Crk Adapter Proteins Promote an Epithelial–Mesenchymal-like Transition and Are Required for HGF-mediated Cell Spreading and Breakdown of Epithelial Adherens Junctions

Louie Lamorte,* Isabelle Royal,^{†‡} Monica Naujokas,[†] and Morag Park^{*†§||}

Molecular Oncology Group, McGill University Hospital Center, Departments of *Biochemistry,
[†]Medicine and [§]Oncology, McGill University, Montreal, Quebec, Canada H3A 1A1

Submitted October 1, 2001; Revised December 20, 2001; Accepted January 18, 2002
Monitoring Editor: Daniel Goodenough

Activation of the Met receptor tyrosine kinase through its ligand, hepatocyte growth factor (HGF), promotes an epithelial–mesenchymal transition and cell dispersal. However, little is known about the HGF-dependent signals that regulate these events. HGF stimulation of epithelial cell colonies leads to the enhanced recruitment of the CrkII and CrkL adapter proteins to Met-dependent signaling complexes. We provide evidence that signals involving CrkII and CrkL are required for the breakdown of adherens junctions, the spreading of epithelial colonies, and the formation of lamellipodia in response to HGF. The overexpression of a CrkI SH3 domain mutant blocks these HGF-dependent events. In addition, the overexpression of CrkII or CrkL promotes lamellipodia formation, loss of adherens junctions, cell spreading, and dispersal of colonies of breast cancer epithelial cells in the absence of HGF. Stable lines of epithelial cells overexpressing CrkII show enhanced activation of Rac1 and Rap1. The Crk-dependent breakdown of adherens junctions and cell spreading is inhibited by the expression of a dominant negative mutant of Rac1 but not Rap1. These findings provide evidence that Crk adapter proteins play a critical role in the breakdown of adherens junctions and the spreading of sheets of epithelial cells.

INTRODUCTION

The acquisition of a mesenchymal phenotype by epithelial cells, a process termed epithelial–mesenchymal (EM) transition (Boyer *et al.*, 2000), is required for morphogenesis and tissue remodeling during development (Guarino, 1995). EM transitions are regulated in part through the actions of growth factors, the extracellular matrix, and cell–cell adhesion proteins (Prieto and Crossin, 1995; Boyer *et al.*, 2000). Although tightly controlled during development, nonscheduled EM transitions in the adult organism can lead to the development and progression of human malignancies. In some cancers, this occurs through downregulation or muta-

tion of E-cadherin or β -catenin (Behrens, 1999). In addition, the dissolution of cadherin-based junctional complexes is also promoted by growth factors and their receptors and oncogenes such as Ras (Boyer *et al.*, 2000).

Hepatocyte growth factor (HGF) is a multifunctional factor, which, in addition to promoting epithelial cell growth and survival (Matsumoto and Nakamura, 1997), is a potent modulator of EM transition (Weidner *et al.*, 1993; Zhu *et al.*, 1994). In two-dimensional cultures, HGF stimulates the breakdown of cell–cell junctions and dispersal of sheets of epithelial cells, increasing their invasiveness (Stoker *et al.*, 1987; Weidner *et al.*, 1990). HGF and its receptor, the Met receptor tyrosine kinase, are deregulated or overexpressed in many human tumors (Lamorte and Park, 2001). For example, amplification of the Met receptor tyrosine kinase has been found in gastric carcinomas (Nakajima *et al.*, 1999) and gliomas (Koochekpour *et al.*, 1997). Activating point mutations within the kinase domain of the Met receptor tyrosine kinase have been characterized in hereditary and sporadic papillary renal carcinomas (Schmidt *et al.*, 1997). Moreover, overexpression of HGF and/or the Met receptor is associated with a poor prognosis for breast cancer patients (Jin *et al.*, 1997; Ghoussoub *et al.*, 1998; Camp *et al.*, 1999) and transgenic mice overexpressing HGF develop tumors with

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.01–10–0477. Article and publication date are at www.molbiol-cell.org/cgi/doi/10.1091/mbc.01–10–0477.

^{||} Corresponding author. E-mail address: morag@lan1.molonc.mcgill.ca.

[†] Present address: Ste-Justine Hospital, Department of Pediatrics, University of Montreal, Montreal, Quebec, Canada.

Abbreviations used: EM, epithelial–mesenchymal; FBS, fetal bovine serum; HGF, hepatocyte growth factor; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3'-kinase; SH2, Src homology 2; SH3, Src homology 3.

metastatic lesions (Takayama *et al.*, 1997; Otsuka *et al.*, 1998). These results underscore the importance of defining the mechanisms involved in promoting HGF-dependent EM transitions.

At the cellular level, stimulation of the Met receptor tyrosine kinase induces the remodeling of the actin cytoskeleton, cell spreading, and the breakdown of cell-cell junctions (Ridley *et al.*, 1995; Royal and Park, 1995; Potempa and Ridley, 1998; Royal *et al.*, 2000). Rac1 and Cdc42 are small GTP binding proteins involved in the remodeling of the actin cytoskeleton (Hall, 1998) and are activated in response to HGF (Royal *et al.*, 2000). Rac1 mediates the formation of lamellipodia and membrane ruffles, whereas Cdc42 regulates the formation of filopodia (Hall, 1998). Both Rac1 and Cdc42 are critical for the spreading of Madin-Darby canine kidney (MDCK) epithelial cells stimulated with HGF (Ridley *et al.*, 1995; Royal *et al.*, 2000). Rac1 activation in response to HGF is dependent on phosphatidylinositol 3'-kinase (PI3K), as the pretreatment of cells with inhibitors of PI3K blocks HGF-stimulated activation of Rac1, cell spreading, and dispersal (Royal and Park, 1995; Royal *et al.*, 2000). In addition, inhibition of PI3K prevents the breakdown of adherens junctions after the stimulation of MDCK cells with HGF (Royal and Park, 1995; Potempa and Ridley, 1998).

Studies using different model systems have shown that overexpression of CrkII or CrkL enhances cell migration (Klemke *et al.*, 1998; Uemura and Griffin, 1999; Cho and Klemke, 2000; Petit *et al.*, 2000; Spencer *et al.*, 2000). The CrkII adapter protein was originally identified as a viral oncogene, *v-crk* (Mayer *et al.*, 1988) and is composed of one Src homology 2 (SH2) and two Src homology 3 (SH3) domains (SH2-SH3-SH3; Reichman *et al.*, 1992). A second Crk-like gene, CrkL, was later isolated and it contains a similar modular structure as CrkII (ten Hoeve *et al.*, 1993). The Crk SH2 domain binds several tyrosine-phosphorylated proteins: p130Cas, paxillin, Cbl, and Gab1 (Feller, 2001), whereas the amino terminal SH3 domain binds C3G, DOCK180, and Abl (Feller, 2001). More recently, it was shown that DOCK180 can bind and activate Rac1 (Kiyokawa *et al.*, 1998; Nolan *et al.*, 1998), and genetic studies in *Caenorhabditis elegans* have demonstrated a role for CrkII and DOCK180 in the activation of Rac1 and cell migration (Reddien and Horvitz, 2000).

Although previous studies have examined the involvement of CrkII or CrkL in the migration of single cells, they did not address the involvement of CrkII or CrkL in the dispersal of sheets of epithelial cells, an event critical for metastasis. We demonstrate a role for CrkII and CrkL in the formation of lamellipodia, the spreading of colonies, and the breakdown of adherens junctions in epithelial MDCK cells in response to HGF. In addition, we show that in the absence of HGF, CrkII, or CrkL overexpression promotes lamellipodia formation, cell spreading, and the breakdown of adherens junctions in MDCK cells and cell dispersal in well-differentiated breast carcinoma cells.

