The function of the Crk adapter proteins in cell migration and invasion

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Abstract

Epithelial morphogenesis is an important signaling program in normal embryonic development involving cell proliferation, migration, invasion and cell matrix turnover and also during tumourigenesis. The Crk adaptor family of proteins has been implicated as promoters and mediators of such biological responses downstream of various extracellular signals. Our laboratory has defined the involvement of Crk adaptor proteins in the signaling of Met-dependent anchorage-independent growth, cell dispersal, cell invasion and epithelial morphogenesis. In an MDCK epithelial cell model CrkII overexpression was sufficient to promote cell spreading. This initial biological response was dependent on Rac activity. In Chapter 3 I demonstrate that MDCK cells overexpressing CrkII unstimulated, or stimulated with HGF, exhibit higher levels of activated Rac1. In an attempt to validate the function of Crk during cell migration and invasion I have developed targeted siRNAs to CrkI/II and CrkL and assessed their impact in the disruption of migration and invasion of cancer cell lines. In Chapter 4 I demonstrate that siRNAs promote a specific decrease in the level of Crk adaptor proteins. Notably this decrease impairs motility and invasion of several human tumor cell lines. I provide evidence that a role for Crk during cell migration or invasion is to promote the formation of cell matrix adhesion contacts necessary for cell spreading and migration. These studies have established that Crk adaptor proteins are required and may be key promoters of cell migration events during tumorigenesis.

Resume

La morphogenèse épithéliale joue un rôle clé dans le développement embryonnaire et requiert la prolifération, la migration et l'invasion cellulaire, ainsi que le renouvellement de la matrice cellulaire et aussi durant le développement des cancers. La famille de protéines adaptatrices Crk agit en tant que médiatrice de la morphogenèse épithéliale en aval de plusieurs signaux extracellulaires. Des études effectuées dans notre laboratoire ont démontré l'implication des protéines Crk au niveau de la croissance, de la dispersion et de l'invasion cellulaire ainsi que de la morphogenèse épithéliale de manière dépendante du récepteur Met et indépendante de l'ancrage cellulaire. Dans les cellules épithéliales MDCK, la surexpression de CrkII induit l'étalement cellulaire, l'étape initiale de la dispersion cellulaire induite par l'HGF et qui requiert l'activité de Rac. Dans le troisième chapitre, je démontre que dans les cellules MDCK surexprimant Crk II, ou des cellules qui ont ete stimule par HGF, le niveau d'activation de Rac1 est plus élevé. Pour élucider la fonction des protéines Crk durant la migration et l'invasion cellulaire, j'ai développé des siARNs ciblés contre CrkI/II et CrkL. J'ai évalué leur impact sur l'inhibition de la migration et l'invasion de lignées cellulaires cancéreuses. Dans le quatrième chapitre, je démontre que les siARN diminuent spécifiquement le niveau des protéines Crk. Cette diminution nuit à la motilité et l'invasion de plusieurs lignées cellulaires dérivées de tumeurs humaines. Je présente plusieurs résultats qui suggèrent qu'un des rôles de Crk dans la migration et l'invasion cellulaire est d'induire la formation de contacts d'adhérence liés à la matrice

cellulaire nécessaires à l'étalement et la migration cellulaire. Ces études ont démontré que les protéines adaptatrices Crk sont nécessaires à la migration cellulaire, un évènement clé de la tumorigenèse.

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Publications

1. Lamorte L, S Rodrigues, M Naujokas and M Park. 2002. *Crk synergizes with epidermal growth factor for epithelial invasion and morphogenesis and is required for the met morphogenic program.* JBC. 2002 Oct 4; **277**(40): 37904-11.

   I contributed figure 4B – work described in Chapter 3.

2. Lamorte L, S Rodrigues, V Sangwan, C Turner and M Park. *Crk Associates with a Multimolecular Paxillin/GIT2/{beta}-PIX Complex and Promotes Rac-dependent Relocalization of Paxillin to Focal Contacts* MCB 2003 Jul; **14**(7): 2818-2831.

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Chapter I Introduction

The ability of tumours to metastasize and grow at distant sites is dependent on the acquired migratory and invasive mechanisms activated in cancer cells. Depending on their extracellular environment epithelial sheets or individual cells can transit between different molecular migratory programmes in order to invade surrounding tissue. Examples of some of these programmes include epithelial-mesenchymal transitions, mesenchymal-amoeboid transitions, collective-amoeboid transitions and mesenchymal-epithelial transitions (reviewed in [1]). Most of these transitions usually take place during physiological processes such as embryonic development, wound healing and organogenesis, but it is the unregulated onset of such programmes that may contribute to malignancy. The dissection of molecular signals required for cell migration and invasion is an important step towards the development of therapeutics to target tumor metastases.

2 EMT Dynamics

The epithelial-mesenchymal transition programme is a critical step during embryogenesis in which polarized epithelial cells lose their polarity and undergo cytoskeletal changes allowing them to acquire mesenchymal markers such as Vimentin expression and the internalization of E-cadherin that are required for motility and the permeation of the surrounding extracellular matrix. This process is characterized by the loss of the typical apical-basal polarized epithelium. This

occurs through the stepwise dissociation of cell-cell contacts such as adherens junctions followed by the breakdown of tight junctions, as well as the reorganization of the cytoskeleton and microtubule network to allow for the redistribution of organelles. This also includes the formation of membrane protrusions towards a leading edge concomitant with the formation of a trailing edge, which provide for a new re-polarized morphology that is more suitable for cell motility (See fig 1).

Madin-Darby canine kidney (MDCK) epithelial cells are used as a model to study epithelial mesenchymal transitions and has provided major contributions in dissecting the molecular mechanisms involved in EMT [2]. One of the predominant factors that promotes EMT in MDCK cells is the hepatocyte growth factor/scatter factor and its receptor, the Met tyrosine kinase [3].

However extracellular components such as collagens and laminins, as well as soluble factors such as EGF and members of the FGF and TGFβ families signal through their interaction with receptor tyrosine kinases and other transmembrane receptors such as integrins to activate of signalling pathways that will lead to the transcriptional regulation of genes involved in the EMT programme such as the downregulation of E-cadherin expression [4].

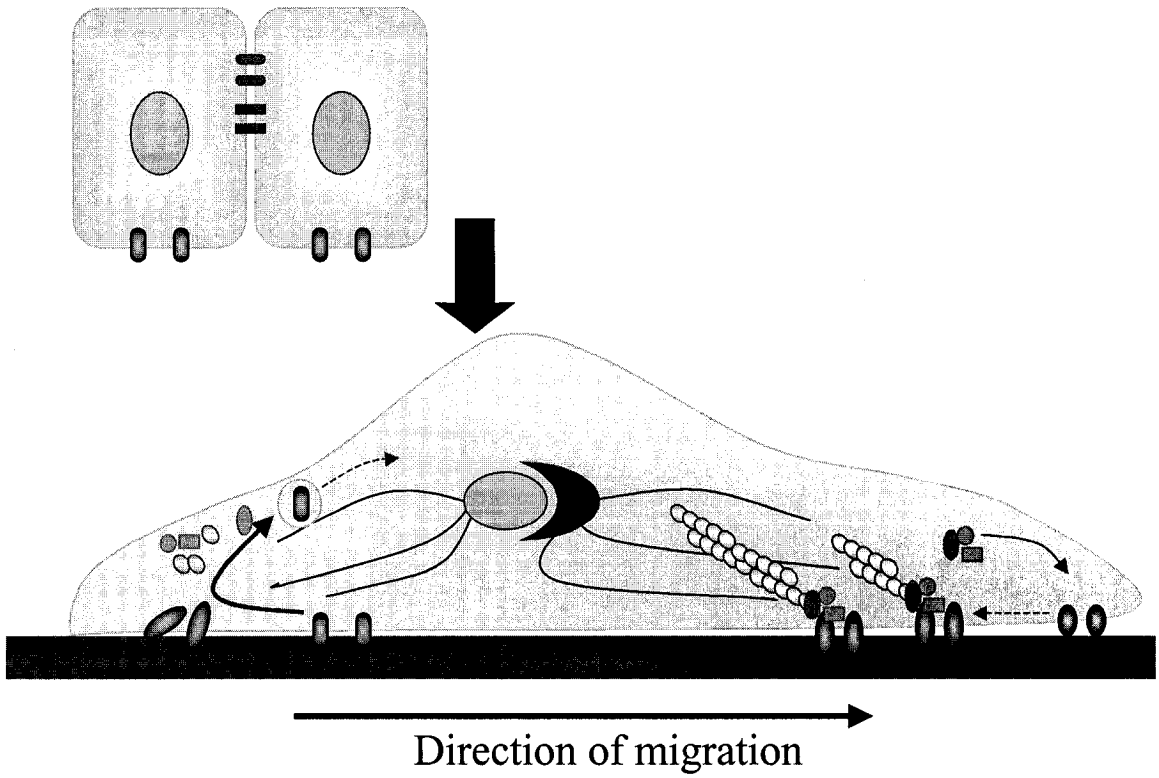

Figure1: EMT/cell migration dynamics

3 Cell-cell adhesions

A polarized epithelium is maintained mainly through cell-cell contacts, which maintains integrity of the colony and polarity through direct anchorage to the actin cytoskeleton. These include adherens junctions, tight junctions and desmosomes. One of the earlier steps during tumorigenesis includes the breakdown of cellular junctions. Furthermore, some of the components of these junctions have been used as pathological markers for invasive carcinomas, such as the internalization or loss of expression of E-cadherin.

The formation of adherens junctions is a tightly regulated process, which begins with the vesicular recruitment of β-catenin and E-cadherin dimmers to the plasma membrane [5]. At the plasma membrane β-catenin links the cadherin zippers to the actin cytoskeleton via α-catenin. α-catenin can bind directly to the actin filaments or indirectly through vinculin. Actin polymerization at these sites has been reported to be mediated by VASP a protein that can also bind to vinculin [6]. The formation and stability of adherens junctions may occur through the endocytic recycling of E Cadherin, mediated through Arf6 activity [7, 8], an increase of Rac activity [9] and its effector IQGAP [10], which in turn can interfere with the cadherin complex by competitive binding to β-catenin [11] and still through p120-catenin, which has been shown to be both a positive and negative regulator of cadherin clustering [12]. Recently, IQGAP has been also recognized as an intermediate between E-cadherin adhesions and the microtubule network by its interactions with CLIP-170 [13].

At cell- cell contacts, desmosomes alternate with adherens junctions along the cell borders. The assembly of either of these structures is calcium dependent [14]. Desmosomes are heterotypic structures of desmosomal cadherins (desmogleins and desmocollins). These transmembrane receptors are linked to the intermediate filaments network through the association with desmoplakin [15]. Alternatively, desmogleins can also bind the intermediate filament network through plakoglobin, which contains structural similarities with β-catenin, possibly why it also binds the cytoplasmic tail of e-cadherin [14].

Tight junctions are specialized plasma membrane microdomains that form a network of continuous fibrils around each epithelial cell providing not only a structural support to maintain cell polarity but particularly a selective barrier for diffusion of molecules and ions across the epithelium and also a barrier between apical and basolateral lipids and proteins [16]. The ZO-1 adaptor protein complexes, which link tight junctions to the actin cytoskeleton, occludins and claudins are transmembrane proteins responsible for the maintenance of these structures [16]. Recently signaling complexes, such as Rab small GTPases [17], have been found to associate at these structures. These are thought to regulate endocytic trafficking, which may be responsible for the recruitment of protein complexes. The Par3-Par6 complex, which regulates Cdc42 GTPase activity is localized to these sites and plays a role in the maintenance of cell polarity [18] most likely mediated by microtubule orientation.

4 Cell-matrix adhesions

Adhesions with the substrate are fundamental not only for the propagation of migratory signals, intracellularly, but also to provide the anchoring and tension required to physically propel the cell body forward. These structures are complex molecular assemblies bridging the extracellular matrix to the actin cytoskeleton via transmembrane receptors known as integrins [19].There are different types of adhesion foci according to their components, their strength or elasticity, their duration and their spatio-temporal location. These characteristics may depend on cell type, intracellular molecular signaling and composition of transmembrane receptors just as much as the composition of the extracellular matrix to which the cell is adhering.

There are several types of cell-matrix adhesion foci. These include the well-characterized focal adhesion, the short-lived focal complexes, fibrillar adhesions and podosomes [20]. Initial contact with the substrate at the leading edge originates formation of focal contacts, which are small dot-like structures ~60nm thick and ~ 1 μm long [21, 22]. These structures have a fast turnover and appear to be Rac dependent. Their constituents are mostly tyrosine phosphorylated proteins, and scaffolds such as paxillin and talin, low levels of vinculin and FAK and are totally devoid of zyxin and tensin [20, 23], [24], [25]. Focal contacts are predominantly enriched with $\alpha_V\beta_3$ integrins and apply stronger traction during cell migration than mature focal adhesions [26]. Pak a downstream effector of both Rac and Cdc42 appear to be a major regulator of focal complexes turnover. This serine kinase phosphorylates LIM kinase, which

in turn phosphorylates and deactivates actin depolymerizing proteins ADF/cofilin [27]. Focal complexes can then mature into longer, denser structures known as focal adhesions via Rho activation. Rho activity leads to further polymerization and stabilization of actin stress fibers through its effectors ROCK and mDia activities. These structures are ~2-5 μm long, they are found at the cell periphery and are mainly constituted of FAK, Vinculin, Zyxin, Paxillin and α_V integrins. Interestingly there appears to be a shift between Paxillin containing protein complexes and Zyxin containing protein complexes when transitioning between focal complexes into focal adhesions respectively [23]. Furthermore, the traction and adhesive properties of the focal contact may vary due to the nature of its integrin and cytoskeletal proteins components. Fibrillar adhesions in contrast arise from focal adhesions during actomyosin induced contractibility and contain predominantly α_5 integrins and Tensin [28]. These are structures of variable length, from 1-10 μm long found in the central region of the cell.

The initial response of a cell to a migration-promoting agent is the formation of membrane protrusions through the extension of the actin filaments towards a leading edge (Figure1). Large and broad membrane ruffles known as lamellipodia or spike-like membrane extensions known as filopodia are examples of some of these protrusions. Integrin and receptor tyrosine kinase receptor clustering at these edges allow for further actin polymerization and maturation of cell-matrix contacts required for cell motility [1, 29]. The clustering of integrins at the leading edge signals the recruitment of scaffolds and adaptor

proteins that interact with integrins through their cytoplasmic tail. Such adaptor proteins form protein complexes through their SH2 and SH3 domains and contain regulatory molecules such as PI3K or GEFs such as C3G or SOS, and GAPs such as GC-GAP, which regulate Rho GTPases and actin binding proteins that allow for the bridging of integrins to the actin cytoskeleton [1]. Surface proteases are recruited to focal contacts and initiate cleavage of extracellular matrix components such as collagens, fibronectin and laminins, as well as pro-matrix metalloproteinases to generate active soluble MMPs necessary for the degradation of the surrounding extracellular matrix [1, 30]. The physical extension of the cell's cytoskeleton generates tension required for the contractibility of the cell body. The contractability of the actin cytoskeleton is mediated through active myosin II binding to actin filaments forming the actomyosin complex. Myosin II is activated by MLCK phosphorylation of the myosin light chain. This process is regulated by MLC phosphatase, which in turn can be inhibited by phosphorylation from ROCK, an effector of Rho.

