVert 135 microscope and image analysis was carried out using Northern Eclipse software. C. Quantification of fixed time points in Fig. 6B). Cell numbers were acquired by manual counting using Northern Eclipse.
Adhesion to the extracellular matrix is mediated at focal adhesion sites through clustering of integrins and cytoskeletal linking proteins, such as vinculin, filamin, α-actinin, actopaxin/parvin, and paxillin (reviewed in ref. 41). Paxillin localization at these sites is concomitant with the ability to form strong or stable focal adhesions required during cell migration and adhesion (4, 42-44). Crk adaptor proteins form a complex dependent on tyrosine phosphorylation with both paxillin and p130Cas, two proteins recruited to focal adhesions (4, 45, 46). Moreover, the overexpression of Crk enhances the formation of a complex of Crk with paxillin, Git2 and Pix, a Rac exchange factor, as well as elevated Rac activity in response to exogenous growth factor stimulation (4, 5). In support of reduced adhesion of Crk knockdown in MDA-231 cells, the analysis of endogenous paxillin and vinculin by confocal microscopy showed a decrease in the redistribution of paxillin and vinculin to focal adhesions, and a decrease in overall focal adhesions following the plating of cells on fibronectin (Fig. 7). Consistent with a reduced number of focal adhesions, we observed an overall decrease in bundled actin in cells transfected with siCrk (Fig. 7). Hence, knockdown of Crk proteins decreases the recruitment or maintenance of cytoskeletal proteins, such as paxillin, to focal adhesions, thereby reducing complexes required for the establishment of strong focal points and actin bundling. A similar phenotype was observed for paxillin- and p130Cas-null fibroblasts (42, 47, 48). In agreement with a role for Crk in the formation and/or maintenance of focal adhesions, epithelial Madin-Darby canine kidney cells that overexpress Crk contain enhanced numbers of focal adhesions (4).

The observation that cell migration and invasion of multiple human cancer cells is specifically decreased in the presence of Crk siRNA establishes Crk as a key integrator of signals involved in cell migration and invasion of highly invasive human cancer cells. This has important implications where elevated levels of Crk are observed in various human cancers, including glioma (26), lung cancer (28), as well as breast cancers (Fig. 1). In the breast cohort series, elevated Crk immunostaining was identified in breast adenocarcinoma and was evident in the cytoplasm and nucleus of the cancer cells (Fig. 1). Using this staining protocol, elevated Crk levels were detected in 60% of the tumors, which included stage I and stage III disease (Fig. 1). This supports the data of Miller et al. (28), who showed a similar relationship between Crk and lung cancer at the mRNA level. The relationship between elevated Crk and a worse overall survival in lung carcinoma patients could reflect a role for Crk in the development of a more clinically aggressive phenotype. Hence,
our findings, demonstrating a role for Crk as a key integrator of signals for cell migration and invasion, identify a potential role for Crk in metastatic progression. Our results also highlight the need for further analysis of Crk status in human tumors.

Materials and Methods
Reagents and Antibodies
Monoclonal antibodies that recognize both Crkl and CrkII and monoclonal antibodies raised against paclitaxel were purchased from BD Transduction Laboratories (Lexington, KY). Antibodies against vinculin were purchased from Sigma-Aldrich (Oakville, Canada). Alexa Fluor 555-phalloidin as well as secondary goat antibodies against mouse conjugated Alexa Fluor 488 were purchased from Molecular Probes (Eugene, OR). Complete human cDNAs were purchased from Open-Biosystems (Huntsville, AL). The CrkII clone used, BC008506, was inserted in the mammalian expression vector pCMVSPORT6. The pOTB7-CrkI clone used was BC009837. Two siRNA duplex sequences were designed to target the CrkI/II gene. The siRNA sequences targeting the CrkI/II human mRNA corresponded to the coding region 264-284, 5'-AAUAGGAGAUCAGAGUUAUUA-3' and 5'-UCAAUCUCUUGTUCUCUTU-3' [duplex previously published in Nagashima et al. (29)], or coding region 465-485, 5'-AGGAGACAUCUGAGAAUCdTdT-3' and 5'-GAUUCUCAAGUGUCUCUTdTdT-3' (designed using siRNA Design Center from Dharmaco Research, Inc., Lafayette, CO). The nontargeting RNA-induced silencing complex–free siRNA used as a control was designed by Dharmaco. This construct is chemically modified to impair processing by the RNA-induced silencing complex. All of the siRNA duplexes were synthesized, formulated to impair processing by the RNA-induced silencing complex, annealed, and purified by Dharmaco Research using 2'-ACE protection chemistry. Cy3 labeling of siRNAs was done using a Cy3-siRNA labeling kit (Ambion, Austin, TX).

Immunohistochemistry
Paraffin-embedded tissues were sectioned and pretreated in a microwave for 5 minutes for antigen retrieval. Immunostaining was done with a Ventana Immunostainer (Ventana Medical System, Inc., Tucson AZ), using the monoclonal Crk antibody that recognizes both Crkl and CrkII isofoms, diluted 1:200 in EDTA (BD Transduction Laboratories) as the primary antibody. Peroxidase-labeled anti-rabbit immunoglobulin was used as secondary antibody and diaminobezidin as substrate. Each individual tumor was stained for Crk and scored for intensity (none, low, moderate, and high) separately by a pathologist and a surgeon. Controls of adjacent normal epithelium were used. A statistically significant difference was set at \( P < 0.05 \).