MATERIALS AND METHODS

Materials and Antibodies

HGF was provided by Dr. George Vande Woude (Van Andel Research Institute, Grand Rapids, MI), and Dr. Michel Tremblay (McGill University) provided a polyclonal p130Cas antibody. Crk antibodies recognizing both CrkI and CrkII were purchased from

BD Transduction Laboratories (Lexington, KY). Crk antibodies raised against either CrkII (C-18) or CrkL (C-20) along with Cbl, C3G, and Rap1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Paxillin and Rac1 antibodies and a phosphotyrosine antibody conjugated to horseradish peroxidase (RC20H) were purchased from BD Transduction Laboratories. HA.11 and c-Myc (9E10) antibodies were obtained from Berkeley Antibody Company (Berkeley, CA). ZO-1 antibodies were purchased from Zymed Laboratories, Inc. (South San Francisco, CA). Vinculin antibodies were purchased from Sigma (Oakville, ON, Canada). AlexaFluor488 phalloidin, Texas Red-X phalloidin, and secondary antibodies conjugated to AlexaFluor488 were purchased from Molecular Probes (Eugene, OR). Secondary antibodies conjugated to CY3 were obtained from Jackson ImmunoResearch Labs (West Grove, PA). All molecular biology products were purchased from New England BioLabs Inc. (Mississauga, ON, Canada).

Plasmids

Dr. Bruce Mayer (University of Connecticut Health Center, Farmington, CT) provided pEBB, pEBB-CrkI W170K, pEBB-CrkI R38K/W170K, and pEBB-Crk II. CrkII was subcloned as a *Bam*HI/*Not*I fragment into pLXSH and used for retroviral infection of T47D cells. Dr. John Groffen (Children's Hospital of Los Angeles Research Institute, Los Angeles, CA) provided SV40-CrkL. Dr. Michel Tremblay (McGill University) provided pcDNA3-p130Cas. Dr. Alan Hall (University College London, London, United Kingdom) provided pRK5-mycN17Rac1 and pRK5-mycN17Rap1.

Cell Culture

MDCK and T47D cells were maintained in DMEM containing 10% fetal bovine serum (FBS) and 50 µg/ml gentamicin (Invitrogen Canada Inc., Burlington, ON, Canada). To generate MDCK cells overexpressing CrkII, cells were transfected in a six-well plate with 1.8 µg of pEBB-CrkII and 200 ng of pSV2neo using GenePorter (Gene Therapy Systems, San Diego, CA). Cells were selected in 400 µg/ml Geneticin (Invitrogen Canada Inc.), and stable clones were isolated and screened by Western blotting. Populations of T47D cells expressing CrkII or empty vector were obtained by retroviral infection with pLXSH-CrkII or pLXSH, respectively, as described in Rodrigues and Park (1993). Cells were selected in 125 ng/ml Hygromycin B (Roche Diagnostics, Laval, PQ, Canada), and several hundred colonies were pooled together. 293T transient transfections were performed using calcium phosphate. Cells were serum starved 48 h later for 20 h in DMEM containing 0.1% FBS and lysed the next day in 1.0% Triton X-100 lysis buffer as described below.

Microinjection

MDCK cells (7×10^3) were plated on glass coverslips (Bellco Glass, Vineland, NJ) 3 days before microinjection. DNA plasmids were diluted in phosphate-buffered saline (PBS) as indicated in the figure legends. Occasionally, rabbit immunoglobulin G (Pierce, Rockford, IL) was included at a concentration of 0.6 µg/µl to detect injected cells. Small colonies of 10–50 cells were injected using an Eppendorf Micromanipulator (Eppendorf Scientific, Westbury, NY). Microinjected cells were incubated for 4 hours before stimulation with HGF for an additional 4 hours. For experiments with CrkII or CrkL, cells were incubated for 5 hours after microinjection and fixed as described below.

Indirect Immunofluorescence

Cells were fixed for 15 min in 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. For vinculin staining, cells were incubated for 10 min in 0.25× CSK buffer (2.5 mM 1,4-piperazinediethanesulfonic acid, pH 7.0, 75 mM sucrose, 12.5 mM NaCl, 0.75 mM MgCl₂, and 0.125% Triton X-100) at room temperature and then fixed as described above. Nonspecific binding sites on the cells were

blocked with 1% bovine serum albumin for 30 min. Primary and secondary antibodies were added successively, each for 30 min, with extensive washing between each incubation. HA.11 antibodies were diluted 1:300, 9E10 antibodies were diluted 1:800, vinculin antibodies were diluted 1:400, and Crk, CrkL, β -catenin, and E-cadherin antibodies were diluted 1:200. Secondary antibodies were diluted 1:1000. Both AlexaFluor488 phalloidin and Texas Red-X phalloidin were used at a 1:1000 dilution. All reagents were diluted in PBS supplemented with 1 mM $MgCl_2$ and 1 mM $CaCl_2$, with the exception of phalloidin, which was diluted in PBS supplemented with 0.2% Triton X-100. For experiments where cells were microinjected with rabbit immunoglobulin G, donkey α -rabbit antibodies conjugated to AlexaFluor488 were used to detect injected cells. Coverslips were mounted onto glass slides using Immunofluore mounting medium (ICN, St. Laurent, PQ, Canada). Images were acquired using a Retiga 1300 digital camera (QIMAGING, Burnaby, BC, Canada) and a Zeiss AxioVert 135 microscope (Carl Zeiss Canada Ltd., Toronto, ON, Canada). Image analysis was carried out using Northern Eclipse version 6.0 (Empix Imaging, Mississauga, ON, Canada).

Immunoprecipitation and Western Blotting

For coimmunoprecipitations, MDCK cells were serum starved for 20 h in DMEM containing 0.02% FBS, stimulated with 100 U/ml HGF, and lysed in 1.0% Triton X-100 lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM EGTA, 1.5 mM $MgCl_2$, 1 mM PMSF, 1 mM Na_3VO_4 , 50 mM NaF, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin. Immunoprecipitations and Western blotting were performed as described previously (Fixman *et al.*, 1996). The preparation of NP40-soluble and -insoluble fractions was carried out as described in (Potempa and Ridley, 1998) and equal amounts of protein were used for Western blotting with E-cadherin antibodies.

Rac1 and Rap1 Pulldown Assays

Cells were grown for 36 h in DMEM containing 10% FBS and serum-starved in DMEM containing 0.02% FBS for 4 h before HGF stimulation (100 U/ml). Cells were lysed in 25 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1% NP-40, and 5% glycerol for the Rac1 pulldowns and in 50 mM TrisCl, pH 7.5, 10 mM $MgCl_2$, 200 mM NaCl, 1% NP-40, and 10% glycerol for the Rap1 pulldowns. The lysis buffers included 1 mM PMSF, 1 mM Na_3VO_4 , 50 mM NaF, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin. GST-CRIB or GST-Ral-GDS fusion proteins were used for Rac1 or Rap1 pulldowns, respectively. Fusion proteins were prepared from bacteria as described previously (Royal *et al.*, 2000), and 20 μ g of GST fusion protein was coupled to glutathione-Sepharose beads for 30 min at room temperature. After two washes of the glutathione-Sepharose beads, 700 μ g of cell lysate was added to the coupled fusion proteins and incubated for 60 min at 4°C while rocking. Glutathione-Sepharose beads were washed four times with lysis buffer, and proteins were eluted by boiling in 2× Laemmli sample buffer containing 100 mM DTT. Samples were subjected to SDS-PAGE and Western blotted with Rac1 or Rap1 antibodies. Dr. John Collard (The Netherlands Cancer Institute) provided GST-CRIB and Dr. Johannes Bos (University Medical Center Utrecht) provided GST-RalGDS.