At the trailing edge, membrane retraction occurs through a combination of mechanisms. Capping proteins such as cofilin bind to monomeric actin preventing actin polymerization and inducing filament dissociation. The protease calpain cleaves cytoplasmic tails of integrins. Phosphatase activity can also promote disassembly by dephosphorylation of cytoskeletal proteins and consequently disassembly of protein complexes at focal adhesion sites. Once the focal adhesion disintegrates integrins are internalized by endocytic vesicles

and recycled towards the leading edge where new focal contacts will generate[1, 31].

5 Cell migration and invasion signaling

The interaction of a cell with the extracellular matrix not only provides a substrate for cell anchorage and guidance during embryogenesis and wound repair induced migration, but it also acts as a medium by which the cell receives environmental signals essential for proliferation, differentiation and apoptosis. Cell-matrix contact sites become therefore signaling domains where protein complexes agglomerate to activate various signaling cascades. In order to better understand how some of these signals are regulated researchers have tried to dissect the protein complexes responsible for transmitting them.

5.1 Integrins

Integrins are heterodimeric transmembrane receptors containing an α and a β chain assembled noncovalently. To date, 18 α, 8 β subunits and more than 20 heterodimers have been identified. Integrins recognize several extracellular matrix ligands, such as fibronectin, collagens and laminins, as well as receptors on neighbouring cells [32]. Similarly, one ligand can be recognized by several integrin heterodimers. For example, $\alpha 2\beta 1$ expression in platelets is specific for collagen and not laminin [33]. The $\beta 1$ chain in particular can associate with 12 α chains [34]. Knockout studies of integrins have highlighted their importance in development. $\beta 1$ chain knockouts show very early embryonic

lethality, while α chain knockouts have demonstrated these receptors play an important role in the development of the nervous system [35, 36]. In vitro studies have not only demonstrated the specific affinities between integrin heterodimers and their extracellular matrix ligands but also mechanisms and biological responses downstream of particular integrin dimers. For example, while α5β1 integrins are mainly found in small focal complexes, αVβ3 are only found in large focal adhesions at the ends of stress fibers [37]. On the other hand, in immortalized b1 null cells replaced with aVb3 show reduced RhoA activity accompanied with few actin stress fibers and few peripheral focal adhesions [38]. Although their cytoplasmic tails are short and devoid of enzymatic activity, integrin clustering at the cell membrane signals the recruitment of cytoplasmic kinases, adaptor proteins, growth factor receptors and actin binding proteins. Integrins can also associate laterally with membrane proteins such as caveolin-1. The abrogation of this interaction leads to the suppression of integrin signaling and of formation of focal adhesions [39]. Integrins are also required for proper signaling of certain growth factors, and have been shown to directly associate with some of them, examples are VEGF, EGF, Insulin receptor, PDGF [40]. This cross-activation may lead to enhanced clustering of integrins at the cell surface and amplification of survival, migration, and growth signals. Importantly, upon ligand binding cytoskeletal proteins (Talin, tensin, α-actinin,Vinculin, Paxillin) bridge integrins to the actin cytoskeleton and to downstream effectors of intra-cellular signaling pathways such as FAK, Src, and SHC and PKC. Activated Fak and src bind to several

signaling molecules mediating integrin activation of the Erk and Ras pathways. These proteins, once active can phosphorylate p130Cas and promote the assembly of p130Cas-Crk-Dock180 complexes leading to the activation of the small GTP-binding protein Rac [41]; [42].

5.2 Focal adhesion molecules

Integrins can link to the actin cytoskeleton through a variety of pathways. Direct linkers include ILK, α-actinin, Talin and Tensin [43]. Talin is a cytoskeletal linker between β1, β2 and β3 integrins to FAK, vinculin and F-actin [44]. This large (270kDa) protein also associates and activates PIPKIγ leading to an increase in PIP2 expression, which in turn amplifies integrin clustering and association with Talin [45, 46]. These complexes are tightly regulated by growth factor signaling [47]. Talin association with vinculin occurs both at focal adhesions and cell –cell contacts and it is also regulated by PIP2 activity [43]. In vivo talin-deficient flies show integrin association with the extracellular matrix but deficient integrin clustering and association with the actin cytoskeleton [48]. Talin-1 defficient stem cells also show impaired cell adhesion and spreading on collagen and laminin due to inability to form focal adhesion structures and polarize actin [49].

Vinculin is a large scaffold protein that contains binding domains for several cytoskeletal proteins such as actin, α-actinin, talin, paxillin, VASP, ponsin, vinexin and PKC [50]. Its structure resembles the structure of many kinases in the sense that it can maintain an open or closed conformation (see

figure2 in [44]. Interestingly, the "closed" conformation of the protein masks the binding sites to several proteins like talin, VASP, F-actin and a-actinin but not the paxillin binding site [51]. Recent studies from the Burridge lab suggest that vinculin may act directly on the initiation of actin polymerization at focal complexes by direct association with the Arp2/3 complex and its recruitment to these sites [52]. This interaction is transient and dependent on PIP2 activity and Rac activation of Arp2/3. Although vinculin is important for the stabilization and formation of focal adhesion in fibroblasts, genetic studies in mouse embryonic stem cells have shown that it is not essential for focal adhesion assembly [49]. Vinculin knockout mouse embryos showed heart and brain defects and died by day E10 [53]. Such findings underscore its role in cell adhesion signaling required during embryogenesis.

FAK is a cytoplasmic tyrosine kinase that has been shown in several cell types to be an integral downstream effector upon integrin ligand binding [54]. Although FAK binds directly to β1 and β3 integrins tails, this association does not seem to be required for FAK activation or its localization to focal adhesions [55]. FAK phosphorylation by Src enhances its catalytic activity [56]. Further support for a Src dependent activation of FAK are the findings of Tom Parsons that src expression in FRNK expressing cells, an inhibitor of FAK, rescues cell spreading and motility [57]. FAK null fibroblasts also exhibit a rounded morphology, increase in cell-matrix contacts and decreased cell motility [58]. Activation of FAK leads to the phosphorylation and recruitment of several adaptor protein complexes important for the initiation and maintenance of focal

adhesions. For example, a p130Cas-Crk-Dock180 complex responsible for Rac activation for lamellipodia formation and Jnk activation leading to cell cycle progression is assembled post FAK phosphorylation of p130Cas [59]. Also FAK can indirectly activate Rho-GTPase-activating GEFs via PI3K or Paxillin –Git2-βPix complexes which in turn activate Rac and Cdc42 [60], [61], [62] [63]. FAK binding to Paxillin, an integral focal adhesion scaffold (will be further discussed later on) has been suggested to be an important step in the recruitment of Fak to focal adhesions [64]. FAK mediated cell adhesion is also responsible for the activation of the protein kinase B/Akt pathway via PI3K in prevention of anoikis [65].

Other enzymes at focal adhesion sites include serine/threonine kinases such as ILK, PKC and PAK, and phosphatases such as SHP-2 and SHIP-2 [20]. Eventhough, proteins such as ILK appear to have more of a scaffolding and F-actin reorganization function than a catalytic function at focal contacts [20]. ILK appears to be involved in the recruitment of various protein complexes, which modulate actin dynamics. Examples of these are the ILK–Pinch complex, which interacts with Nck, a mediator between growth factor and integrin signaling, and the actopaxin-β-Pix-Paxillin complex which in turn activates Rac and Cdc42 and may also be a direct link to F-actin [20].

Many other proteins such as tensin, α-actinin and filamin provide a linking bridge between integrins and F-actin, although it still remains unknown whether these molecules merely provide structural anchoring points for other

signaling protein complexes or whether they are important for the propagation of signaling pathways.

5.3 Rho GTPases

The Rho family of small guanosine triphosphate GTP-binding proteins (Rho-GTPases) is pivotal in the regulation of the actin organization, nucleation and extension and adhesion turnover events during cell migration. Rho proteins cycle between an active GTP-bound form, and an inactive GDP-bound form. Regulators of GTP loading and hydrolysis of Rho-GTPases include guanine-nucleotide-exchange factors (GEFs) and GTP-ase-activating proteins (GAPs) respectively [66]. Furthermore, guanine nucleotide dissociation inhibitors (GDIs) bind to the GDP-bound forms to prevent nucleotide dissociation thereby keeping GTPases inactivated [67]. The Rho GTPase family is comprised of more than 20 members, of which RhoA/B, Rac1/2 and Cdc42 have been well characterized as key modulators of the actin remodeling dynamics [66].

Each of these three Rho GTPases have distinct effects on the actin cytoskeleton. Rac activity is responsible for the formation of large lamellipodia and membrane ruffles, while Cdc42 induces the formation of filopodia during early sensory events of cell migration and Rho activity promotes maturation of focal adhesions and formation of stress fibers [68]. The major actin nucleation targets of Rac and Cdc42 are the WASP/WAVE family of proteins. Pak and PI5K are some other important Rac downstream effectors, which play a role in actin reorganization at the leading edge. PI5K activation leads to uncapping of

actin filaments [69]. Pak, a serine/threonine kinase, phosphorylates and activates LIM kinase, which inactivates actin depolymerization proteins ADF/cofilin downstream of Rac and Cdc42 [27]. Recently several reports have suggested that Cdc42 may also participate in the reorientation of the microtubule-organizing center (MTOC) and the golgi during directional cell migration [70-72]. A Par6, Par3, αPKC complex mediates this event [73]. Other reports have suggested that the microtubule polymerization at the leading edge and its de-polymerization at the trailing edge may be responsible for further activation of Rac and RhoA at each respective site [70]. RhoA main downstream effectors during actin remodeling are mDia and ROCK. Rho kinase 1 and 2 (ROCK1/2) act on various targets, including LIM kinase, ERM proteins (ezrin/radixin/moesin) and others, all of which enhance actin polymerization [26]. ROCK regulates cell contractibility by acting on myosin-II regulatory light chain (MLC) dynamics [26]. Dia molecules once activated by Rho-GTP interact with profilin, src and IRSp53 through their exposed FH (formin homology) domain [74]. mDia has also been attributed a role in microtubule reorientation towards a wounded edge of fibroblasts [75]. ROCK and mDia play complementary roles in Rho dependent formation of focal contacts and stress fibers. mDia is required for profilin mediated filament assembly, enhancing focal contacts and stress fibers formation in normal cells but these are inhibited once ROCK activity is blocked [76].

6 Regulation of cell migration

Current models of cell migration agree that cell migration signaling relies on extracellular stimuli for activation. Nevertheless, it has been postulated that aggressive/invasive tumours have acquired the ability to constitutively activate intracellular pathways involved in cell migration and invasion. In normal morphogenic responses EMT signaling activation relies on the interaction of growth factors, cytokines, hormones and extracellular matrix elements with their cell surface receptors. Receptor activation triggers signaling cascades involved in cell growth and differentiation, cell migration and invasion, cell survival or apoptosis [77].

6.1 RTK family

Receptor tyrosine kinases are a large family of transmembrane receptors that incorporates 20 subfamilies characterized by their structure, ligand affinity, and their biological responses [78]. All members of these subfamilies consist of a similar structure containing an extracellular ligand-binding domain, connected to the cytoplasmic domain through a single transmembrane helix. The cytoplasmic domain contains a well-conserved catalytic tyrosine kinase domain and other auto-phosphorylation and protein docking sites [79]. With the exception of the Insulin receptor family of RTKs, which exists as a disulfide-linked dimmer, ligand binding to the receptor promotes receptor dimerization that leads to a conformational change resulting in phosphorylation of the tyosines in the active loop and kinase activation. Once active, the kinase

autophosphorylates tyrosines outside the kinase domain that results in binding of SH2 or PTB domain containing proteins required for the propagation of the signal [77]. The recruitment of adaptor and scaffolding proteins, which are now in complex with the activated receptor provides docking sites for the activation and phosphorylation of additional downstream effectors. In this manner not only the signal is transduced from the cell membrane to the nucleus but also cross-talk between signaling pathways occurs [80].

Although various RTKs activate similar downstream effectors, specific biological responses arise from the activation of different RTKs. It has been suggested that this event could be due to the intrinsic qualities of the signal, be it duration or association of different intracellular signals, or due to cross-talk between an RTK signal and cell specific pre-existing signaling pathways [81]. Examples of the later would be FGFR1 activation during early development induces cell migration while at later stages in neuronal cells it leads to cell survival signals and proliferation [81]. It becomes important therefore to dissect what kind of signaling complexes are formed at each step of the different signaling cascades and take into account localization of activation, cell types, and the duration of different signals. For example, the ErbB family of RTKs will initiate a distinct signaling pathway by recruiting different SH2 containing proteins [82]. On the other hand, recruitment of similar proteins to different receptors initiates consistent biological responses. PI3K recruitment to RTKs leads in the most part to cytoskeletal reorganization events and it also plays a role in trafficking [77]. Most RTKs lead to the activation of the Ras/MAPK

pathway as well[83]. These studies would suggest that RTKs play a role in the initiation process of various signaling pathways that may already be programmed by other signals in the cell. Deregulated RTK activation would bring about the constitutive activation of various downstream signaling programmes such as cell migration, proliferation, growth and survival. Deregulation of RTK activity occurs through genetic amplification, formation of an autocrine loop, chromosomal translocations and point mutations [78]. ErbB2/Her2 is found to be amplified in about 30% of breast cancers [84]. A point mutation in the transmembrane domain of ErbB2/Her2 promotes receptor constitutive dimerization, which leads to a ligand-independent kinase activation [85]. The poor prognosis associated with ErbB2 amplification [84] may be due to elevated activation of migratory signals. HGF overexpression is also a poor prognostic marker in breast cancer [86] /and various in vitro studies [87] and mouse models [88] have demonstrated its scattering and invasive qualities. Also, several RTKs, including members of the Trk family, Ret, FGFR1, and PDGFRβ have been reported to show chromosomal translocations in various tumours [77]. Tpr-Met (translocated promoter region fused to Met), initially isolated in 1980 from an human osteogenic cell line treated with a carcinogen, *N*-methyl-*N'*-nitronitrosoguanidine [89] is another member of these oncoproteins which has been well characterized to have a role in promoting cell scattering during tumorigenesis.

6.2 Met signaling and epithelial remodeling

As mentioned above, the mutant form of the Met receptor, Tpr-Met was the initial oncogene isolate of Met identified in 1980 [89]. This oncoprotein results from a chromosomal translocation in which a leucine zipper dimerization domain from the translocated promoter region of chromosome 1 is fused to the Met cytoplasmic region in chromosome 7 [90]. Since Tpr-Met does not contain the transmembrane and the juxta-membrane domains of Met, it colocalizes in the cytoplasm, although, due to the inherited receptor dimerization mediated by the leucine zipper, its kinase is found constitutively activated[90]. Independent studies identified a potent motility factor (scatter factor, SF [91]) and a mitogenic factor for rat hepatocytes (hepatocyte growth factor, HGF[92]). It was only later on that HGF would have been identified as the ligand for the Met receptor [93] and that SF and HGF were the same molecule [94].

The Met receptor is expressed ubiquitously in epithelial tissues, but it is also expressed in other cell types such as neuronal cells, hematopoetic cells, melanocytes, endothelial cells and a variety of tumour cells from various origins [95]. In normal mammary development, mesenchymal cells produce HGF and other growth factors to initiate tubulogenesis in a controlled paracrine manner [96]. However, the overexpression of HGF or Met has been identified in a variety of invasive cancers, including breast carcinoma [96]. The Met receptor belongs to a subfamily of RTKs, which includes Ron and Sea receptors. This family of oncoproteins has been implicated in the activation of proliferation, cell motility, cell morphogenesis and differentiation [97, 98]. Furthermore, HGF/Met

signaling is believed to play an important role in epithelial-mesenchymal interactions required during organogenesis and morphogenic differentiation programmes [99]. Examples include Met and HGF/SF signaling during migration and development of muscle and neuronal precursors [100, 101], liver and placenta organogenesis [102, 103], tissue regeneration [104], and its role in angiogenesis [105-107]. In vitro HGF/Met signaling has been characterized as a potent modulator of branching epithelial morphogenesis [108], wound healing[109], cell scattering and invasion in 3D matrices[3, 110], all biological responses dependent on epithelial-mesenchymal transitions.