Western Blot
Cell lines were grown to 80% confluency and lysed in 1% Triton X-100 lysis buffer containing 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EGTA, 1.5 mmol/L MgCl2, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na3VO4, 50 mmol/L NaF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. Whole cell lysates were resolved by SDS-PAGE in 12% gels. Western blot was done as previously described (49) by transferring the separated proteins onto a Hybond-enhanced chemiluminescence nitrocellulose membrane from Amersham Biosciences (Baie d’Urfe, Canada).

Cell Culture and Transfections
HeLa, H1299, KB, T47D, T47D-CrkII, and MDA-231 cells were maintained in culture in DMEM, whereas MDA-435s cells were maintained in Leibowitz media, all containing 10% fetal bovine serum and 50 μg/mL gentamicin (Invitrogen Canada, Inc., Burlington, Canada). Cells (3 \times 10^5 cells/mL) were plated in 12-well plates 24 hours before transfection. A total of 100 pmol/L of siRNA was transfected per well using Lipofectamine and Plus reagents (Invitrogen) as per protocol of the company. Transfected cells were trypsinized and used in all assays 48 hours posttransfection. For immunofluorescence, 1.5 \times 10^4 cells/mL were seeded on glass coverslips in 24-well plates and the amount of siRNA transfected per well was 60 pmol/L.

Migration and Invasion Assays
Cells (5 \times 10^4) were counted and seeded directly onto 6.5-mm Corning Costar transwells for migration or transwells coated with 100 μg/cm² matrigel (BD Biosciences, San Jose, CA) for the invasion assays. Complete media was added to both the top and bottom wells and cells were incubated at 37°C overnight. For HGF stimulations, 34 ng/mL of HGF was added to the bottom wells. Following the overnight incubation, cells on both sides of the transwells were fixed using formalin phosphate for 20 minutes at room temperature. After washing with double-distilled water, cells were stained with 0.1% crystal violet in 20% methanol for 20 minutes at room temperature. Cells on the top layer were scraped and membranes were left to dry overnight. Images were captured using a Retiga 1300 digital camera (QIMAGING, Burnaby, Canada) and a Zeiss AxioVert 135 microscope (Carl Zeiss Canada Ltd., Toronto, Canada). Image analysis of these assays was carried out using Northern Eclipse version 6.0 (Empix Imaging, Mississauga, Canada) and quantification was done by using a constant threshold for all images and measuring pixels using Scion Image-NIH equivalent program for Microsoft Windows (Scion Company, Frederick, MD).

Adhesion and Spreading Assays
MDA-231 cells were plated on coverslips (for time point experiments) or 35-mm dishes (time lapse) previously coated with 20 μg/mL fibronectin (Ville Mont-Royal, Quebec, Canada) in PBS (for 30 minutes). Coverslips were washed with PBS at each time point and fixed. Images were acquired using a Retiga 1300 digital camera (QIMAGING) and a Zeiss AxioVert 135 microscope (Carl Zeiss Canada). Image analysis was carried out using Northern Eclipse version 6.0 (Empix Imaging). Time-lapse microscopy done during adhesion assays was captured using Northern Eclipse.

Indirect Immunofluorescence
Cells were fixed in 4% paraformaldehyde for 20 minutes and permeabilized using 0.2% Triton X-100 in PBS for 10 minutes. Coverslips were rinsed with 100 mmol/L glycine and blocked with 2% bovine serum albumin buffer (containing 0.2%
Triton X-100 and 0.05% Tween 20 in PBS) for 30 minutes. Primary antibodies diluted in blocking buffer were incubated for 1 hour. Paxillin antibody was diluted 1:200 and vinculin antibodies were diluted 1:400. All secondary antibodies were diluted 1:1,000 as well as Alexa 488-phalloidin. To counterstain nuclei, cells were incubated with 0.5 ng/mL 4′,6-diamidino-2-phenylindole for 5 minutes. Coverslips were mounted on glass slides using Immumount (Fisher Scientific, Nepean, Canada). All steps were carried out at room temperature. Images were acquired using a Retiga 1300 digital camera (QIMAGING) and a Zeiss confocal microscope.

Acknowledgments

We thank members of the Park laboratory for helpful comments and support. HGF was kindly provided by Dr. George Vande Woude, Van Andel Research Institute, Grand Rapids, Michigan.

References


7. Matsuda M, Reichman CT, Hanafusa H. Biological and biochemical activity of c-Crk homologue of DOCK180/CED-5, is required in a Rac-specific phospholipase C-


Crkl and CrkII Function as Key Signaling Integrators for Migration and Invasion of Cancer Cells


Updated version  Access the most recent version of this article at: http://mcr.aacrjournals.org/content/3/4/183

Cited articles  This article cites 49 articles, 29 of which you can access for free at: http://mcr.aacrjournals.org/content/3/4/183.full#ref-list-1

Citing articles  This article has been cited by 15 HighWire-hosted articles. Access the articles at: http://mcr.aacrjournals.org/content/3/4/183.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://mcr.aacrjournals.org/content/3/4/183. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.