RESULTS

CrkII-associated Proteins in MDCK Cells

To examine the molecular mechanisms that regulate EM transitions, we have used MDCK kidney epithelial cells as a model system. In response to HGF, colonies of MDCK epithelial cells undergo multiple morphological changes leading to an EM transition and cell dispersal. This occurs as a series of sequential events where the centrifugal spreading of cells within the colony is concomitant with

Figure 1. The association of CrkII and CrkL with phosphotyrosine-containing proteins is enhanced after HGF stimulation of MDCK cells. (A) MDCK cells were serum-starved for 20 h and stimulated or not with 100 U/ml HGF for 15 min. Cell lysate (800 μ g) was used for immunoprecipitation of CrkII or CrkL with their respective antibodies. The immunoprecipitates were washed and associated proteins were resolved by SDS-PAGE. Proteins on the gel were transferred to a nitrocellulose membrane, immunoblotted with α -phosphotyrosine, stripped, and reprobed with α -Crk and α -CrkL. (B) MDCK cells expressing HA-Gab1 were serum-starved for 20 h and stimulated or not for the indicated times with 100 U/ml HGF. Cell lysate (3 mg) was used for immunoprecipitation of CrkII with α -Crk, the immunoprecipitates were washed, and associated proteins were resolved by SDS-PAGE. Proteins on the gel were transferred to a nitrocellulose membrane and immunoblotted with α -phosphotyrosine, stripped, and reprobed with α -p130Cas, α -Cbl, α -HA, α -paxillin, and α -Crk.

the breakdown of cell-cell junctions and the acquisition of a motile mesenchymal phenotype (Ridley *et al.*, 1995; Royal and Park, 1995; Potempa and Ridley, 1998). Both the CrkII and CrkL adapter proteins coordinate cell motility (Klemke *et al.*, 1998; Uemura and Griffin, 1999; Cho and Klemke, 2000; Petit *et al.*, 2000; Spencer *et al.*, 2000), yet their involvement in the regulation of EM transitions has not been addressed. We and others have demonstrated that after activation of the Met receptor tyrosine kinase, CrkII and CrkL are recruited into Met-dependent signaling complexes (Garcia-Guzman *et al.*, 1999, 2000; Lamorte *et al.*, 2000; Sakkab *et al.*, 2000). Both CrkII and CrkL are expressed in MDCK epithelial cells and in well-differentiated breast cancer cell lines that retain the ability to grow as colonies (see Figures 1 and 6 and our unpublished results). To assess the role of the CrkII and CrkL adapter

Figure 2. A Crkl SH3 domain mutant impairs cell spreading, lamellipodia formation, and loss of cortical actin in HGF-stimulated MDCK cells. (A) Vector (200 ng/ μ l) and rabbit immunoglobulin G (0.6 μ g/ μ l, a–c), Crkl W170K (200 ng/ μ l, d–f), or Crkl R38K/W170K plasmids (200 ng/ μ l, g–i) were microinjected into the nuclei of MDCK cells. After a 4-h incubation, cells were stimulated with 10 U/ml HGF and fixed 4 h later. Cells were double-stained with α -rabbit-AlexaFluor488 (a) or α Crk/ α -mouse-AlexaFluor488 (d and g), together with Texas Red-X Phalloidin (b, e, and h). Corresponding phase contrast images (c, f, and i) are shown. Lamellipodia in b are indicated by an arrow. Note the lack of lamellipodia in cells microinjected with Crkl W170K (e, arrowhead), whereas noninjected cells have lamellipodia (e, arrow). (B) 293T cells were transiently transfected with 500 ng of pXM-Tpr-Met and 5.5 μ g of pEBB or pEBB-Crkl W170K. Serum-starved cells were lysed, and 400 μ g of cell lysate was used for CrkII or CrkL immunoprecipitation. The immunoprecipitates were washed, and associated proteins were resolved by SDS-PAGE. Proteins on the gel were transferred to a nitrocellulose membrane, immunoblotted with α -phosphotyrosine, stripped, and reprobed with α CrkII and α CrkL. Whole cell lysate (30 μ g) was resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and Western blotted with α Met and α Crk to detect expression of Tpr-Met and Crkl W170K, respectively.

proteins in HGF-induced EM transition, we initially examined the profile of phosphotyrosine-containing proteins bound to CrkII and CrkL in serum-starved and HGF-stimulated MDCK cells. In the absence of stimulation, CrkII and CrkL were associated with tyrosine phosphorylated proteins, and these interactions were enhanced after HGF stimulation (Figure 1A). Both CrkII and CrkL immunoprecipitates displayed similar phosphotyrosine profiles, with the predominant tyrosine phosphorylated proteins displaying a molecular weight of 110–130 kDa. We examined the ability of CrkII to associate with known Crk interacting proteins such as Gab1, Cbl, and p130Cas, all of which are 110–130 kDa in size. For these experiments, MDCK cells overexpressing Gab1 were used

in order to detect CrkII/Gab1 coupling. CrkII displayed a similar phosphotyrosine content in both MDCK (Figure 1A) and MDCK cells overexpressing Gab1 (Figure 1B). Complexes of CrkII with Gab1, Cbl, and p130Cas (Figure 1B) were present at low levels in the absence of HGF stimulation and were enhanced in the presence of HGF (Figure 1B). Coupling of CrkII with each of these proteins showed distinct kinetics suggesting that in response to HGF, CrkII is recruited into multiple protein complexes. Paxillin, a known Crk-interacting protein of 68 kDa, was associated with CrkII in the absence of stimulation, and its association was enhanced after HGF stimulation (Figure 1B). Similar levels of CrkII were immunoprecipitated in each sample (Figure 1B).

Figure 3. Microinjection of CrkII or CrkL plasmids induce remodeling of the actin cytoskeleton. CrkII (50 ng/ μ l, a–c), CrkL (50 ng/ μ l, d–f), or p130Cas plasmids (200 ng/ μ l, g–i) were microinjected into the nuclei of MDCK cells. Cells were fixed after a 5-h incubation and double-stained with α Crk/ α -mouse-AlexaFluor488 (a), α CrkL/ α -rabbit-AlexaFluor488 (d) or α Myc/ α -mouse-AlexaFluor488 (g) together with Texas Red-X Phalloidin (b, e, and h). Corresponding phase contrast images (c, f, and i) are shown. Lamellipodia in b and e are indicated by arrows. Note the lack of lamellipodia and cell spreading in cells microinjected with p130Cas plasmids (h, arrow).

A CrkI SH3 Domain Mutant Impairs HGF-induced Cell Spreading

To examine the potential role of CrkII and CrkL in the early biological events after HGF stimulation, MDCK cells were microinjected with empty vector or with plasmids encoding a CrkI protein with a mutation in its SH3 domain (CrkI W170K) or with mutations in both its SH2 and SH3 domains (CrkI R38K/W170K). CrkI is an alternatively spliced form of CrkII that lacks the carboxy-terminal SH3 domain. The CrkI W170K proteins contains a point mutation in its amino-terminal SH3 domain, which abolishes Crk binding to proline-rich containing proteins (Tanaka *et al.*, 1995). This Crk mutant contains a functional SH2 domain that interacts with tyrosine-phosphorylated proteins and acts as a dominant negative mutant, inhibiting the activation of ERK-1 in cells expressing an oncogenic form of Abl (Tanaka *et al.*, 1995). In colonies of MDCK cells microinjected with vector (Figure 2Aa) or the CrkI SH2/SH3 mutant (CrkI R38K/W170K, Figure 2Ag), HGF stimulation (10 U/ml) promotes lamellipodia formation, centrifugal spreading, and loss of cortical actin (Figure 2A, b and h). In contrast, cells microinjected with the CrkI SH3 domain mutant (W170K, Figure 2Ad) failed to form lamellipodia or spread in response to HGF (Figure 2Ae, arrowheads), whereas uninjected cells in the same colony form lamellipodia and spread in response to HGF (Figure 2Ae, arrows). Moreover, cells microinjected with the CrkI SH3 domain mutant retained cortical actin and showed a decreased ability to form actin stress fibers in response to HGF (Figure 2Ae). This suggests that the ability of Crk adapter proteins (CrkII or CrkL) to couple tyrosine-phosphorylated proteins with downstream effectors is important for lamellipodia formation and cell spreading in response to HGF. To confirm that the CrkI SH3 domain mutant competes with endogenous CrkII or CrkL for binding to tyrosine-phosphorylated proteins, 293T cells were

transiently transfected with an oncogenic form of Met (Tpr-Met) together with empty vector or the CrkI SH3 domain mutant (W170K). The association of CrkII and CrkL with tyrosine-phosphorylated proteins was enhanced in the presence of Tpr-Met (Figure 2B), as observed in HGF-stimulated MDCK cells (Figure 1, A and B). However, in the presence of CrkI W170K, the coupling of CrkII and CrkL with tyrosine-phosphorylated proteins was significantly diminished, in particular, with proteins of 110–130 kDa (Figure 2B). The expression levels of Tpr-Met and CrkI W170K are shown in Figure 2B.