The role of HGF and the Met receptor in the regulation of cell motility has been extensively demonstrated in cell culture and mouse models. During development progenitor cells have to migrate for long distances to reach their target where they will differentiate and undergo organogenesis. In Met-homozygous-mutant mouse embryos, these progenitor cells where unable to undergo transient EMT and invade the surrounding limb buds resulting in the absence of skeletal muscles of the limbs [100]. The release of progenitor cells during development to distant sites involves the activation of an EMT event similar to that occurring during tumour progression when invasive cells are released from the primary tumour mass to metastasize to distant sites [111]. In cell culture models, HGF promotes cell scattering and an increase in cell motility. This transient EMT is characterized by a loss of cell polarity, breakdown of cell-cell junctions resulting in colony spreading and dispersal [112-115]. In search of the signaling involved in early events during cell scattering,

researchers have found that inhibition of either the PI3K or Erk/MAPK pathways abrogated colony scattering [116]. Gab1, the primary substrate of the Met receptor, promotes transcriptional activation and the activation of regulatory adhesion molecules via Shp2 and the Erk/MAPK pathway [117-120]. Actin cytoskeletal rearrangement occurring during HGF-induced cell scattering is mediated by Rho GTPases activation. In an MDCK epithelial cell model HGF treatment induces Rac-mediated membrane ruffling and breakdown of cell-cell junctions [114, 121]. Furthermore, dominant-negative mutants of Rac or Cdc42 inhibit HGF-induced cell scattering [114, 121]. Gab1 recruitment of adaptor protein Crk leads to the activation of Rap1 via Crk's association with C3G[122, 123]. Rap1 activation downstream of the Met receptor may be involved in promoting integrin clustering at the leading edge during cell motility. Recent studies from our lab have also elucidated other adhesion protein complexes (Paxillin-Git2-Pix) assembled upon HGF treatment, which are required in the actin remodeling during cell scattering [61, 121]. Cell spreading requires turnover of the cell-matrix adhesions. Recent data suggests that Met can directly interact with β4-integrins and ezrin, a cytoskeletal linker protein from the ERM family[124, 125]. Moreover, HGF has been suggested to promote the assembly of a protein complex between Met and E-cadherin/catenin proteins in prostate cancer cells [126]. Such interactions may be important in the clustering of the receptor at particular subcellular localizations for the assembly of specific receptor-protein complexes. Collagen/matrigel assays are 3D in vitro models that have been used extensively in the search for molecular regulators

downstream of HGF/Met mediated epithelial-morphogenesis. Well-differentiated epithelial cells are seeded in a collagen or matrigel matrix and allowed to form organized polarized hollow cysts. HGF treatment induces a partial-EMT, which involves the extension of branching tubules containing cells that are undergoing major cytoskeletal rearrangement. When cells regain their polarity tubules are formed. Tubulogenesis is a complex morphogenic programme that occurs in vivo during the differentiating steps of organs like the lungs or kidneys[111]. These models have been successful for the dissection of mechanisms required for epithelial morphogenesis and tumorigenesis. For example, the deregulated activation of other RTKs such as the Erb2 family in addition to a normal differentiation signal from Met induces the transition between a partial-EMT to a permanent-EMT in MDCK cells [127]. Also, HGF induced Gab1 protein complexes that are required for cell scattering have been found to also play a role during epithelial-morphogenesis using these same models [117, 118, 128-130].

7 Crk adapter proteins

SH2/SH3 domain containing adaptor family of proteins are critical modulators of tyrosine kinase signaling pathways. Members of this family include adaptor proteins Grb2, Nck and Crk. Although these proteins do not contain enzymatic activity they play a key role in the recruitment of proline-rich motif containing effector molecules to tyrosine phosphorylated kinases or scaffolds (Figure 2). The formation of multi-protein complexes at particular

cellular compartments is crucial for the activation of signaling cascades. HGF/Met mediated cell scattering and epithelial morphogenesis are biological responses dependent on the assembly of Gab1 protein complexes [121, 122]. Various reports have identified the Crk family of adaptor proteins as key modulators of morphogenic and tumorigenic programmes (reviewed in [131]).

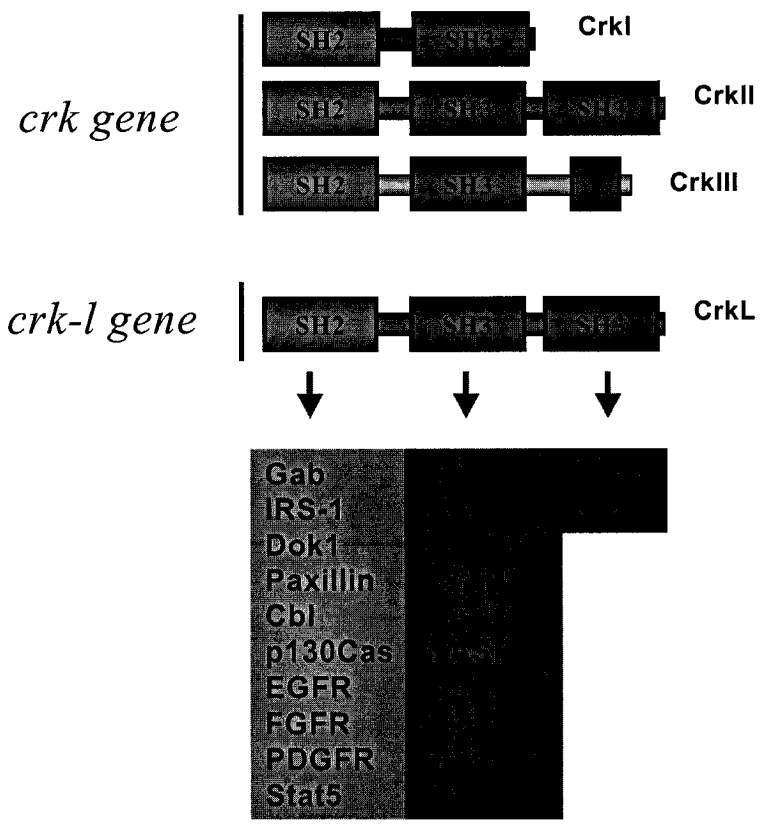

Figure 2: Crk family of adaptor proteins and their interacting proteins

7.1 History

The Crk adaptor protein was originally isolated as an oncogenic viral form, v-Crk, from a chicken sarcoma [132-134]. This novel oncogene encoded a fusion gene containing the viral gag gene and a cellular gene, which encoded a

protein that shared homology to PLCγ. This oncoprotein was named v-Crk, viral CT10 regulator of kinase, because of the increased tyrosine phosphorylation levels observed in its transformed cells, despite its lack of catalytic activity [132, 133, 135]. Shortly after the initial identification of p47gag-Crk from the CT10 sarcoma virus, another laboratory isolate the same oncogene from another avian sarcoma virus, ASV1 [134]. The sequencing of the cellular homologs of v-Crk, revealed that v-Crk was indeed a fusion of the viral gag protein and the shorter form of cellular Crk, CrkI [136, 137]. The Crk proto-oncogene maps to human chromosome 17p13 [138], and it generates two protein products, CrkI and CrkII by alternative splicing [136]. Cellular Crk I is a 28 kDa protein that consists of one SH2 domain and one SH3 domain. Crk II, a 38-42 kDa protein, depending on tyrosine phosphorylation and cell type, consists of one SH2 domain and two SH3 domains separated by a spacer region of about 50 amino acids. In 1993, a protein sharing 60 % amino acid homology to Crk II was identified as Crk-like (CrkL) protein [139]. This protein like CrkII contains one SH2 domain and two SH3 domains. CrkL is highly phosphorylated and the major substrate for Bcr-Abl in CML cells[140-142]. Crk II is the most abundant form of Crk in all cell types and it is also found as a double band in western blots due to its phosphorylated species [137]. Although Crk II expression levels appear to be higher than CrkI in most cell types, CrkI has a higher transforming activity than CrkII [136]. These results are consistent with the findings that CrkII contains a regulatory tyrosine residue (tyr221) in the spacer region [143]. This tyrosine, once phosphorylated, binds intramolecularly to the SH2 domain

promoting folding of the protein that impairs SH3 interactions [144, 145]. CrkL contains a similar regulatory tyrosine residue at position 207 [146]. Abl binds to either CrkII or CrkL's SH3 domains and phosphorylates this regulatory tyrosine, downregulating their signaling [143, 146].Unlike CrkII and CrkL, CrkI and v-Crk lack this tyrosine residue due to their truncation. It's been suggested that the transforming and oncogenic activities of these two proteins result from an increase in protein-protein interactions and activation of downstream signaling [131, 143, 147, 148]. Various cell culture based studies have highlighted the regulatory role of this tyrosine in complex assembly and biological responses downstream of Crk proteins [149-153]. An additional site that may serve as a regulatory region in Crk II is the DE loop fold, a proline-rich region that bulges out of the SH2 domain and may act as a transient ligand to the SH3 domain. This structure has been recently shown to interact directly with the SH3 domain of Abl by NMR, independently of tyr221 [154].

7.2 Crk SH2 domain binding proteins

Src homology 2 domains usually bind short protein motifs that contain a phosphotyrosine followed by specific residues. Crk's SH2 domain binds specifically to pY-x-x-P motifs [155]. Crk and CrkL share similar SH2 domain binding partners due to their high homology in their SH2 domains. Some of their SH2 binding partners include Gab1/2 and IRS docking proteins, Paxillin, p130Cas, Cbl, receptor tyrosine kinases such as EGFR, FGFR and PDGFR, and nuclear proteins such as Stat5 and Wee1 (reviewed in [131]).

Paxillin is a 70kDa protein that serves as a scaffold of multi-protein complexes at focal adhesion sites. This protein contains various protein binding sites, LD motifs, LIM motifs, SH2 and SH3 interacting sites. LIM domains provide anchorage points for it to bind to the plasma membrane while LD domains appear to interact with cytoskeletal proteins required for its recruitment. Paxillin is phosphorylated and binds to Fak and src. Paxillin phosphorylation is required for proper assembly of focal adhesions and formation of stress fibers [156, 157]. Crk adaptor proteins bind to phosphorylated tyr31 and tyr118 of paxillin. This interaction is important for the assembly and turnover of focal contacts [61, 158]. Recently, paxillin was also shown to be ser/thr phosphorylated by Erk downstream of HGF, which resulted in the recruitment and activation of FAK and subsequently increased cell spreading and adhesion [159]. Paxillin also associates with phosphatase PTP-PEST through its LIM domains. This interaction is thought to be important for p130Cas (substrate of Crk proteins in complex with paxillin) dephosphorylation and mechanistically help the turnover of focal adhesion sites at the leading edge of migrating cells [160-162]. Paxillin knockout mice show developmental defect and are embryonic lethal. Fibroblasts derived from these mice exhibit impaired cell spreading and cell motility due to inadequate formation of focal adhesions [64].

Gab 1 and Gab 2 docking proteins, like certain IRS family members, interact and recruit Crk adaptor molecules to the plasma membrane in response to receptor activation [122, 123, 163-165]. Gab1-Crk interactions appear to be

important for HGF/Met induced epithelial morphogenesis [118, 121, 122, 128]. Furthermore, Gab-Crk interactions lead to activation of Rap1 and Jnk downstream of activated Met [122, 163]. These signals may be important for anchorage independent growth and activation of certain matrix metalloproteinases [123, 163]. Gab deficient mice show developmental defects in placenta, heart and skin, pathological defects similar to those of receptor tyrosine kinase knockouts [166]. MAPK activation levels were low and cells isolated from these mice showed reduced migration capacity[166].

Similar in structure to docking proteins such as Gab, IRS-family, and DOS, p130Cas contains 15 Y-x-x-P motifs that can interact with the Crk SH2 domain [167, 168]. The P130Cas family includes Hef1/Cas-L and Efs/Sin homologous proteins. These docking proteins recruit various adaptor proteins such as Crk, Grb2 and Nck, tyrosine kinases such as FAK and src, a variety of phosphatases like PTP1B and Shp2, and in the case of p130Cas even C3G, a guanine nucleotide exchange factor that also binds to the SH3 domain of Crk. The ability of Cas to form large multi-protein complexes dependent on stimuli, localization and association with heterologous proteins validates its pivotal role as a signalling regulator. Cas-Crk and Cas-src complexes regulate actin cytoskeleton rearrangement, cell migration and adhesion, and cell proliferation processes. For example, Cas-Crk interactions are pivotal for the intracellular transmission of integrin signals [169]. Furthermore, these complexes are important for the activation of Rho GTPase Rac at membrane protrusions during cytoskeletal remodelling required for cell migration [42, 170]. P130Cas

deficient embryos show cardiovascular defects, retarded growth and an inability to form actin stress fibers[171].

7.3 SH3 domain binding proteins

Crk adaptor proteins are recruited to upstream signaling proteins through the ability of their SH2 domains to bind a phosphotyrosine residue in the context of its surrounding amino acids. Crk proteins recruit downstream effectors through the ability of their SH3 domains to bind to proline-rich sequences. The Crk SH3(1) domain binds preferentially to the consensus sequence P-x-L-P-x-K[172]. A Lys-Arg mutant still binds to the first SH3 domain of Crk but with a much lower binding affinity, and an increased affinity to the SH3 domain of Grb2 [173]. Most Crk adaptor proteins SH3 domain interactions are mediated through the fisrt SH3 domain of Crk. To date there have only been two reports of c-terminal SH3 binding proteins, Cd34 and Crm1. Cd34 is a transmembrane proteins expressed during lymphohematopoetic development that is involved in cell adhesion. This protein appears to have specific affinity to CrkL and not CrkII [174]. Crm1 is a nuclear export factor that can form a complex with Crk and a nuclear tyrosine kinase Wee1. A mutant of Crk that can no longer bind to Crm1 induces apoptosis of mammalian cells[175]. The recent finding of CrkIII further highlighted the importance of Crk's second SH3 domain. The C-terminal SH3 domain of CrkIII is a truncated version of the SH3 domain of its homologs CrkII and CrkL, Overexpression of CrkIII does not enhance insulin-induced Erk activity as does CrkII implicating this domain in signal transduction [176]. The

N-terminal SH3 domain interacts with various regulatory proteins. Recently, Rac was shown to bind to this domain and this interaction was important for the localization of Rac to membrane ruffles and for its signaling [177]. There have also been reports of interactions with this domain that do not follow the P-x-L-P-x-K consensus sequence. Examples include the interaction ofa P-x-I-P-x-K motif in Jnk with the SH3(1) domain of CrkI [178] which is essential for the recruitment of Jnk to membrane ruffles. This also includes the interaction of WASP (an integral recruiter of the Arp2/3 complex during actin remodeling) [179], and DOCK2 (a haematopoietic cell-specific CDM protein essential for lymphocite migration with CrkL [180].