Overexpression of CrkII or CrkL Promotes the Spreading of MDCK Cells

The ability of the CrkI SH3 domain mutant to block HGF-mediated rearrangement of the actin cytoskeleton and cell spreading (Figure 2A) prompted us to examine whether CrkII or CrkL could modulate these responses independently of HGF. MDCK cell colonies were microinjected with plasmids encoding wild-type CrkII or CrkL and fixed 5 hours later. In the absence of HGF, the microinjection of CrkII (Figure 3a) or CrkL plasmids (Figure 3d) promoted the formation of distinct membrane extensions resembling lamellipodia in cells at the edge of the colony (Figure 3, b and e, arrows) and the spreading of cells within the colony (Figure 3, b and e). Moreover, cells microinjected with CrkII or CrkL plasmids displayed the loss of cortical actin and formation of actin stress fibers (Figure 3, b and e). Because the overexpression of CrkII or p130Cas enhanced cell motility after transient overexpression in COS cells (Klemke *et al.*, 1998), we examined the ability of p130Cas to promote cell spreading in MDCK cells. In contrast to CrkII or CrkL, the microinjection of p130Cas plasmids (Figure 3g) did not promote lamellipodia formation or cell spreading (Figure 3h,

arrow), although the myc-tagged p130Cas was overexpressed as detected with anti-myc immunostaining (Figure 3g). Hence, when overexpressed, CrkII or CrkL but not p130Cas mimic the early morphological events that occur after HGF stimulation.

Stable Overexpression of CrkII in MDCK Cells Activates Downstream Effectors, Rac1 and Rap1, and Promotes Cell Spreading

To confirm the data obtained by microinjection, we generated stable lines of MDCK cells overexpressing CrkII (Figure 4, A and B). When compared with parental MDCK cells (Figure 4Aa), cells overexpressing CrkII displayed enhanced cell spreading (Figure 4Ab) and possessed large lamellipodia (Figure 4Ab, arrows) in the absence of HGF stimulation. The Crk SH3 domain associates with DOCK180 and C3G, which activate Rac1 (Kiyokawa *et al.*, 1998; Nolan *et al.*, 1998) and Rap1 (Gotoh *et al.*, 1995), respectively. Although we were unable to detect the association of DOCK180 with CrkII or CrkL (our unpublished results), the association of C3G with CrkII was enhanced in MDCK cells overexpressing CrkII (Figure 4D). To establish if downstream effectors were activated in MDCK cells overexpressing CrkII, we evaluated Rac1-GTP levels by pulldown assays using GST-PAK and Rap1-GTP levels by pulldown assays using GST-RalGDS. In MDCK cells overexpressing CrkII, Rac1-GTP levels (Figure 4C) and Rap1-GTP levels (Figure 4E) were elevated, in comparison with parental cells.

Rac1 but not Rap1 Is Required for CrkII-induced Lamellipodia Formation and Cell Spreading

The activation of Rac1 is required for HGF-induced lamellipodia formation and cell spreading (Ridley *et al.*, 1995; Royal *et al.*, 2000), CrkII-stimulated cell migration (Klemke *et al.*, 1998), and CrkII-stimulated lamellipodia formation in single cells (Dolfi *et al.*, 1998; Klemke *et al.*, 1998). In addition, Rap1 regulates integrin-mediated cell adhesion (Bos *et al.*, 2001) and cell spreading (Ohba *et al.*, 2001). We proceeded to determine whether Rac1 and/or Rap1 were required for CrkII-induced cell spreading in MDCK cells. MDCK cells were microinjected with CrkII plasmids together with empty vector (Figure 5, a–c), plasmids encoding a dominant negative mutant of Rac1 (N17 Rac1, Figure 5, d–f) or plasmids encoding a dominant negative mutant of Rap1 (N17 Rap1, Figure 5, g–i). After fixation, cells were stained with CrkII (Figure 5a) or Myc (9E10) antibodies (Figure 5, d and g) to detect expression of dominant negative myc-tagged Rac1 or Rap1. Expression of N17 Rac1 (Figure 5e) but not N17 Rap1 (Figure 5h) abolished the ability of CrkII to mediate cell spreading and lamellipodia formation. In contrast to cells microinjected with CrkII and empty vector or N17 Rap1 (Figure 5, b and h), cells microinjected with CrkII and dominant negative Rac1 failed to lose their cortical actin and were unable to form stress fibers (Figure 5e). Thus, Rac1 but not Rap1 is an essential mediator of CrkII-induced cytoskeletal rearrangements and cell spreading.

Figure 4. Overexpression of CrkII in MDCK cells promotes cell spreading and activation of Rac1 and Rap1. (A) MDCK (a) and MDCK cells overexpressing CrkII (b) were grown in DMEM containing 10% FBS and photographed. Arrows indicate lamellipodia in MDCK cells overexpressing CrkII. Bar, 50 μ m. (B) Whole cell lysate (30 μ g) from MDCK and MDCK cells overexpressing CrkII was subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with α Crk. (C) MDCK or MDCK cells overexpressing CrkII were stimulated with 100 U/ml HGF for 15 min or left unstimulated. Cells were lysed, and 700 μ g of protein lysate was incubated for 60 min with GST-CRIB fusion proteins bound to glutathione-Sepharose beads. The beads were washed extensively and bound proteins, together with 20 μ g of whole cell lysate, were resolved on a 12% SDS-polyacrylamide gel. Proteins on the gel were transferred to a nitrocellulose membrane and immunoblotted with α Rac1. The Western blots were scanned, and the relative intensity of the bands is indicated. (D) Lysates were prepared from serum-starved MDCK or MDCK cells overexpressing CrkII and subjected to immunoprecipitation with α Crk. The immunoprecipitates were washed, and associated proteins, together with 20 μ g of whole cell lysate, were resolved by SDS-PAGE. Proteins on the gel were transferred to a nitrocellulose membrane and immunoblotted with α C3G (top panel) and α Crk (bottom panel). (E) Rap1 activity was measured using GST-RalGDS exactly as described in C for Rac1.

CrkII Promotes Loss of β -catenin from Cell–Cell Junctions

To establish whether CrkII-induced cell spreading is unique to MDCK cells or common to other epithelial cell lines that grow as colonies, we generated CrkII-overexpressing populations of a highly differentiated breast cancer epithelial cell line, T47D. Interestingly, populations of T47D cells overexpressing CrkII demonstrated enhanced membrane ruffling

Figure 5. Rac1 but not Rap1 is required for CrkII-induced lamellipodia formation, cell spreading, and loss of cortical actin. CrkII plasmids (50 ng/ μ l) were coinjected into the nuclei of MDCK cells with vector (70 ng/ μ l, a–c), N17 Rac1 (20 ng/ μ l, d–f), or N17 Rap1 plasmids (70 ng/ μ l, g–i). Cells were fixed after a 5-h incubation and double-stained with α Crk/ α -mouse-CY3 (a) or α Myc/ α -mouse-CY3 (d and g) together with AlexaFluor488-Phalloidin (b, e, and h). Corresponding phase contrast images (c, f, and i) are shown. Arrows indicate lamellipodia in b and h. Note the lack of lamellipodia and cell spreading in cells microinjected with CrkII and N17 Rac1 plasmids (e, arrow).

(Figure 6Ab, arrows), and 82% of the colonies were dispersed (Figure 6Ab), whereas only 17% of vector-containing cells were dispersed (Figure 6Aa). Colonies of epithelial cells contain adherens junctions composed of E-cadherin and β -catenin and tight junctions containing ZO-1 protein complexes (reviewed in Gumbiner, 2000). The breakdown of adherens junctions is a necessary prerequisite for cell dispersal (Tsukamoto and Nigam, 1999). Therefore, we compared the localization of β -catenin in vector and CrkII-overexpressing T47D cells. Although vector containing T47D cell colonies exhibited well defined β -catenin staining at cell–cell junctions (Figure 6Cb), β -catenin staining was absent from the membrane of cells in dispersed T47D colonies overexpressing CrkII (Figure 6Cd, arrowhead). Moreover, reduced β -catenin staining was observed at cell–cell junctions in nondispersed T47D cells overexpressing CrkII (Figure 6Cd, arrow). Hence, the overexpression of CrkII promotes the loss of β -catenin-containing adherens junctions in T47D colonies and enhances cell dispersal.