The interaction of Crk with C3G, a guanine nucleotide exchange factor for Ras-family members H-Ras [181], Rap1 [182-184] and R-Ras [185], was the means through which C3G was isolated[186]. Crk-C3G interactions mediate activation of Rac1, Jnk and H-Ras via R-Ras in NIH-3T3 fibroblasts and 293T cells. Furthermore, an R-Ras dominant negative mutant blocked v-Crk induced transformation in NIH-3T3 cells [185].

DOCK180 (downstream-of-crk 180 kDa protein) was initially identified by Far Western as an unknown prominent binder of the Crk SH3(1) domain [187, 188]. It's homolog in *C.elegans*, CED-5, was identified in a complex with CED-2 (CrkII homolog) and CED-10 (Rac1 homolog) that regulates phagocytosis and migration in C. elegans [189]. The exchange factor activity of DOCK180 for Rac1 was found to be dependent on the binding of yet another protein ELMO/CED-12 [190]. The recruitment of this complex to the plasma membrane

appears to be also dependent on DOCK180 interaction with PtdIns(3,4,5), although this interaction had no effect on Rac1 activity [191].

Binding partners for the Crk SH3(1) domain include members of the endocytic pathway such as Eps15 and Eps15R [192]. Eps15 is involved in EGF receptor recycling, and CrkII can also bind directly to EGFR through its SH2 domain, so it has been suggested that CrkII-Eps15 interactions may play a role in the regulation of receptor endocytosis [192]. Another endocytic pathway member that interacts with SH3(1) domain of Crk is Reps1. This RalBP1 (a GAP for Cdc42 and Rac) associated protein is phosphorylated upon EGF stimulation and interacts both with the SH3 domains of both Crk and Grb2 [193]. Recently CrkL was also found to bind directly an Arf-GAP protein, ASAP1 in an SH3(1) dependent manner[194]. The recruitment of ASAP1 to focal adhesions is dependent on this association. GC-GAP, a Rho GTPase-activating protein mostly present in the nervous system, was identified as a Gab1/2 binding partner by yeast-two hybrid, and shown to interact with Crk's SH3 domain. It was proposed that Gab-Crk-GC-GAP complexes may regulate Rac and Cdc42 activity during dendritic morphogenesis [195].

Another important Crk SH3 domain binding partner is PI3K p85 subunit. The interaction of Crk with PI3K mediates anti-apoptotic signals. Furthermore, the formation of Crk stable complexes with p85 and Cbl at the cell membrane increase PI3K activity [196].

7.4 Biological Role

Crk adaptor proteins act as central modulators of various signaling cascades.

7.4.1 Differentiation and development

To date, CrkL is the only member of the Crk adaptor family of proteins to which true knockout studies have been performed [197]. Null mutants of CrkoL (mouse homolog of CrkL) generated by homologous recombination in embryonic stem cells were embryonic lethal at day E16.5.. Heterozygous and up to 2% of homozygous null mice survived and showed substantial neural crest developmental defects. Some of which included, cranial ganglia defects, thymus, aortic arch arteries defects, parathyroid glands and craniofacial structure defects [197]. Since this phenotype is similar to symptoms to DiGeorge syndrome and CrkL locus maps to the chromosomal region that is usually deleted in DGS patients (chromosome 22q11) [198-200], these results suggest that DGS may arise from defects in CrkL molecular signaling. Furthermore, it is suggested that such defects are post-migration of neural crest cells and arise from aberrant differentiation and survival signals during the development of the neural crest. CrkII mutants have been generated by gene-trap insertional mutagenesis, although, the mutants expressed a protein containing an SH2 and an SH3 domain, similar in structure to CrkI [201]. Since this protein was similar in structure to CrkI, the mutants were considered to be CrkII knockouts only. The mice did not reveal any obvious developmental

abnormalities which may suggest that CrkL, CrkI and CrkII act redundantly, or that CrkII is not essential for embryonic development.

These were not the only studies that have implicated Crk adaptor proteins with developmental and differentiation programmes. In neuronal PC12 cells, the overexpression of Crk induces neurite formation and it is dependent on a functional Crk protein [202]. Independent reports have suggested that NGF-induced neurite outgrowth may be mediated by a Crk-C3G-Rap1 activation of MAPK [203, 204].

7.4.2 Cell migration and invasion

Since early studies on v-Crk identified it as a potent oncogenic protein in chickens [134], it wouldn't be surprising that Crk adaptor proteins would be involved in cell migration and remodeling signaling. In rat fibroblast studies, v-Crk induced stress fiber formation in a Rho dependent manner and regulated cell motility [205]. Similar results were obtained in PC12 cells, where overexpression of v-Crk promoted cell spreading and formation of β1-integrin, Fak and Paxillin containing focal adhesions [206]. The role of Crk and CrkL proteins in the promotion of cell migration and cell invasion signals has been widely established. As mentioned in previous sections Crk adaptor proteins are required during HGF-induced cell scattering and epithelial morphogenesis [61, 121, 122]. Our lab has also established that overexpression of Crk adaptor proteins may complement EGF signaling to induce cell scatter, cell invasion and epithelial morphogenesis in MDCK cells, while EGF signaling alone is not

sufficient to promote such biological responses [130]. These results suggest that Crk adaptor proteins complexes may work as "molecular switches" for cell migration and invasion. The coupling of Crk with p130Cas, for example, is downstream of various stimuli in mediating single cell migration. The of Richard Klemke has shown using dominant negative constructs that Cas-Crk complexes are required for ErbB2 dependent migration of cancer cells [207], and integrin-matrix dependent signaling [208]. By dissection of pseudopodia extensions of single migratory cells, Crk-Cas complexes were found to mediate Rac1 localization to the pseudopodia and this Rac activity appears to act as a feedback-loop to maintain Cas-Crk complexes and the extension of the pseudopod [209]. CrkII tyrosine de-phosphorylation by PTP1B also modulates p130Cas/FAK mediated cell migration [210]. CrkL can also associate with p130Cas and is translocated to focal adhesions in a src dependent manner. A CrkL mutant that contains a focal adhesion targeting region induces translocation of Dock1, a Rac1 activator, and enhanced Rac1 and Cdc42 activity. On the other hand, mouse embryonic fibroblasts that do not express CrkL show impaired integrin-mediated motility [211]. Interestingly, when analyzing knockouts of some of Crkbinding partners, all show defects in cell motility or adhesion events. For example, Paxillin knockout fibroblasts show reduced cell motility, inability to translocate FAK to focal adhesions, abnormal focal adhesions and slower spreading to fibronectin [64]. P130Cas knockout fibroblasts show impaired motility and inability to form stable actin stress fibers [171, 212].

7.4.3 Apoptosis

Apoptosis is a programmed cell death pathway that is highly regulated and may be initiated by a variety of stimuli. Tumour cells acquire the ability to bypass this controlled cell death programme when they undergo uncontrolled proliferation. Crk adaptor proteins were initially identified as potential regulators for apoptosis in the Xenopus egg extract system. In these early experiments, the immunodepletion of endogenous Crk protein from the egg extracts, or the use of Crk antisera prevented apoptosis [213]. Later studies elucidated that cell-cycle protein Wee1 specifically bound to Crk's SH2 domain and that this interaction could restore apoptosis in egg extracts which had been depleted of Crk SH2 interacting proteins [214]. Recently, the same authors further clarified how this regulation occurs. The C-terminal SH3 interacting protein, Crm1, which is a nuclear export factor, when bound to Crk prevents it from accumulating in the nucleus. In the nucleus CrkII interacts with nuclear tyrosine kinase Wee1 and further activates apoptosis. It was suggested that the accumulation of Crk-Wee1 complexes in the nucleus is a pro-apoptotic event. A Crk mutant lacking the nuclear export sequence that interacts with Crm1, promotes apoptosis [175]. Additional independent studies have further confirmed Crk's role in apoptosis. Uncoupling of Crk-Cas complexes in COS-7 and NIH-3T3 cells not only prevented migration but also induced apoptosis [208]. In a more recent study, Abl phosphorylation of CrkII at tyr 221, caused dissociation of Crk-Cas

complexes leading to inhibition of invasion of ECM and to cell death due to cytoskeletal disruption [215].

7.4.4 Tumorigenesis

Crk adaptor proteins participate in the signaling required for cell migration, invasion, cell proliferation and growth, cell survival, and even angiogenesis, all of which are events that take place during tumour progression. For example, CrkL-C3G activation of Rap1 in addition to src activity is required for cAMP induced inhibition of cell growth[216]. Crk association with insulin receptor substrate-4 promotes cell proliferation downstream of IGF [217, 218]. CrkII association with the beta subunit of Met enhances cell proliferation, which can be abolished when blocking Sos activity, implying that such a biological response is mediated through the Ras pathway [219]. Furthermore, VEGFR-1 increased DNA synthesis and proliferation was abolished in porcine aortic endothelial cells when expression VEGFR-1 mutants that could no longer induce phosphorylation of Shp-2, Crk and Erk1/2 [220].

Immunohistological analysis of CrkII in various human tumours exhibited elevated expression in colon and lung cancers, although PCR analysis showed that there were no c-Crk mutations which could be correlated to tumourigenesis [221]. More recently, Affymetrix analysis of human lung carcinomas showed significantly higher expression of CrkI and CrkII in poorly differentiated tumours [222]. Analysis of mRNA levels in normal brain vs. glioblastoma tissue revealed that CrkII mRNA is detectable in normal and glioblastoma tissue, while CrkI

mRNA levels in normal tissue are quite low and are overexpressed in glioblastomas. [223]. In EphA3-expressing melanoma cell lines knockdown of CrkII protein levels by siRNA or the use of a Crk SH3 mutant ablated cell rounding, and detachment induced by Ephrin-A5 stimulation, which was dependent on CrkII activation of RhoA. CrkL transgenic mice exhibited higher tumour incidence and Rap1 enhanced activity in mammary carcinomas [224]. These mice developed several tumours, including lymphoma, fibrosarcoma, and mammary adenocarcinoma. The mammary carcinomas were highly metastatic and the biochemical analysis of such tumours revealed an increased constitutive CrkL-C3G complex. The co-expression of CrkL and Bcl-Abl in mice lead to an increase in incidence of leukemia and lymphoma associated with a decrease in survival [225]. However, further studies revealed that Bcr-Abl CrkL-/- mice still developed leukemia, most likely due to compensation by CrkII signalling since CrkII was found in complex with Bcr-Abl in those tumours [225].

8 RNAi: emerging tool for protein function analysis

RNA interference (RNAi) refers to sequence specific gene silencing mediated by double stranded RNA (dsRNA). RNAi is a post-transcriptional event that occurs naturally in plants, animals and fungi to maintain the integrity of the genome by preventing accumulation of DNA repeats in the germline, regulation of endogenous gene expression and it is also thought to be used as a defense against viral infections. This method of gene silencing emerged as a

cheaper and faster alternative to the established methods of gene function analysis.

8.1 History

In a search to induce gene silencing by introducing both sense and antisense RNA strands into C. elegans, Fire and Mello found that injection of both strands was ten-fold more efficient than either strand alone at silencing gene expression [226]. They named this double-stranded induced gene silencing as RNA interference. Subsequent studies in plants established that small double stranded RNA species (about 21-25 nucleotide long) were the effector molecules that signaled the initiation of PTGS (post-transcriptional gene silencing) [227]. Drosophila in vitro studies established that the double stranded RNA introduced to embryo extracts was cleaved into 21-23nt segments in an ATP dependent manner and that these segments were responsible for triggering mRNA degradation [228-230]. The same year, Elbashir and colleagues report the first successful transfection of 21nt siRNAs into mammalian cells (Hela cells and 293 cells) [231]. They did so by co-transfecting a luciferase siRNA and a luciferase reporter construct into various mammalian cell lines and showing loss of luciferase activity. Small interfering RNA segments were also found in fly embryos [232], and c. elegans [233] post-introduction of a double stranded RNA sequence.

Although siRNA-mediated gene silencing appears to be highly efficient and has proven to be an immediate response, in mammalian cells where there

is no siRNA replication (unlike plants and worms) gene silencing becomes limited to the transient nature of transfection. Various groups have been successful at overcoming such limitations by generating plasmid constructs that will express the siRNA segments in vivo. Plasmids incorporating RNA polymerase III promoters U6 or H1 have been successfully used to establish stable expression of RNA hairpins that once transcribed are cleaved into siRNAs by the Dicer enzyme in the cytoplasm. P53 expression was knocked down with similar efficiency to siRNA transfections, using a short hairpin expression vector containing the H1 promoter [234]. Furthermore, the siRNAs may also be expressed as two separate strands from separate promoters. The HIV-rev gene was targeted using this technology and its efficiency was established using a rev-GFP fusion protein[235]. Plasmid transfection into mammalian cells is an efficient method to generate stable knockouts but the transfection efficiency is still not as high as using siRNAs. In order to overcome transfection limitations, the use of retrovirus to introduce siRNAs into cells has grown in popularity. The expression of p53 in haematopoetic stem cells was efficiently knocked down using short hairpins targeting the gene at various sites, which were delivered in a oncoretrovirus containing a U6 cassette (pBabe-puro) [236]. The use of lentivirus has also been successful in infection of primary dendritic cells with short hairpins [237]. In vivo studies are now starting to emerge. Fertilized eggs from transgenic mice endogenously expressing GFP were infected with a lentivirus vector expressing siRNA targeted to GFP and resulted in blastocysts with significantly reduced GFP expression [238].

8.2 Mechanism

To date the exact mechanism of RNAi is still not completely understood, although it is widely accepted that RNA interference, as well as other post-transcriptional gene silencing mechanisms such as quelling act post-transcriptionally and target the endogenous mRNA for degradation [239]. Studies analyzing the requirements for siRNA structures demonstrated that siRNAs must be 21-23 nt double stranded duplexes with symmetric 2-3nt 3' overhangs and 5'-phosphate and 3'hydroxyl groups for it to be recognized by RNase III Dicer enzyme [230, 240, 241]. The first step of RNAi/PTGS is an ATP dependent step in which Dicer recognized the double stranded RNA sequence in the cytoplasm and cleaves it to produce small interfering RNA duplexes [242](Figure 2). Obviously this step is excluded when siRNA duplexes are introduced directly to the cell. The siRNAs trigger the recruitment of a multi-protein complex known as RISC (RNA-inducing silencing complex). In order for the siRNA to be recognized and incorporated into RISC it requires to be 5'-phosphorylated [243]. Following incorporation of the siRNA into RISC to form the siRNA-protein complex (siRNP), unwinding of the duplex takes place in an ATP dependent manner and the single-stranded antisense strand guides the RISC complex to the endogenous complementary mRNA sequence for endonucleolytic cleavage. The target mRNA is cleaved at a single site, about 10nt from the 5'-end of the siRNA sequence [244]. Although siRNAs don't naturally occur in mammalian cells, microRNAs (miRNAs) have been found in

various mammalian cell types when imperfect hairpin structures are expressed [245].