In contrast to T47D cells (Figure 6), MDCK cells overexpressing CrkII are not dispersed (Figure 4A), yet they displayed reduced β -catenin staining at cell–cell junctions (Figure 7Ad) when compared with parental cells (Figure 7Ab). Consistent with this, E-cadherin was translocated from an NP40-insoluble to an NP40-soluble compartment in MDCK cells overexpressing CrkII (Figure 7B). The ability of CrkL to promote the breakdown of epithelial adherens junctions was evaluated by microinjecting MDCK cells with plasmids encoding CrkL. Reduced β -catenin staining at cell–cell junctions was observed in cells microinjected with CrkL plasmids (Figure 7Cb, arrows), compared with noninjected cells that showed no reduction in β -catenin at cell–cell junctions (Figures 7Cb). Rac1 is present at cell–cell junctions in MDCK cells (Royal *et al.*, 2000), and Rac1 activity is involved in the assembly and breakdown of adherens junctions (Braga, 2000). In cells microinjected with CrkII plasmids (Figure

7Da), Rac1 localization to cell–cell junctions was decreased and displayed a diffuse cytoplasmic localization (Figure 7Db), whereas in noninjected cells, endogenous Rac1 was retained at cell–cell junctions (Figure 7Db). The data presented in Figures 6 and 7 demonstrate that both CrkII and

Figure 6. T47D cells overexpressing CrkII are dispersed and display reduced β -catenin staining at the membrane. (A) Populations of T47D (a) and T47D cells overexpressing CrkII (b) were grown in DMEM containing 10% FBS and photographed. Arrows indicate membrane ruffling in T47D cells overexpressing CrkII. Bar, 50 μ m. (B) Whole cell lysate (30 μ g) from T47D and T47D cells overexpressing CrkII was subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with α Crk. (C) T47D and T47D cells overexpressing CrkII were grown on glass coverslips in DMEM containing 10% FBS for 48 h before fixation. Cells were stained with β -catenin/ α -mouse-CY3 (b and d). Corresponding phase contrast images (a and c) are shown.

Figure 7. MDCK cells overexpressing CrkII or microinjected with CrkL plasmids display reduced β-catenin staining at cell-cell junctions. (A) MDCK and MDCK cells overexpressing CrkII were grown on glass coverslips in DMEM containing 10% FBS for 48 h before fixation. Cells were stained with β-catenin/α-mouse-CY3 (b and d). Corresponding phase contrast images (a and c) are shown. (B) NP40-soluble lysates were prepared, and the insoluble material was solubilized in SDS-containing buffer. Cell lysate (10 μg) was subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with E-cadherin antibodies. (C) CrkL plasmids (50 ng/μl) were microinjected into the nuclei of MDCK cells. Cells were fixed after a 5-h incubation and stained with αCrkL/α-rabbit-AlexaFluor488 (a) and β-catenin/α-mouse-CY3 (b). Corresponding phase contrast image (c) is shown. Note the loss of β-catenin at cell-cell junctions in cells microinjected with CrkL plasmids (arrow in b). (D) CrkII plasmids (50 ng/μl) and rabbit immunoglobulin G (0.6 μg/μl) were microinjected into the nuclei of MDCK cells. Cells were fixed after a 5-h incubation and stained with α-rabbit-AlexaFluor488 (a) and αRac1/α-mouse-CY3 (b). Corresponding phase contrast image (c) is shown. Note the loss of Rac1 staining at cell-cell junctions in MDCK cells microinjected with CrkII.

CrkL can stimulate the loss of epithelial adherens junctions and alter Rac1 localization.

ZO-1 Is Retained at Cell-Cell Junctions in MDCK Cells Overexpressing CrkII

We have previously shown that in MDCK cells, the HGF-stimulated turnover of adherens junctions corresponds with cell spreading and precedes the loss of tight junctions (Royal

and Park, 1995). Although MDCK cells overexpressing CrkII displayed extensive cell spreading, ZO-1 was retained at cell-cell junctions (Figure 8Ad), as with the parental MDCK cells (Figure 8Ab). However, a minority of MDCK cells microinjected with CrkII plasmids dispersed (Figure 8Bc). These cells exhibited loss of ZO-1 staining at cell-cell contacts (Figure 8Bb, arrow) and showed punctate ZO-1 staining within the cytoplasm (Figure 8Bb). In contrast, T47D

Figure 8. MDCK cells overexpressing CrkII retain ZO-1 containing tight junctions. (A) MDCK and MDCK cells overexpressing CrkII were grown on glass coverslips in DMEM containing 10% FBS for 48 h before fixation. Cells were stained with αZO-1/α-rabbit-AlexaFluor488 (b and d). Corresponding phase contrast images (a and c) are shown. (B) CrkII plasmids (50 ng/μl) were microinjected into the nuclei of MDCK cells. Cells were fixed after a 5-h incubation and stained with αCrk/α-mouse-CY3 (a) and αZO-1/α-rabbit-AlexaFluor488 (b). Corresponding phase contrast image (c) is shown. Note the loss of ZO-1 staining at the membrane in a cell that has dispersed (arrow in b).

Figure 9. Reorganization of actin and focal adhesions in MDCK and T47D cells overexpressing CrkII. MDCK (a–c) and MDCK cells overexpressing CrkII (d–g) or T47D (h–j) and T47D cells overexpressing CrkII (k–n) were grown on glass coverslips in DMEM containing 10% FBS for 48 h. Cells were solubilized with 0.25× CSK buffer for 10 min and later fixed. Cells were double-stained with α -vinculin/ α -mouse-CY3 (a, b, d–f, h, i, k, l, and m) and AlexaFluor488-Phalloidin (c, g, j, and n). Images highlighted in the boxes from a, d, h, and k were enlarged and presented in b, e, i, and l, respectively. The regions indicated by arrows in d and k were enlarged and presented in f and m, respectively. Note the presence of vinculin staining in lamellipodia (arrows in f) and the colocalization of vinculin with actin (arrows in m). Large focal adhesions are highlighted by arrows in e and l. Large bar, 25 μ m; small bar, 5 μ m.

cells lack ZO-1 containing tight junctions (our unpublished results) and the loss of β -catenin containing adherens junctions in cells overexpressing CrkII promotes cell dispersal and conversion to a mesenchymal like phenotype (Figure 6A).

Reorganization of Focal Adhesions in Cells Overexpressing CrkII

The spreading and dispersal of epithelial colonies requires the reorganization of the actin cytoskeleton and the assembly of nascent focal adhesions/complexes to strengthen adhesion to the extracellular matrix (Sastrý and Burridge, 2000). MDCK and T47D cells overexpressing CrkII were stained with vinculin antibodies and phalloidin to visualize focal adhesions and the actin cytoskeleton, respectively. In colonies of MDCK cells, vinculin-

containing adhesion complexes are prominent on cells at the edge of the colony, and only small vinculin-containing complexes are observed throughout the colony (Figure 9a). Consistent with the increased spreading and lamellipodia formation observed in MDCK cells overexpressing CrkII, vinculin-containing focal adhesions were reorganized (Figure 9, a vs. d) and were larger in these cells (Figure 9, b vs. e, arrows). In addition, vinculin-containing focal complexes were visible at the edge of the lamellipodia (Figure 9, d and f, arrow). Focal adhesions present in T47D cells overexpressing CrkII were also larger in comparison with vector containing T47D cells (Figure 9, h and i vs. k and l, arrows) and colocalized with membrane extensions (Figure 9m, arrows). From these results we concluded that enhanced expression of CrkII mediates the reorganization of the actin cytoskeleton and promotes enhanced cell-matrix interactions.

DISCUSSION

The modulation of epithelial junctions and cell migration occurs during normal embryonic development and participates in the dispersal of tumor cells (Boyer *et al.*, 2000). The Met receptor tyrosine kinase is deregulated in many human tumors (Lamorte and Park, 2001) and is one of the predominant mediators of EM transition (Weidner *et al.*, 1993; Zhu *et al.*, 1994). Although several receptor tyrosine kinases that are deregulated in human tumors contribute to the modulation of epithelial junctions, the precise mechanism regulating this loss is not completely understood (Boyer *et al.*, 2000). The results presented here demonstrate that the CrkII and CrkL adapter proteins play an important role in the spreading and breakdown of epithelial cell-cell adherens junctions in colonies of epithelial cells (Figure 10), processes critical for epithelial cell dispersal.