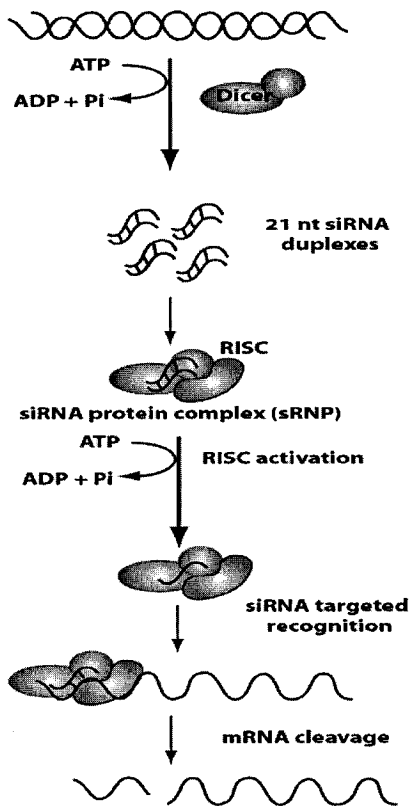

Figure 3: RNAi step-wise mechanism

The quick amplification of gene silencing that occurs in c. elegans when introducing dsRNAs has lead to generation of amplification models. It is thought that siRNAs may prime the synthesis of additional dsRNA by RdRP (RNA-directed RNA polymerase) [246], although, other models appear to be more convincing. Exposure to dsRNA induces alterations in chromatin structure, it is possible that this effect may lead to the expression of aberrant mRNA that become substrates for RdRPs [247]. In plants however, it's been established

that siRNA silencing can be amplified by transmission of siRNAs through cell-cell junctions, known as plasmodesmata and that long range amplification occurs as a relay amplification that requires activity of a putative RNA-dependent RNA polymerase, SDE1 and a putative RNA helicase SDE3 [248].

RNA interference has been widely established as a potential genetic-based therapy. Several efforts have recently been made to introduce siRNAs to specific tissues by use of plasmid and viral vectors for transcription of short-hairpin RNAs in vivo and in vitro. Recently, 21nt siRNAs against Caspase 8 were efficient at attenuating acute liver damage in mice treated with Jo2 [249]. Furthermore, fluorescein-labelled siRNAs were successfully delivered into mice by cationic lyposomal intravenous and intraperitoneal delivery [249]. These studies underscore the potential for development of therapeutic gene silencing as well as the efficiency of gene-function analysis that RNAi technology may offer.

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Chapter 2 Materials and Methods

Materials and Antibodies

Monoclonal antibodies that recognize both CrkI and CrkII and monoclonal antibodies raised against Paxillin were purchased from BD Transduction Laboratories (Lexington, KY). A polyclonal antibody to CrkL was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Vinculin were purchased from Sigma-Aldrich. AlexaFluor488 phalloidin and AlexaFluor 555 phalloidin as well as secondary goat antibodies against mouse or rabbit conjugated to AlexaFluor488 or AlexaFluor555 were purchased from Molecular Probes (Eugene, OR).

Plasmids

Complete human cDNAs were purchased from OpenBiosystems. The CrkII clone used was BC008506 inserted in the mammalian expression vector pCMVSport6. The pOTB7-CrkI clone used was BC009837. pcDNA3.1-GS-CrkL corresponding to Clone ID RG001552 (NM_005207) clone was obtained from Invitrogen.

siRNA constructs

Two small interfering RNA duplex sequences were designed to target each gene. The siRNA sequences targeting the Crk human mRNA corresponded to the coding region 264-284 5'-AAUAGGAGAUCAAGAGUUUGA-3' and 5'-UCAAACUCUUGTUCUC- CUTUU-3' (duplex previously published in *Nagashima et. al. MBC Dec. 2002*) or coding region 465-485 5'-AGGAGACAUCUUGAGAAUCdTdT-3' and 5'-

GAUUCUCAAGA- UGUCUCCUdTdT -3' (designed using siRNA design centre from Dharmacon Research, Lafayette, CO). Two siRNA sequences targeted human CrkL coding regions 547-565 (5'-AUAUGUACGGACUCUGUAUdTdT-3' and 5'-AUAC AGAGUC CGUA CAUA UdTdT-3') and 613-631(5'-GAUCCU AGUGAU AAUAGA GdTdT-3' and 5'-CUCUAUU AUCA CUAGGAUCdTdT-3'); both duplexes designed using siRNA design centre from Dharmacon Research, Lafayette, CO. All of the siRNA duplexes were synthesized annealed and purified by Dharmacon Research Inc. (Lafayette, CO) using 2'-ACE protection chemistry.

Western Blotting

Cell lines were grown to about 80% confluence and lysed in 1% Triton X-100 lysis buffer containing 50mM HEPES pH7.5, 150mM NaCl, 2mM EGTA, 1.5mM $MgCl_2$, 1mM PMSF, 1mM Na_3VO_4, 50mM NaF, 10μg/ml aprotinin and 10μg/ml leupeptin. Whole cell lysates were resolved by SDS-PAGE in 12% gels. Western blotting was performed as previously described (Fixman, 1996).

Cell culture and transfections

Hela and MB-MDA-231 cells were maintained in culture in DMEM containing 10% fetal bovine serum (FBS) and 50□g/ml gentamicin (Invitrogen Canada Inc., Burlington, ON, Canada). $3x10^4$ cells/ml were plated in 12 well plates 24 hours prior to transfections. A total of 100pM of siRNA was transfected per well using Lipofectamine Plus reagents (Invitrogen, USA) following the company's protocol. Transfected cells were trypsinized and used in all assays 48 hours post-transfection. For immunofluorescence, $1.5x10^4$

cells/ml were plated on glass coverslips in 24 well plates and the amount of siRNA transfected per well was 60pM. H1299 cells and KB cells, were maintained in DMEM containing 10% fetal bovine serum (FBS) and 50μg/ml gentamicin (Invitrogen Canada Inc., Burlington, ON, Canada). MB-MDA-435s cells were maintained in Leibowitz media containing 10% fetal bovine serum (FBS) and 50μg/ml gentamicin.

Migration and invasion assays

Cells were grown for 48 hours post transfection with siRNA. $5x10^4$cells were counted and plated directly on 6.5 mm Corning Costar Transwells for migration or on transwells coated with 100μg/cm^2 Matrigel (BD Biosciences) for the invasion assays. Complete media was added to both the top and bottom wells and cells were incubated at 37°C overnight. The next day media was removed and cells on both sides of the transwells were fixed using Formalin Phosphate for 20 minutes at room temperature. After washing with ddH_2O, cells were stained with 0.1% crystal violet in 20% MetOH for 20 minutes at RT. Using a Q-Tip top or bottom layers were scraped off and membranes were left to dry overnight at RT. Using a Retiga 1300 digital camera (QIMAGING, Burnaby, BC, Canada) and a Zeiss AxioVert 135 microscope (Carl Zeiss Canada Ltd, Toronto, ON, Canada) several images were taken from each condition. Image analysis of these assays was carried out using Northern Eclipse version 6.0 (Empix Imaging, Mississauga, ON, Canada) and Scion Image for Windows

2000 based on NIH image for MacIntosh of NIH, USA. The results for each cell line are representative of at least three separate experiments.

Adhesion and spreading assays

MDA-231 cells were transfected as described above. 48 hours post-transfection cells were plated on coverslips (for time point experiments) or 35mm dishes (for movies) previously coated with 20μg/ml Fibronectin (VWR) in PBS (for 30 minutes). In the time point experiments coverslips were washed with PBS at each time point and fixed. Images were acquired using a Retiga 1300 digital camera (QIMAGING, Burnaby, BC, Canada) and a Zeiss confocal microscope. Image analysis was carried out using Northern Eclipse version 6.0 (Empix Imaging, Mississauga, ON, Canada).

Chapter 3 Crk adaptor proteins downstream of Receptor tyrosine kinases are required for the activation of morphogenic programmes

3.1Background

Epithelial morphogenesis is a tightly regulated programme that is vital during embryonic development, wound healing and even cell shape differentiation such as phagocytic processes. This programme involves the activation of proliferation, migration, invasion and turnover of the surrounding matrix signals. Epithelial growth factors are established signalling activators of the morphogenic programme of epithelial cells. Normal sheets of epithelia possess cell-cell contacts, which provide for structural barriers and at the same time communication channels for the integrity of the epithelial sheet. Epithelial morphogenesis is characterized by the loss or remodelling of cell-cell contacts and the acquisition of cell motility, which result in the scattering of the epithelial sheet [1]. Hepatocyte growth factor (HGF) and its receptor Met, are potent activators of epithelial remodelling in vitro [2] and in vivo [3]. HGF was initially isolated as a "scatter factor" [4]. HGF and the activation of the Met receptor can also stimulate epithelial morphogenesis and remodelling required during tubulogenesis. However other growth factors, such as EGF or the insulin growth factor are incapable of promoting tubulogenesis on their own [5, 6].

Epithelial remodelling and cell scattering involve two essential steps: the breakdown of epithelial cell-cell contacts and the rearrangement of the cytoskeleton to allow for formation of new cell-matrix contacts [1]. Previous

studies at our lab have begun to elucidate some of the Met-dependent signals required during epithelial morphogenesis. Using Met-receptor chimeras our lab had previously demonstrated that Met-induced morphogenic programmes are dependent of Grb2 recruitment to tyr1456 of Met [7]. Grb2 recruitment to Met mediates the indirect binding of Met to docking protein, Gab1. However, Gab1 overexpression is sufficient to rescue epithelial branching morphogenesis in Met-mutants' stable cell lines[8]. Lock et. al. established that this rescue may be due to the ability of Gab1 to bind directly to Met [9, 10]. Furthermore, the recruitment of Gab1 to EGF is completely dependent on Grb2, which may reflect the inability for EGF mediated signals to induce prolonged activation of Erk, a signal that has been associated with Met-dependent epithelial morphogenesis. Phosphorylation of Gab1 enables binding to SH2 or PTB domain containing proteins, such as PLCγ, SHP2, p85 and Crk adaptor proteins. [11-13]. Of these Crk appears to be a predominant effector of Met-induced cell scattering. In Fr3T3-Tpr-Met transformed fibroblasts Crk association with Gab1 was responsible for Jnk activation and these complexes were maintained in the absence of cell-matrix adhesion [14]. However, Gab1ΔCrk mutants did not abrogate completely Jnk activity downstream of Tpr-Met (constitutively activated cytoplasmic form of Met), suggesting that there may be other mechanisms by which a constitutively activated Met may induce Jnk activation. Further studies carried out using Crk-SH2 and Crk-SH3 domain mutants confirmed that functional Crk proteins are required for HGF-dependent cell scattering. The overexpression of these mutants in MDCK cells inhibited the

breakdown of cell junctions and the formation of lamellipodia needed during cell scattering [15]. Moreover, CrkII overexpression in breast cancer cell lines was sufficient to induce these same biological responses. Crk mediated cell spreading and scattering comprised higher activity levels of Rac1 and Rap1. Previous studies had already implicated the activation of small RhoGTPases in mediating cytoskeleton changes during HGF-induced epithelial morphogenesis [16]. However in these studies PI3K was essential for the activation of both Rac1 and Cdc42, as PI3K inhibitors blocked HGF-stimulated breakdown of cell-cell junctions, cell spreading, and cell dispersal. Since studies of single-cell migration and also some c.elegans studies had lead to models in which Crk-Dock180-Rac1 complexes were required for cytoskeleton remodelling during cell migration [17-19], our goal was to try and dissect the mechanisms by which Crk adaptor proteins promoted HGF dependent epithelial morphogenesis..

3.2 Crk synergizes with EGF for epithelial invasion and morphogenesis and is required for the Met morphogenic program

In a search for the mechanisms by which HGF promotes cell dispersal and epithelial morphogenesis, our lab has used MDCK cells as a prototypic epithelial model. Upon HGF stimulation MDCK cells, which usually form polarized and well organized colonies in 2D culture, undergo cell dispersal in a stepwise process. Initially cell colonies will spread through the breakdown of cell-cell adherens junctions, which is characterized by the internalization of E-cadherin/β-Catenin complexes, and proceed to dispersal by breakdown of tight

junctions, which occurs with the loss of ZO-1 localization at cell-cell junctions (see fig1A in appendix 1). In contrast, although MDCK cell colonies start to spread in response to EGF they fail to disperse. This phenotype is consistent with the inability of EGF to induce breakdown of cell-cell junctions as seen by analysis of β-catenin and ZO-1 localization [20]. Over-expression of CrkII in well-differentiated MDCK epithelia triggers the initial events that take place during HGF-dependent cell scattering, and the stimulation of these cells with low levels of HGF induces cell dispersal. Furthermore, MDCK cells overexpressing CrkII dispersed in response to EGF (fig 1B in appendix 1). 3D collagen assays further confirmed that CrkII can synergize with EGF signals to promote invasive and morphogenic responses (fig.2 in appendix 1).

HGF-dependent morphogenic programmes require activation of the MAPK and of PI3K pathways. The inhibiton of Mek1 or PI3K activities blocks HGF-induced cell spreading and breakdown of cell junctions [21, 22]. In CrkII-overexpressing MDCK cells, only the inhibition of Mek1 was efficient at blocking HGF or EGF mediated cell dispersal. However, the overexpression of the CrkII adaptor protein did not extend the usually transient Erk1/2 activity in response to EGF. These results suggested that although CrkII synergy with EGF to promote cell dispersal is dependent on MEK1, it is not dependent on a sustained activation of Erk1/2, as in HGF-mediated cell dispersal.

3.3 CrkII overexpressing cells exhibit elevated Rac activation

Since previous studies had confirmed that HGF breakdown of cell-cell junctions and cell remodelling required during cell dispersal where events dependent on the activity of small RhoGTPases such as Rac1 and Cdc42, it was only logical to examine whether an increased activity of either of these actin regulators was behind the ability of CrkII to promote early-EMT events. In CrkII- over-expressing MDCK cells Rac activity was significantly increased compared to normal MDCK cells (see figure1). Furthermore, upon HGF or EGF stimulation, Rac levels were significantly higher in Crk-overexpressing cells compared to parental MDCK cells (figure1 and [20]).

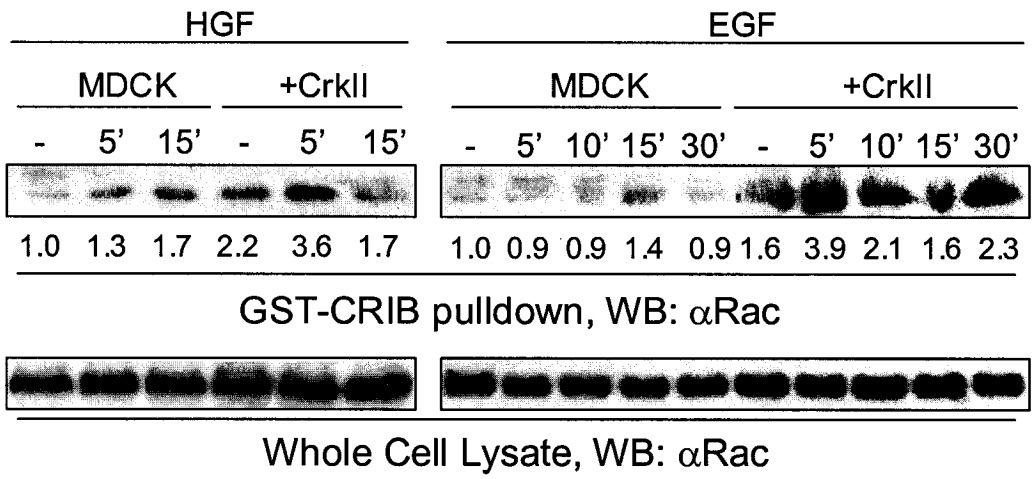

Figure 1: Rac activity is increased in CrkII overexpressing MDCK cells.

MDCK and MDCK cells overexpressing CrkII were stimulated for the indicated various time points using 70 units/ml of HGF or 70ng/ml of EGF. Cells were lysed and 700 mg of protein lysate was incubated for 1 hour with GST-CRIB constructs bound to glutathione-sepharose beads. The pulldowns were washes extensively and bound proteins were separated in a 12% SDS-PAGE gel. 20mg of whole cell lysate was run in parallel. Proteins were transferred to a nitrocellulose membrane and western blotted using an a-Rac monoclonal antibody.