Crk Adapter Proteins Are Required for HGF-mediated Cell Spreading

The ability of a CrkI SH3 domain mutant (W170K) to block HGF-induced cell spreading and lamellipodia formation (Figure 2A) suggests that the coupling of Crk SH2 and SH3 domains with upstream and downstream binding proteins is an important event required for HGF-induced cell spreading. The CrkI SH3 mutant contains an SH2 domain but lacks functional SH3 domains, thereby acting as an isolated SH2 domain. Consistent with this, the CrkI W170K mutant competes with CrkII and CrkL for the binding to tyrosine-phosphorylated proteins (Figure 2B). The SH2 domains of CrkII and CrkL prefer a YXXP motif and bind the same proteins where tested (Feller, 2001). This is further supported by our data demonstrating that both CrkII and CrkL bind similar phosphotyrosine-containing proteins in MDCK cells (Figure 1A). Our loss-of-function approach using the CrkI SH3 mutant demonstrated that both CrkII and CrkL participate in HGF-stimulated cell spreading and breakdown of adherens junctions. This is further supported by complementary gain-of-function experiments demonstrating the ability of CrkII or CrkL to promote spreading of colonies of MDCK cells (Figures 3 and 4A) and dispersal of colonies of T47D breast carcinoma cells (Figure 6A) in the absence of HGF stimulation.

Figure 10. In the initial 2–4 h after HGF stimulation, cells form lamellipodia and new focal adhesions while spreading and losing their adherens junctions. Similar changes are observed in MDCK cells overexpressing CrkII or CrkL. A dominant negative mutant of Rac1 or a CrkI SH3 domain mutant blocks HGF-mediated cell spreading and the breakdown of adherens junctions. A dominant negative mutant of Rac1 blocks Crk-mediated cell spreading and breakdown of adherens junctions.

The Crk SH3 domain interacts with several proteins including DOCK180, which binds and activates Rac1 (Erickson *et al.*, 1997; Kiyokawa *et al.*, 1998), and C3G, an exchange factor for Rap1 (Gotoh *et al.*, 1995). Consistent with a role for Crk in HGF-dependent cell spreading, Rac1 activity is required for both CrkII- and HGF-stimulated cell spreading and remodeling of the actin cytoskeleton (Figure 5 and Ridley *et al.* [1995] and Royal *et al.* [2000], respectively). Moreover, Rac1 activity is elevated in MDCK cells overexpressing CrkII, to levels similar to those observed after HGF stimulation (Figure 4C). Although DOCK180 has been shown to activate Rac1 (Kiyokawa *et al.*, 1998; Nolan *et al.*, 1998), we have been unable to coimmunoprecipitate either CrkII or CrkL with DOCK180 in MDCK or T47D cells (our unpublished results). Moreover, a farnesylated form of DOCK180 that promotes cell spreading in fibroblasts (Kiyokawa *et al.*, 1998) failed to do so when microinjected into MDCK cells (our unpublished results). Alternatively, Crk SH3 binding protein(s) distinct from DOCK180 may be involved in the activation of Rac1 in MDCK cells. Consistent with its ability to interact with C3G, overexpression of CrkII in MDCK cells promoted the enhanced association of CrkII with C3G (Figure 4D) and enhanced Rap1 activity (Figure 4E). However, whereas a dominant negative Rac1 mutant inhibited Crk- or HGF-induced cell spreading, the overexpression of a dominant negative Rap1 mutant failed to do so (Figure 5). Although we show that activation of Rap1 is not required for cell spreading downstream of CrkII, Rap1 may be important for cell migration, morphogenesis, and/or sustained ERK activation (Bos *et al.*, 2001), processes not evaluated here.

We have shown that in MDCK cells, CrkII binds multiple phosphotyrosine-containing proteins including Cbl, Gab1, paxillin, and p130Cas (Figure 1B) and that these interactions are increased with distinct temporal profiles after HGF stimulation (Figure 1B). In response to HGF, the binding to one or all of these proteins may target CrkII to the membrane and/or other cellular compartments, where Crk SH3 binding proteins can activate Rac1 and Rap1. CrkII and p130Cas each promote the formation of lamellipodia (Dolfi *et al.*, 1998; Klemke *et al.*, 1998) and enhance cell migration when overexpressed in dispersed COS cells (Klemke *et al.*, 1998). However, overexpression of p130Cas in adhesive colonies of MDCK cells does not promote cell spreading or lamellipodia formation (Figure 3). Thus, although the coupling of Crk with p130Cas may be important for cell migration once cells have adopted a mesenchymal phenotype, overexpression of p130Cas is not sufficient for lamellipodia formation and cell spreading in colonies of epithelial MDCK cells. Similarly, the microinjection of plasmids expressing Gab1 or Cbl failed to induce cell spreading and lamellipodia formation in the absence of HGF (our unpublished results).

MDCK cells overexpressing CrkII or CrkL display numerous actin stress fibers (Figures 3 and 9) and larger focal adhesions (Figure 9), suggesting that RhoA is activated downstream of Crk adapter proteins. In addition, prominent actin stress fibers are present in control cells stimulated with HGF but not in cells microinjected with a Crk SH3 domain mutant (Figure 2Ae). Hence, the coupling of Crk adapter proteins with SH2 and SH3 domain binding proteins may be required for RhoA activation

after HGF stimulation. This is consistent with the ability of CrkII SH2 or SH3 domain mutants to abolish stress fiber formation in fibroblasts (Nakashima *et al.*, 1999). Moreover, PC12 cells expressing v-Crk display enhanced stress fiber and focal adhesion formation and v-Crk activated Rho-kinase (Altun-Gultekin *et al.*, 1998). Pharmacological inhibition of Rho-kinase using Y27632 decreased stress fiber formation in MDCK cells microinjected with CrkII or CrkL but failed to inhibit cell spreading (our unpublished results). We concluded that Rho-kinase activity and stress fiber formation are dispensable for CrkII and CrkL-stimulated cell spreading.

Crk Adapter Proteins Stimulate the Breakdown of Epithelial Adherens Junctions

We have demonstrated for the first time that both CrkII and CrkL induce the loss of epithelial adherens junctions when overexpressed (Figures 6C and 7). Moreover, cells microinjected with the CrkI SH3 mutant and stimulated with HGF retain cortical actin (Figure 2Ae), demonstrating that this mutant blocks the HGF-mediated loss of adherens junctions. In support of a role for Crk-dependent loss of adherens junctions, less E-cadherin and β -catenin are present in the insoluble compartment of MDCK cells overexpressing CrkII, in comparison with control cells (Figure 7B and our unpublished results). Rac1 is involved in both the assembly and disassembly of adherens junctions (Braga, 2000) and regulates the clathrin-independent endocytosis of E-cadherin (Akhtar and Hotchin, 2001). In colonies of MDCK cells, Rac1 is localized to cell-cell junctions and is translocated to lamellipodia after stimulation of cells with HGF (Royal *et al.*, 2000). In cells microinjected with CrkII, Rac1 is lost from cell-cell junctions and translocates to the cytoplasm (Figure 7Db). ARF6 is required for the targeting of Rac1 to the membrane (Radhakrishna *et al.*, 1999) and for HGF-stimulated E-cadherin internalization and migration (Palacios *et al.*, 2001). Moreover, overexpression of ARNO, an ARF6 exchange factor, stimulates Rac1 activity, lamellipodia formation, and cell dispersal in MDCK cells (Santy and Casanova, 2001). Hence, it will be important to establish the ability of CrkII and CrkL to regulate ARF activity through its ability to associate with protein complexes such as paxillin, which binds ARF GTPase-activating proteins (reviewed in Turner *et al.*, 2001).

The ability of epithelial cells to detach from neighboring cells is a prerequisite for tumor cell invasion. Although the overexpression of CrkII in stable lines of MDCK cells promotes cell spreading (Figure 4A) together with the loss of adherens junctions (Figure 7A), MDCK cells remain in colonies and retain tight junctions as visualized by ZO-1 (Figure 8A). In contrast, the overexpression of CrkII in a well-differentiated breast carcinoma cell line T47D promotes cell dispersal (Figure 6A). Notably, T47D cells lack tight junctions containing ZO-1 (our unpublished results), and the loss of adherens junctions after CrkII overexpression (Figure 6C) is sufficient to promote cell dispersal and a mesenchymal transition (Figure 6A). Several invasive breast cancer cell lines show loss of ZO-1 (Sommers *et al.*, 1994), and one study demonstrated loss of heterozygosity in ZO-1 in 23% of breast cancer patients and reduced or no ZO-1 staining in 69% of patients (Hoover *et al.*, 1998). Hence, the loss of adherens junctions and ZO-1 containing tight junctions may

predispose cells to dispersal after deregulation of signaling pathways involving Crk.