Although Rac1 activity was elevated in Crk-overexpressing cells upon HGF or EGF stimulation, Rac1 was still not the exclusive reason why EGF can promote cell dispersal in Crk overexpressing cells since the overexpression of WT-Rac1 in MDCK cells was not sufficient to promote EGF-dependent dispersal (Lamorte unpublished data). These results suggest that there may be other Crk-dependent signals responsible for cell dispersal.

3.4 Crk associates with a multimolecular Paxillin/GIT2/beta-PIX complex and promotes Rac-dependent relocalization of Paxillin to focal contacts

The epithelial remodelling that is involved in cell scattering is a tightly regulated process that initiates with the breakdown of cell junctions and the reorganization of the actin cytoskeleton and advances with the constant turnover of cell-matrix contacts. The activation and targeting of specific structural and signalling complexes ensue proper actin and microtubule network remodelling. From the previous results it became clear that Crk adaptor proteins are fundamental components during spreading, breakdown of junctions and actin remodelling that takes place in HGF-mediated cell scattering. However, the exact mechanism was still unclear. CrkII or CrkL overexpression was sufficient to promote formation of lamellipodia, cell spreading and the breakdown of adherens junctions. Such biological responses have been described as characteristic of Rac1 activation. Rac1 cycling between GTP and GDP forms regulates E-cadherin clathrin-idependent endocytosis [23, 24].

Furthermore, the activity of Arf-6, a member of the ADP-ribosylation factor family of GTPases, has been described to mediate activated Rac1 localization from cell-cell junctions to the leading edge during cell migration. Rac1 dependent lamellipodia formation was also shown to be dependent on Arf-6 activity [25, 26]. Furthermore, there is evidence for Arf-6 activity to mediate β-catenin/E-cadherin complexes' internalization in response to HGF [27]. These reports suggest, for the first time, that membrane recycling and endocytosis are responsible for the translocation of regulatory signalling molecules from one cell compartment to another, such as GTP-Rac1 translocation from cell-cell contacts to focal complexes at the leading edge of a migratory cell. We proceeded onto dissecting the mechanisms by which Crk adaptor proteins may upregulate Rac1 activity in response to HGF.

The analysis by immunofluorescence of focal adhesion protein Paxillin in unstimulated MDCK cells demonstrated that Paxillin is predominantly found in the cytoplasm. However, once these cells are stimulated with HGF Paxillin relocalizes into focal complexes periphery of dispersing colonies (see figure 1 in appendix 2). Microinjection of CrkII or CrkL proteins into MDCK cells elicited a similar response, where a portion of paxillin was found at focal adhesions (see figure 2 in appendix 2). Thus, one of the events that may contribute for enhanced spreading and migratory signals in cells with overexpressed Crk proteins is the relocalization of focal adhesion proteins such as paxillin into focal adhesions. Since Crk adaptor proteins can directly interact with paxillin in a ptyr/SH2 domain dependent manner, it could be possible that this interaction is

responsible for paxillin targeting or for its stability at these sites. The microinjection of CrkI SH2 and SH3 mutants further confirmed our hypothesis. Either of the mutants failed to promote cell spreading and paxillin relocalization to focal complexes (figure 3 in appendix 2).

It had been previously demonstrated that Rac1 activity was essential for cell scattering in response to HGF, as a dominant negative Rac1 construct inhibited HGF-mediated cell dispersal [16]. We had also previously shown that Rac1 activity was enhanced in Crk overexpressing stable cell lines and in response to HGF [20]. In cells co-microinjected with CrkL and N17Rac1 (DN Rac1) paxillin failed to relocalize to focal adhesions (see figure 4 in appendix 2). These results suggested that Rac activity is required for Crk-mediated paxillin targeting to focal complexes.

Current studies in fibroblasts in search of mechanisms responsible for Pak targeting to focal adhesions had demonstrated that Rac and Cdc42 activation promoted the assembly of a paxillin/Git2/β-Pix complex and its targeting to focal adhesions was responsible for Pak localization at these subcellular compartments [29]. This lead us to examine the assembly of such complexes in MDCK cells overexpressing Crk. The association of Paxillin with Git2 and β-Pix was enhanced in MDCK cells overexpressing Crk (see figure 6 in appendix 2). Furthermore, the microinjection of CrkL in MDCK cells demonstrated that a portion of CrkL co-localized with endogenous Paxillin at focal adhesions. Co-microinjection of CrkL with Git2 or β-Pix confirmed a colocalization of these proteins at focal adhesions. Paxillin mutants that can no

longer target to focal adhesions or associate with Git-2 or Crk inhibited Crk-stimulated lamellipodia formation and cell spreading (figure 8 in appendix 2).These results proposed a new model where Crk adaptor proteins promote the relocalization of Paxillin complexes to focal adhesions in a Rac dependent manner.

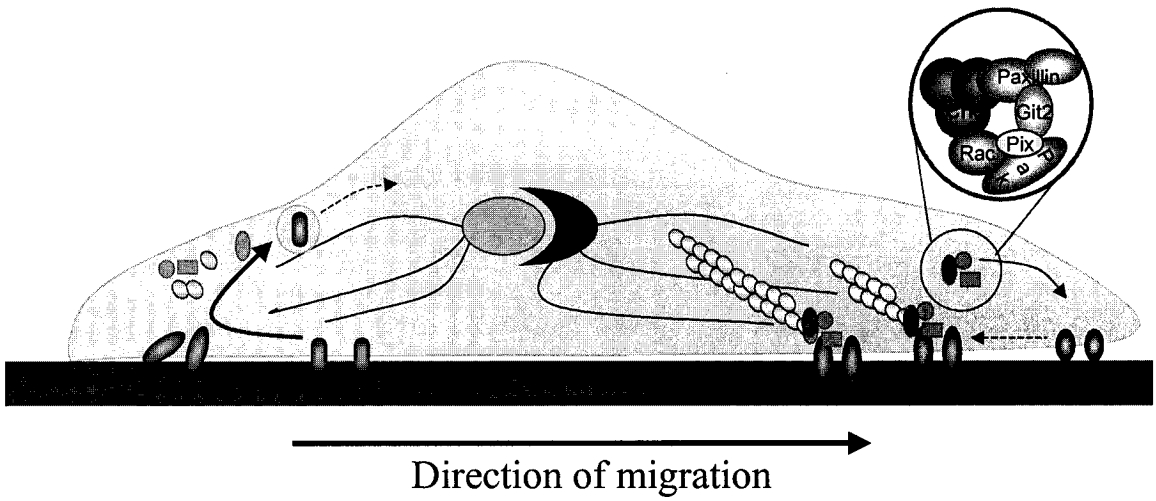

Figure 2: Model for Crk recruitment of a Paxillin/Git2/Pix complex in a Rac depedent manner.

Crk overexpression promotes cell spreading and the formation of lamellipodia, initial steps during HGF-induced migration and cell scattering. Similarly in Crk overexpressing cells Paxillin/Git2/Pix complexes are found at the leading edge in subcellular compartments known as focal complexes, structures required for proper actin cytoskeleton rearrangement and propulsion for cell migration. A model is proposed in which Crk adaptor proteins may recruit and promote the assembly of Crk/Paxillin/Git2/Pix complex to the leading edge and thereby regulate Rac and Arf-Gap activities.

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Chapter 4 Crk adaptor proteins function as a signalling node in the promotion of cell adhesion, migration and invasion

4.1 CrkI/II and CrkL protein levels are decreased in various tumour cell lines following siRNA treatment

A role for the Crk adaptor family of proteins in promoting the assembly of protein complexes important for cell migration, invasion and proliferation has been established (reviewed in [1]). The involvement of these proteins in such pathways may suggest that Crk adaptor proteins play an important role during tumorigenesis and metastasis. Further support for this is provided by recent studies involving human lung cancer cell lines (Nishihara, H et al., 2002) and from immunohistochemical analysis of human lung adenocarcinomas (Miller, C.T. et al., 2003) which revealed that expression of both isoforms of c-Crk (CrkI/II) were associated with an aggressive phenotype. Elevation of Crk adaptor proteins in tumour progression has been reported in various types of cancer. Recently CrkI was identified as a potential modulator of cell migration and invasion in glioblastoma [2]. Also, CrkL transgenic studies showed that an increase in CrkL associated protein complexes was correlated to an increase in incidence of Bcr/Abl induced leukemogenesis, decrease in average survival, and the occurrence in one instance of metastatic mammary carcinoma [3]. Moreover, we had previously published that CrkII overexpression was sufficient to initiate cell spreading and the breakdown of cell adherens junctions, fundamental events in cell motility [4]. The identification of Crk adaptor proteins

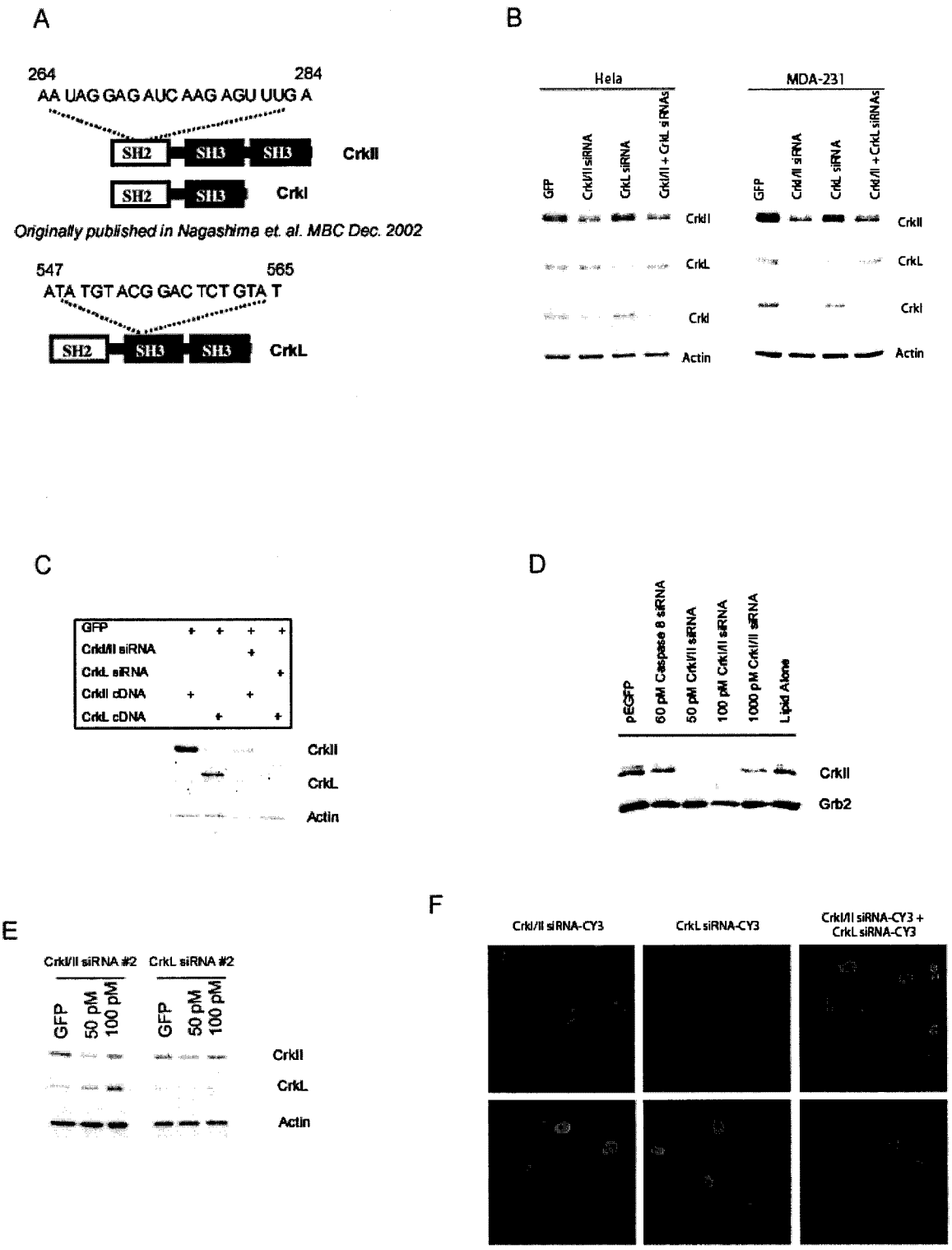
in such various cell and tumour types would suggest that Crk adaptor proteins may play a universal role in tumorigenesis through the association of protein complexes involved in cell migration and invasion, independently of its upstream regulators.

To address the biological importance of Crk adaptor proteins in the cell signalling of various tumour cell lines we used an RNA interference approach. A small interfering RNA duplex has been recently published and shown to be effective by Nagashima's group [5]. Using algorithms generated by Dharmacon (www.dharmacon.com) we constructed a second siRNA duplex against the CrkI/II gene and two other siRNA duplexes against the CrkL gene (Fig. 1A). In order to establish the knockout of Crk adaptor proteins using the siRNA constructs shown in figure 1A and the transfection conditions we tested them in two tumour cell lines, Hela (carcinoma of the cervix) and MDA-231 breast cancer cells (figure 1B). We successfully decreased endogenous levels of CrkI, CrkII and CrkL proteins in both cell lines using 100pM of siRNA (figure 1B). To further analyse the specificity of our small interfering RNA duplexes Hela cells were co-transfected with each duplex together with either CrkII cDNA or CrkL cDNA (Fig. 1C). Western blot analysis of protein lysates with an anti-CrkI/II or anti-CrkL sera revealed that each of the small interfering RNA duplexes was efficient at targeting its respective co-transfected cDNA specifically. Levels of endogenous CrkI/II and CrkL proteins were also knocked down specifically by the CrkI/II siRNA and CrkL siRNA (Fig. 1B). Interestingly the endogenous levels of these proteins seem to be less reduced compared to the overexpressed

proteins by the siRNAs. This result may be due to higher specificity to the EST sequences than endogenous Crk proteins existent in MDA-231 cells or Hela cells. It may be possible that due to their cancerous origin these cells contain Crk proteins with point mutations in their nucleic-acid sequence. The characterization of these cell lines would have to be performed to ensure that Crk proteins expressed in these cancer cell lines show consensus nucleic-acid sequence at the siRNA targeted sites. To further support that the decrease in Crk protein levels that we observed was due to the specificity of the siRNAs and not nonspecific RNA interference or a transfection-induced phenomenon, we transfected Hela cells with an siRNA designed to target Caspase 8 (kindly provided by Dr. Shore at McGill University) and western blotted for CrkII. As shown in figure 1D a non-specific siRNA is unable to decrease CrkII protein expression when compared to a CrkI/II siRNA duplex in Hela cells. Two additional siRNAs targeted to a separate region in their respective CrkI/II and CrkL mRNA sequence showed a similar ability to induce a reduction in the respective Crk protein levels (Figure 1E). Real-time PCR was performed to determine if the Crk or CrkL mRNAs were indeed decreased when transfecting MDA-231 cells with either of the siRNA duplexes (data not shown). Although, complete ablation of detectable protein was not observed we wanted to assess whether this was due to the transfection efficiency. To analyse cell uptake, the different siRNA constructs were labelled with CY3 siRNA tracker labelling kit from Myrus (*Label* IT® siRNA Tracker). As seen in figure 1F approximately 100% of MDA-231 cells or Hela cells are positive following transient transfection

with CY3-labelled siRNAs. Moreover, a decrease in Crk protein levels was observed by western blotting analysis to last for 5-6 days post-transfection (data not shown).

Figure 1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AA UAG GAG AUC AAG AGU UUG A
SH2
SH3
SH3
CrkII
SH2
SH3
CrkI
Originally published in Nagashima et. al. MBC Dec. 2002
547
565
ATA TGT ACG GAC TCT GTA T
SH2
SH3
SH3
CrkL
B
Hela
MDA-231
GFP
CrkI/II siRNA
CrkL siRNA
CrkI/II + CrkL siRNAs
CrkII
CrkL
CrkI
Actin
C
GFP
CrkI/II siRNA
CrkL siRNA
CrkII cDNA
CrkL cDNA
CrkII
CrkL
Actin
D
pEGFP
60 pM Caspase 8 siRNA
50 pM CrkI/II siRNA
100 pM CrkI/II siRNA
1000 pM CrkI/II siRNA
Lipid Alone
CrkII
Grb2
E
CrkI/II siRNA #2
CrkL siRNA #2
GFP
50 pM
100 pM
CrkII
CrkL
Actin
F
CrkI/II siRNA-CY3
CrkL siRNA-CY3
CrkI/II siRNA-CY3 + CrkL siRNA-CY3

Figure 1: a) CrkI/II and CrkL siRNA constructs. b) Hela and MDA-231 cells were transfected with CrkI/II or CrkL or both siRNAs and protein lysates were analysed by western blotting with antibodies against CrkI/II or CrkL. Actin protein levels were used as a loading control. c) Hela cells were transfected with CrkI/II and CrkL siRNAs and CrkII or CrkL expressing plasmids respectively. d) Hela cells were transfected with varying amounts of CrkI/II siRNA and the decrease in protein levels was compared to the effect of a Caspase 8 siRNA transfection. Grb2 protein levels were used as a loading control. e) Two extra constructs of CrkI/II and CrkL siRNAs were designed and their protein "knockdown" efficiency was evaluated by western blot analysis. f) CrkI/II and CrkL siRNAs were labelled with a CY3 marker and transfection efficiency was assessed by indirect immunofluorescence. Top and bottom panels show two separate colonies in each condition. As seen in both examples all cells show CY3 staining, evidencing that CrkI/II and CrkL siRNAs were readily expressed in MDA-231 cells.

4.2 Transfection of siRNA duplexes does not induce cell death or inhibit cell proliferation

Prior to examining a role for Crk in cell migration it was important to establish whether a decrease in Crk protein could promote apoptosis or impair cell proliferation during the time course of the assays. The induction of early and late apoptotic events were assayed by Annexin V staining and Caspase 3 activation respectively in both Hela and MDA-231 cells. 48 hours post-transfection control cells (transfected with lipid alone) and siRNA transfected cells were incubated for 15 minutes with 100μl of Annexin V fluorescein. Positive control cells were exposed to 50J/m^2 UV light and incubated at 37°C overnight, and assayed in parallel with siRNA transfected cells. As seen in figure 2 by Annexin V staining both Hela and MDA-231 cells underwent activation of apoptotic events when exposed to 50J/m^2 UV light. However no significant activation of early apoptotic events were found in either control cells or CrkI/II and CrkL siRNA transfected cells (Fig. 2A and 2B). Similar results were found when either of the siRNAs were transfected alone (data not shown). Further confirmation that siRNA transfection failed to promote cell death was achieved by performing Caspase 3 activity assays (data not shown). Cell proliferation rates were analysed over a 72 hour period in MDA-231 mock cells and siRNA transfected cells. During that period no significant difference in cell number was observed between mock and siRNA transfected MDA-231 cells (data not shown). These results suggest that a siRNA mediated decrease in Crk

adaptor proteins does not induce apoptosis or alter cell proliferation rates. Furthermore they would also suggest that the impairment in cell motility observed when we decrease Crk protein levels results from a direct impact on the cell's motility machinery.

Figure 2

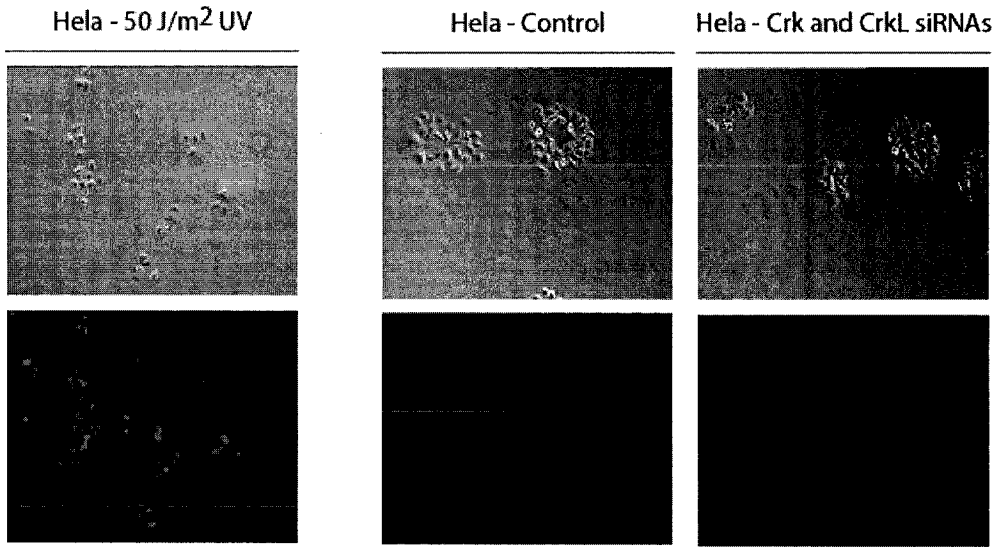


Figure 2A: Hela cells were transfected as previously described on 24 well plates. Positive controls were exposed to 50J/m2 of UV radiation and incubated overnight. 48 hours post-transfection, Annexin V assays were performed according to company's protocol on both siRNA transfected cells, UV irradiated cells and non transfected Hela cells.

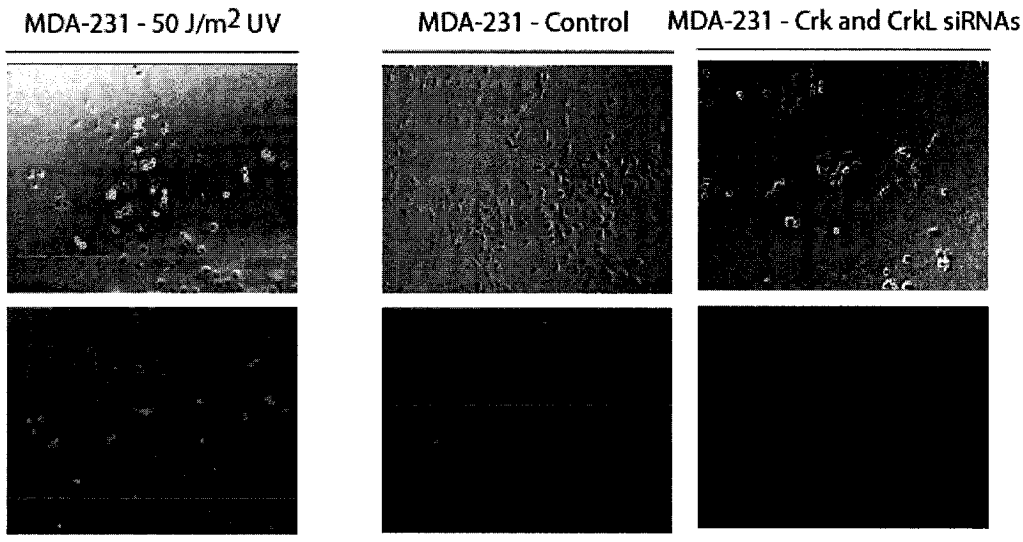


Figure 2B: MDA-231 cells were transfected as previously described on 24 well plates. Positive controls were exposed to 50J/m2 of UV radiation and incubated overnight. 48 hours post-transfection, Annexin V assays were performed according to company's protocol on both siRNA transfected cells, UV irradiated cells and non transfected 231 cells.

4.3 "Knockdown" of either of the Crk family of adaptor proteins inhibits migration and invasion of human tumour cell lines

Previous studies have shown that Crk associated protein complexes are indeed required for the transmission of integrin-dependent or growth factor dependent cell migration signals [6-9]. Moreover we have demonstrated that overexpression of Crk adaptor proteins promotes an EMT like transition in MDCK cells [4] and that Crk association with Paxillin, a focal adhesion scaffold protein, and the localization of such complexes to the cell's leading edge are critical events during cell spreading and migration [10]. All of these reports suggest that signalling mediated by Crk adaptor proteins promotes cell migration in various systems. To assess whether these adaptor proteins are critical regulators for cell migration in human tumour cell lines small interfering RNA duplexes targeting CrkI/II or CrkL, as described above, were transfected into Hela or MB-MDA-231 cells and their effect on cell migration and invasion was assayed. The inherent migration capacity of these cell lines in serum was assayed using Transwell migration assays as previously described [11]. CrkI/II or CrkL specific siRNA duplexes were transiently transfected 48 hours prior to plating cells into the upper Transwell chamber. Cells were allowed to migrate through the polycarbonate porous membranes for 24 hours. The "knockdown" of the Crk gene products, CrkI and CrkII, decreased the migratory capacity of Hela cells about 2-fold relative to control Hela cells (Fig.3A), and about 6-fold relative to control MDA-231 cells (Fig. 3C). CrkL "knockdown" or the

"knockdown" of both Crk and CrkL proteins lead to a similar inhibition of migration in both Hela or MDA-231 cells.

To assay the effect of Crk "knockdown" in the ability of tumour cells to invade an extracellular matrix transwells were coated with 100μg/cm^2 of Matrigel. Hela and MDA-231 cells were plated on top of this matrigel coating and incubated for 24 hours to invade through the matrix. A 5-fold decrease in invasion was observed in Hela cells transfected with either of the siRNA constructs when compared to control Hela cells (Fig.3B). A similar decrease in invasion was observed in MDA-231 cells transfected with the CrkI/II siRNA or with the CrkL siRNA construct (Fig.3D). All of these experiments were performed a minimum of 3 times per cell line, where all showed comparable results with p-values<0.05 (Figure 3). Error bars represent the standard deviation when capturing 5 separate fields of each condition in the representative experiment. These results highlight a requirement for Crk adaptor proteins in cell signaling processes during both cell migration and invasion.

Figure 3

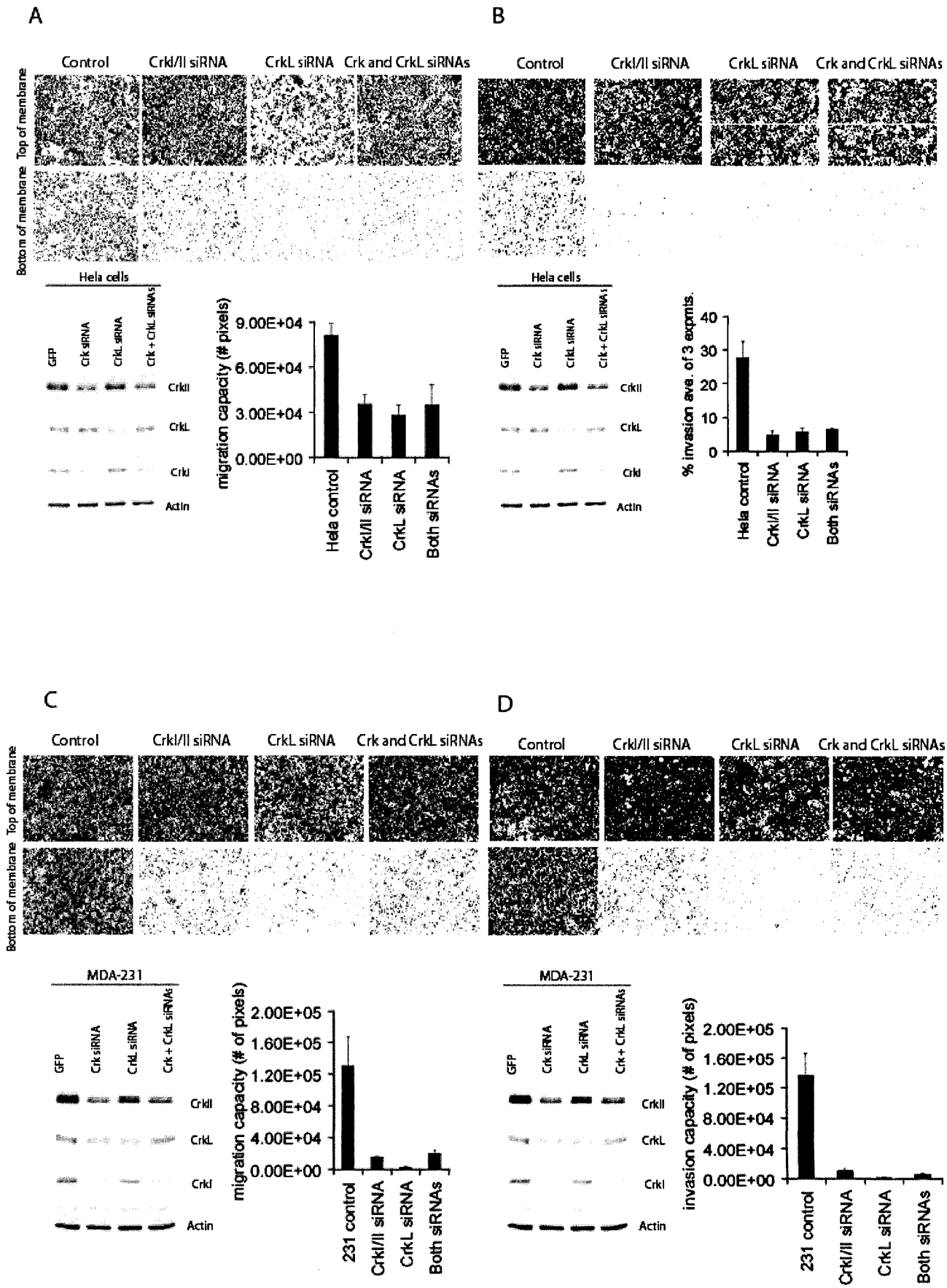

A
B
C
D
Control
CrkI/II siRNA
CrkL siRNA
Crk and CrkL siRNAs
Top of membrane
Bottom of membrane
Hela cells
MDA-231
GFP
Crk siRNA
CrkL siRNA
Crk + CrkL siRNAs
CrkII
CrkL
CrkI
Actin
migration capacity (# pixels)
9.00E+04
6.00E+04
3.00E+04
0.00E+00
Hela control
CrkI/II siRNA
CrkL siRNA
Both siRNAs
% invasion ave. of 3 expmts.
40
30
20
10
0
invasion capacity (# of pixels)
2.00E+05
1.60E+05
1.20E+05
8.00E+04
4.00E+04
0.00E+00
231 control