In conclusion, we have demonstrated that CrkII and CrkL are required for HGF-mediated cell spreading, lamellipodia formation, and breakdown of adherens junctions (Figure 10). Furthermore, we have shown for the first time that CrkII and CrkL each promote the loss of adherens junctions, which contributes to induce an EM-like transition (Figure 10), events critical for tumor cell dispersal and invasion. Our data, together with that demonstrating that CrkII and CrkL enhance cell migration (Klemke *et al.*, 1998; Uemura and Griffin, 1999; Cho and Klemke, 2000; Hemmeryckx *et al.*, 2001), underscore the importance of Crk adapter proteins in cancer and highlights their suitability as therapeutic targets.

ACKNOWLEDGMENTS

The authors thank G. Vande Woude, M. Tremblay, B. Mayer, J. Groffen, A. Hall, J. Collard, J. Bos, and M. Matsuda for reagents provided in this study and C. Maroun, P. Peschard, and V. Sangwan for their insightful comments on the manuscript. L.L. is a recipient of a Canadian Institutes of Health Research studentship. M.P. is a recipient of a Canadian Institutes of Health Research scientist award. This research was supported by an operating grant to M.P. from the Canadian Breast Cancer Research Initiative.

REFERENCES

- Akhtar, N., and Hotchin, N.A. (2001). RAC1 regulates adherens junctions through endocytosis of E-cadherin. *Mol. Biol. Cell* 12, 847–862.
- Altun-Gultekin, Z.F., Chandriani, S., Bougeret, C., Ishizaki, T., Narumiya, S., de Graaf, P., Van Bergen en Henegouwen, P., Hanafusa, H., Wagner, J.A., and Birge, R.B. (1998). Activation of Rho-dependent cell spreading and focal adhesion biogenesis by the v-Crk adaptor protein. *Mol. Cell. Biol.* 18, 3044–3058.
- Behrens, J. (1999). Cadherins and catenins: role in signal transduction and tumor progression. *Cancer Metastasis Rev.* 18, 15–30.
- Bos, J.L., de Rooij, J., and Reedquist, K.A. (2001). Rap1 signaling: adhering to new models. *Nat. Rev. Mol. Cell. Biol.* 2, 369–377.
- Boyer, B., Valles, A.M., and Edme, N. (2000). Induction and regulation of epithelial-mesenchymal transitions. *Biochem. Pharmacol.* 60, 1091–1099.
- Braga, V. (2000). Epithelial cell shape: cadherins and small GTPases. *Exp. Cell Res.* 261, 83–90.
- Camp, R.L., Rimm, E.B., and Rimm, D.L. (1999). Met expression is associated with poor outcome in patients with axillary lymph node negative breast carcinoma. *Cancer* 86, 2259–2265.
- Cho, S.Y., and Klemke, R.L. (2000). Extracellular-regulated kinase activation and CAS/Crk coupling regulate cell migration and suppress apoptosis during invasion of the extracellular matrix. *J. Cell Biol.* 149, 223–236.
- Dolfi, F., Garcia-Guzman, M., Ojaniemi, M., Nakamura, H., Matsuda, M., and Vuori, K. (1998). The adaptor protein Crk connects multiple cellular stimuli to the JNK signaling pathway. *Proc. Natl. Acad. Sci. USA* 95, 15394–15399.
- Erickson, M.R., Galletta, B.J., and Abmayr, S.M. (1997). Drosophila myoblast city encodes a conserved protein that is essential for myoblast fusion, dorsal closure, and cytoskeletal organization. *J. Cell Biol.* 138, 589–603.
- Feller, S.M. (2001). Crk family adaptors-signaling complex formation and biological roles. *Oncogene* 20, 6348–6371.

- Fixman, E.D., Fournier, T.M., Kamikura, D.M., Naujokas, M.A., and Park, M. (1996). Pathways downstream of Shc and Grb2 are required for cell transformation by the tpr-Met oncoprotein. *J. Biol. Chem.* 271, 13116–13122.
- Garcia-Guzman, M., Dolfi, F., Zeh, K., and Vuori, K. (1999). Met-induced JNK activation is mediated by the adapter protein Crk and correlates with the Gab1-Crk signaling complex formation. *Oncogene* 18, 7775–7786.
- Garcia-Guzman, M., Larsen, E., and Vuori, K. (2000). The proto-oncogene c-Cbl is a positive regulator of Met-induced MAP kinase activation: a role for the adaptor protein Crk. *Oncogene* 19, 4058–4065.
- Ghoussoub, R.A., Dillon, D.A., D'Aquila, T., Rimm, E.B., Fearon, E.R., and Rimm, D.L. (1998). Expression of c-met is a strong independent prognostic factor in breast carcinoma. *Cancer* 82, 1513–1520.
- Gotoh, T., Hattori, S., Nakamura, S., Kitayama, H., Noda, M., Takai, Y., Kaibuchi, K., Matsui, H., Hatase, O., Takahashi, H., *et al.* (1995). Identification of Rap1 as a target for the Crk SH3 domain-binding guanine nucleotide-releasing factor C3G. *Mol. Cell. Biol.* 15, 6746–6753.
- Guarino, M. (1995). Epithelial-to-mesenchymal change of differentiation. From embryogenetic mechanism to pathological patterns. *Histol. Histopathol.* 10, 171–184.
- Gumbiner, B.M. (2000). Regulation of cadherin adhesive activity. *J. Cell Biol.* 148, 399–404.
- Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* 279, 509–514.
- Hemmerlyckx, B., van Wijk, A., Reichert, A., Kaartinen, V., de Jong, R., Pattengale, P.K., Gonzalez-Gomez, I., Groffen, J., and Heisterkamp, N. (2001). Crkl enhances leukemogenesis in BCR/ABL P190 transgenic mice. *Cancer Res.* 61, 1398–1405.
- Hoover, K.B., Liao, S.Y., and Bryant, P.J. (1998). Loss of the tight junction MAGUK ZO-1 in breast cancer: relationship to glandular differentiation and loss of heterozygosity. *Am. J. Pathol.* 153, 1767–1773.
- Jin, L., Fuchs, A., Schnitt, S.J., Yao, Y., Joseph, A., Lamszus, K., Park, M., Goldberg, I.D., and Rosen, E.M. (1997). Expression of scatter factor and c-met receptor in benign and malignant breast tissue. *Cancer* 79, 749–760.
- Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T., and Matsuda, M. (1998). Activation of Rac1 by a Crk SH3-binding protein, DOCK180. *Genes Dev.* 12, 3331–3336.
- Klemke, R.L., Leng, J., Molander, R., Brooks, P.C., Vuori, K., and Cheresch, D.A. (1998). CAS/Crk coupling serves as a “molecular switch” for induction of cell migration. *J. Cell Biol.* 140, 961–972.
- Koochekpour, S., Jeffers, M., Rulong, S., Taylor, G., Klineberg, E., Hudson, E.A., Resau, J.H., and Vande Woude, G.F. (1997). Met and hepatocyte growth factor/scatter factor expression in human gliomas. *Cancer Res.* 57, 5391–5398.
- Lamorte, L., Kamikura, D.M., and Park, M. (2000). A switch from p130Cas/Crk to Gab1/Crk signaling correlates with anchorage independent growth and JNK activation in cells transformed by the Met receptor oncoprotein. *Oncogene* 19, 5973–5981.
- Lamorte, L., and Park, M. (2001). The receptor tyrosine kinases: role in cancer progression. *Surg. Oncol. Clin. North Am.* 10, 271–288, viii.
- Matsumoto, K., and Nakamura, T. (1997). Hepatocyte growth factor (HGF) as a tissue organizer for organogenesis and regeneration. *Biochem. Biophys. Res. Commun.* 239, 639–644.
- Mayer, B.J., Hamaguchi, M., and Hanafusa, H. (1988). A novel viral oncogene with structural similarity to phospholipase C. *Nature* 332, 272–275.
- Nakajima, M., Sawada, H., Yamada, Y., Watanabe, A., Tatsumi, M., Yamashita, J., Matsuda, M., Sakaguchi, T., Hirao, T., and Nakano, H. (1999). The prognostic significance of amplification and overexpression of c-met and c-erb B-2 in human gastric carcinomas. *Cancer* 85, 1894–1902.
- Nakashima, N., Rose, D.W., Xiao, S., Egawa, K., Martin, S.S., Haruta, T., Saltiel, A.R., and Olefsky, J.M. (1999). The functional role of CrkII in actin cytoskeleton organization and mitogenesis. *J. Biol. Chem.* 274, 3001–3008.
- Nolan, K.M., Barrett, K., Lu, Y., Hu, K.Q., Vincent, S., and Settleman, J. (1998). Myoblast city, the Drosophila homolog of DOCK180/CED-5, is required in a Rac signaling pathway utilized for multiple developmental processes. *Genes Dev.* 12, 3337–3342.
- Ohba, Y., Ikuta, K., Ogura, A., Matsuda, J., Mochizuki, N., Nagashima, K., Kurokawa, K., Mayer, B.J., Maki, K., Miyazaki, J., and Matsuda, M. (2001). Requirement for C3G-dependent Rap1 activation for cell adhesion and embryogenesis. *EMBO J.* 20, 3333–3341.
- Otsuka, T., Takayama, H., Sharp, R., Celli, G., LaRochelle, W.J., Bottaro, D.P., Ellmore, N., Vieira, W., Owens, J.W., Anver, M., and Merlino, G. (1998). c-Met autocrine activation induces development of malignant melanoma and acquisition of the metastatic phenotype. *Cancer Res* 58, 5157–5167.
- Palacios, F., Price, L., Schweitzer, J., Collard, J.G., and D'Souza-Schorey, C. (2001). An essential role for ARF6-regulated membrane traffic in adherens junction turnover and epithelial cell migration. *EMBO J.* 20, 4973–4986.
- Petit, V., Boyer, B., Lentz, D., Turner, C.E., Thiery, J.P., and Valles, A.M. (2000). Phosphorylation of tyrosine residues 31 and 118 on paxillin regulates cell migration through an association with CRK in NBT-II cells. *J. Cell Biol.* 148, 957–970.
- Potempa, S., and Ridley, A.J. (1998). Activation of both MAP kinase and phosphatidylinositol 3-kinase by Ras is required for hepatocyte growth factor/scatter factor-induced adherens junction disassembly. *Mol. Biol. Cell* 9, 2185–2200.
- Prieto, A.L., and Crossin, K.L. (1995). Cell-cell adhesion molecules in epithelial-mesenchymal transformations. *Acta Anat.* 154, 21–33.
- Radhakrishna, H., Al-Awar, O., Khachikian, Z., and Donaldson, J.G. (1999). ARF6 requirement for Rac ruffling suggests a role for membrane trafficking in cortical actin rearrangements. *J. Cell Sci.* 112, 855–866.
- Reddien, P.W., and Horvitz, H.R. (2000). CED-2/CrkII and CED-10/Rac control phagocytosis and cell migration in *Caenorhabditis elegans*. *Nat. Cell Biol.* 2, 131–136.
- Reichman, C.T., Mayer, B.J., Keshav, S., and Hanafusa, H. (1992). The product of the cellular crk gene consists primarily of SH2 and SH3 regions. *Cell Growth Differ.* 3, 451–460.
- Ridley, A.J., Comoglio, P.M., and Hall, A. (1995). Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac, and Rho in MDCK cells. *Mol. Cell. Biol.* 15, 1110–1122.
- Rodrigues, G., and Park, M. (1993). Dimerization mediated through a leucine zipper activates the oncogenic potential of the met receptor tyrosine kinase. *Mol. Cell. Biol.* 13, 6711–6722.
- Royal, I., Lamarche-Vane, N., Lamorte, L., Kaibuchi, K., and Park, M. (2000). Activation of Cdc42, Rac, PAK, and Rho-kinase in response to hepatocyte growth factor differentially regulates epithelial cell colony spreading and dissociation. *Mol. Biol. Cell* 11, 1709–1725.

- Royal, I., and Park, M. (1995). Hepatocyte growth factor induced scatter of MDCK cells requires phosphatidylinositol 3-kinase. *J. Biol. Chem.* 270, 27780–27787.
- Sakkab, D., Lewitzky, M., Posern, G., Schaeper, U., Sachs, M., Birchmeier, W., and Feller, S.M. (2000). Signaling of hepatocyte growth factor/scatter factor (HGF) to the small GTPase Rap1 via the large docking protein Gab1 and the adapter protein CRKL. *J. Biol. Chem.* 275, 10772–10778.
- Santy, L.C., and Casanova, J.E. (2001). Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. *J. Cell Biol.* 154, 599–610.
- Sastry, S.K., and Burridge, K. (2000). Focal adhesions: a nexus for intracellular signaling and cytoskeletal dynamics. *Exp. Cell Res.* 261, 25–36.
- Schmidt, L., Duh, F.M., Chen, F., Kishida, T., Glenn, G., Choyke, P., Scherer, S.W., Zhuang, Z.P., Lubensky, I., Dean, M., Allikmets, R., Chidambaram, A., Bergerheim, U.R., Feltis, J.T., Casadevall, C., Zamarron, A., Bernues, M., Richard, S., Lips, C.J.M., Walther, M.M., Tsui, L.C., Geil, L., Orcutt, M.L., Stackhouse, T., Lipan, J., *et al.* (1997). Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. *Nat. Genet.* 16, 68–73.
- Sommers, C.L., Byers, S.W., Thompson, E.W., Torri, J.A., and Gelmann, E.P. (1994). Differentiation state and invasiveness of human breast cancer cell lines. *Breast Cancer Res. Treat.* 31, 325–335.
- Spencer, K.S., Graus-Porta, D., Leng, J., Hynes, N.E., and Klemke, R.L. (2000). ErbB2 is necessary for induction of carcinoma cell invasion by ErbB family receptor tyrosine kinases. *J. Cell Biol.* 148, 385–397.
- Stoker, M., Gherardi, E., Perryman, M., and Gray, J. (1987). Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. *Nature* 327, 239–242.
- Takayama, H., LaRochelle, W., Sharp, R., Otsuka, T., Kriebel, P., Anver, M., Aaronson, S., and Merlino, G. (1997). Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor. *Proc. Natl. Acad. Sci. USA* 94, 701–706.
- Tanaka, M., Gupta, R., and Mayer, B.J. (1995). Differential inhibition of signaling pathways by dominant-negative SH2/SH3 adapter proteins. *Mol. Cell. Biol.* 15, 6829–6837.
- ten Hoeve, J., Morris, C., Heisterkamp, N., and Groffen, J. (1993). Isolation and chromosomal localization of CRKL, a human crk-like gene. *Oncogene* 8, 2469–2474.
- Tsukamoto, T., and Nigam, S.K. (1999). Cell-cell dissociation upon epithelial cell scattering requires a step mediated by the proteasome. *J. Biol. Chem.* 274, 24579–24584.
- Turner, C.E., West, K.A., and Brown, M.C. (2001). Paxillin-ARF GAP signaling, and the cytoskeleton. *Curr. Opin. Cell Biol.* 13, 593–599.
- Uemura, N., and Griffin, J.D. (1999). The adapter protein Crkl links Cbl to C3G after integrin ligation and enhances cell migration. *J. Biol. Chem.* 274, 37525–37532.
- Weidner, K.M., Behrens, J., Vandekerckhove, J., and Birchmeier, W. (1990). Scatter Factor: molecular characteristics and effect on the invasiveness of epithelial cells. *J. Cell Biol.* 111, 2097–2108.
- Weidner, K.M., Sachs, M., and Birchmeier, W. (1993). The Met receptor tyrosine kinase transduces motility, proliferation, and morphogenic signals of scatter factor/hepatocyte growth factor in epithelial cells. *J. Cell Biol.* 121, 145–154.
- Zhu, H., Naujokas, M.A., and Park, M. (1994). Receptor chimeras indicate that the Met tyrosine kinase mediates the motility and morphogenic responses of hepatocyte growth/scatter factor. *Cell Growth Differ.* 5, 359–366.