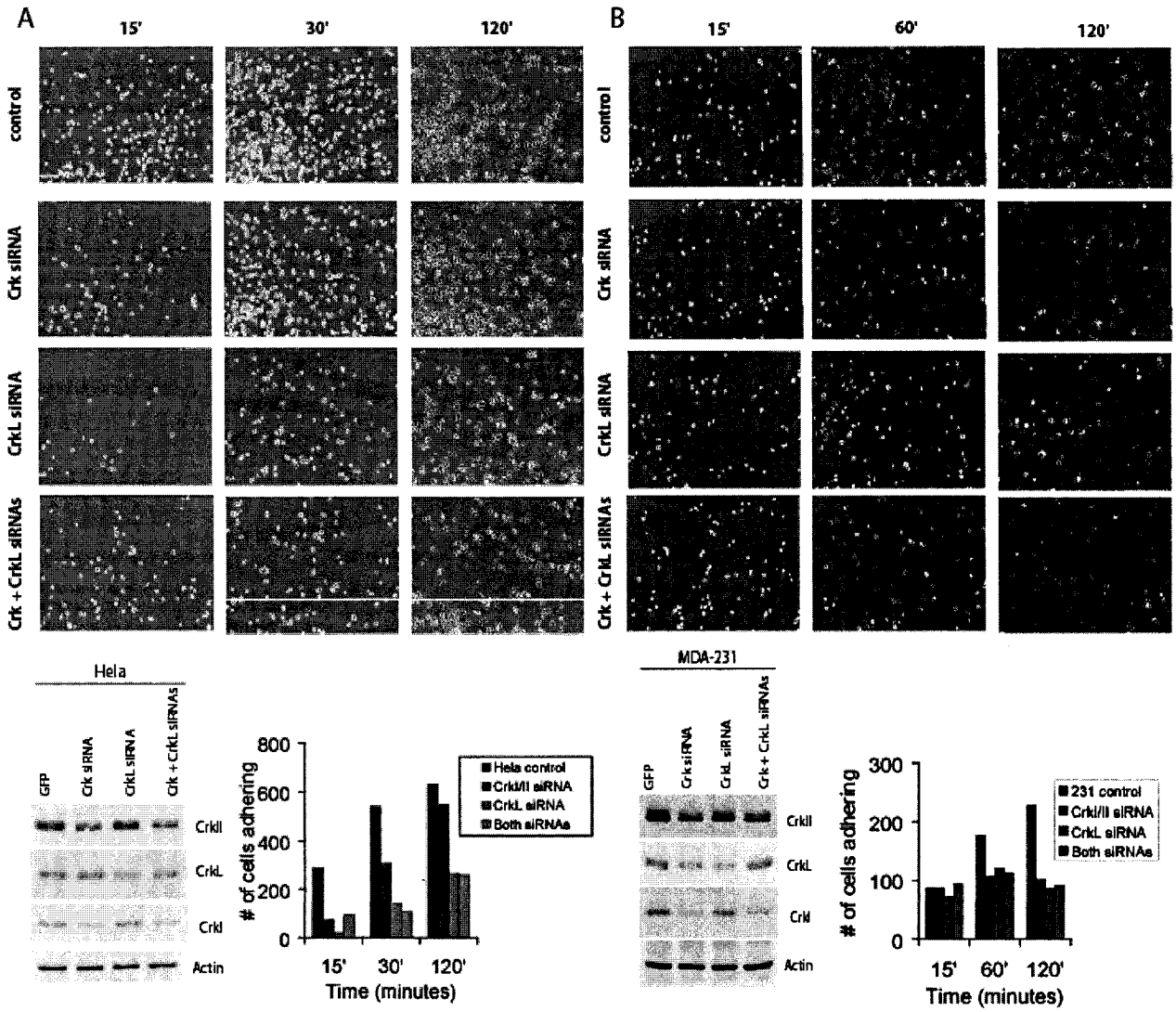
Figure 3: Hela and MDA-231 cells were transfected with CrkI/II or CrkL siRNAs and migration and invasion assays in the presence of serum were performed on these cells 48 hours post-transfection. Cells remaining on un-scraped side of wells were fixed in formalin phosphate, stained with 0.2% crystal violet, washed extensively with H_2O and left to dry overnight. Using a Retiga 1300 digital camera (QIMAGING, Burnaby, BC, Canada) and a Zeiss AxioVert 135 microscope (Carl Zeiss Canada Ltd, Toronto, ON, Canada) top and bottom layers of the transwell were imaged in 5 separate fields for each condition using a 10x magnification in phase contrast. Image analysis of these assays was carried out using Northern Eclipse version 6.0 (Empix Imaging, Mississauga, ON, Canada) and Scion Image for Windows 2000 based on NIH image for MacIntosh of NIH, USA. A minimum of five experiments was performed per cell line.

4.4 Cell adhesion and spreading is delayed in CrkI/II or CrkL siRNA transfected cells

Cell dispersal, migration and invasion are tightly regulated mechanisms that entail spreading of the cell body, and the formation of membrane extensions such as lamellipodia that will allow for the formation of new adhesion contacts with the substrate and retraction of the cell's trailing edge[12, 13]. It is the formation of focal adhesion contacts with the extracellular matrix that promotes the anchoring required to generate a propulsive tension within the actin cytoskeleton in order for the cell to move [13]. Our previous work and others have suggested that Crk adaptor proteins may play a key role in the recruitment of protein complexes, such as Paxillin/Git2/β-Pix, p130Cas, Dock180 or ASAP1, to focal adhesion sites [10, 14-16]. Moreover, I have shown that Crk overexpression is sufficient to induce an increase in Rac activation in MDCK cells [17], a necessary step in the initial cell membrane spreading during cell migration[7, 18]. To establish whether the decrease in cell migration we observed following CrkI/II or CrkL siRNA treatment resulted from an inability of these cells to adhere to the substrate or to spread. Hela cells or MDA-231 cells were plated on fibronectin coated plates 48 hours post transfection with siRNAs. Hela control cells start adhering to the matrix 15 minutes post plating (Fig. 4A). Although less, there are still a significant number of CrkI/II or CrkL siRNA transfected cells that adhered to the matrix, but this is reduced when compared to control cells. Even more dramatic results were seen when using

MDA-231 cells (Fig. 4B). Initially both transfected and control MDA-231 cells adhered to the matrix at similar levels. Although after the first hour the ability of CrkI/II or the CrkL siRNA transfected cells to adhere to the matrix was decreased. At later time points (2 hours) CrkI/II or CrkL "knockdown" cells showed a decreased ability to spread when compared to control cells (Fig.4C). The visualization of MDA-231 cells transfected with siRNA by time-lapse videomicroscopy further confirmed a decreased ability of these cells to spread when compared to control cells (Fig.4D). Moreover, certain cells begin to attach but are unable to spread and round up (Fig.4D). This phenotype is very similar to the recent results obtained with paxillin null primary cells by Sheila Thomas laboratory [19], where they observed a decrease in cell motility and a delayed rate of spreading. Hence it is possible that the reason for such similar phenotypes may be due to the requirement for both of these proteins in the same signaling events during cell migration.

Figure 4


A
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Hela
GFP
Crk siRNA
CrkL siRNA
Crk + CrkL siRNAs
CrkII
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Actin
# of cells adhering
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Time (minutes)
Hela control
CrkI/II siRNA
CrkL siRNA
Both siRNAs
MDA-231
GFP
Crk siRNA
CrkL siRNA
Crk + CrkL siRNAs
CrkII
CrkL
CrkI
Actin
# of cells adhering
300
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Time (minutes)
231 control
CrkI/II siRNA
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Figure 4D

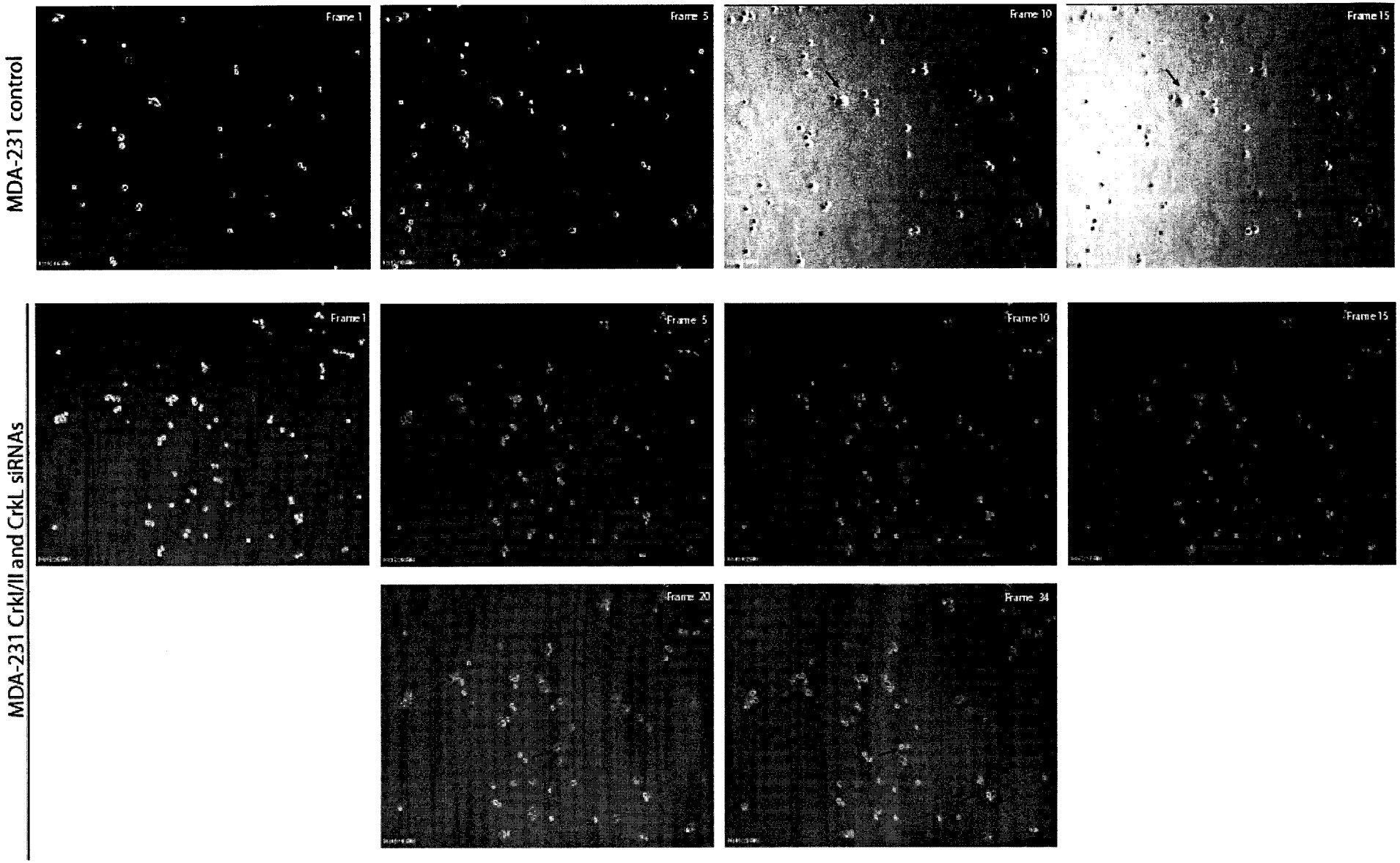

MDA-231 control
Frame 1
Frame 5
Frame 10
Frame 15
MDA-231 CrkI/II and CrkL siRNAs
Frame 1
Frame 5
Frame 10
Frame 15
Frame 20
Frame 34

Figure 4: Adhesion assays – A) Hela cells were transfected with siRNAs as previously described. 48 hours post-transfection cells were trypsinized and re-plated on coverslips coated with 20μg/ml Fibronectin. Cells were washed and fixed in 4% paraformaldehyde at separate time points. Images were captured using a Zeiss AxioVert 135 microscope and image analysis was carried out using Northern Eclipse software. B) MDA-231 cells adhesion assays were performed using the same procedure as for Hela cells. C) MDA-231 cells plated on fibronectin as previously described at time point 120 minutes. a) MDA-231 control cells show beginning of membrane ruffles formation and cell spreading two hours post-platting. b) MDA-231 cells transfected with CrkI/II siRNA are unable to spread on fibronectin at 2hour time point. c) MDA-231 cells transfected with CrkL siRNA also exhibit a slower spreading rate compared to control cells. D) Time-lapse video-microscopy of MDA-231 cells transfected with CrkI/II and CrkL siRNAs adhering to a fibronectin coated 35mm dish. 48 hours post-transfection cells were trypsinized and plated onto fibronectin coated 35mm plates and time-lapse video-microscopy was performed using a Zeiss AxioVert 135 microscope and Northern Eclipse software. Frames were taken every 2 minutes. Transfection efficiency was analysed by both immunofluorescence (see fig. 1F) and western blot.

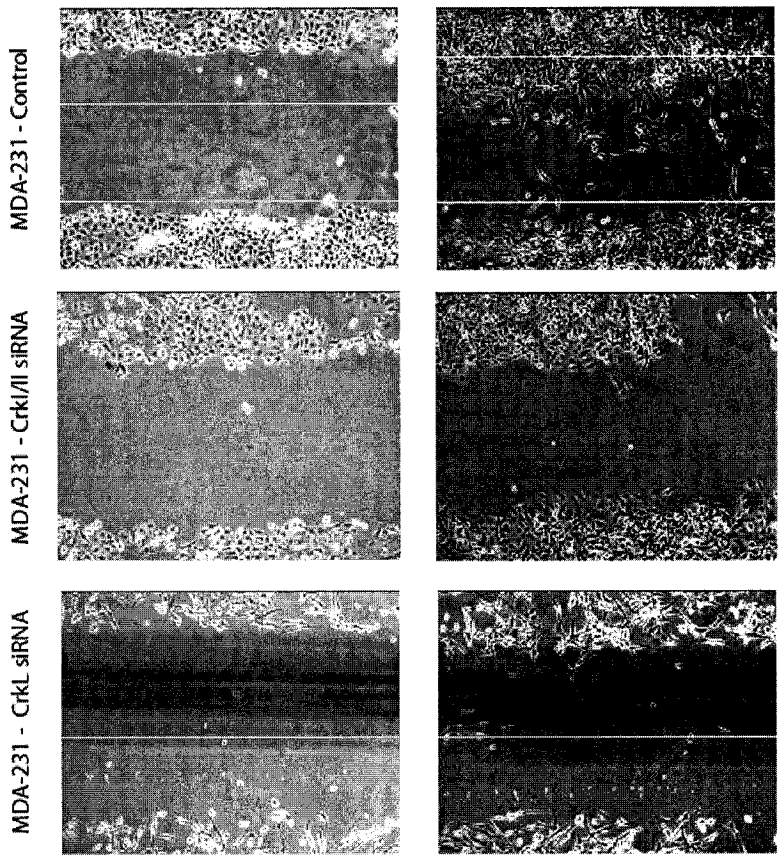
4.5 "Knockdown" of CrkI, CrkII or CrkL expression also impairs directional migration during wound healing

Crk adaptor proteins have been extensively implicated as promoters of single cell migration [7, 11, 20-22]. However, there have been fewer reports analyzing the role of Crk in promoting migration or dispersal of epithelial monolayers [4, 10, 17, 23]. Our lab has previously demonstrated that Crk adaptor proteins are important for the breakdown of cell junctions and cell scattering in HGF driven partial-EMT [4]. Furthermore, the overexpression of Crk SH2 or SH3 mutants blocked HGF-mediated cell scatter, underscoring the importance for Crk SH2 and SH3 interactions in the propagation of growth factor signals.

Since the knockdown of Crk adaptor proteins inhibited single cell migration and the invasion of various human cancer cell lines we proceeded to evaluate its effect in monolayer wound healing. MDA-231 cells were transfected as previously described and grown to confluency. A wound scratch was generated using a p100 pipette tip and 10U of HGF were added immediately. Cells were incubated overnight to fill in the scratch. In an 18 hour period MDA-231 cells mock transfected migrated close to 30μm across the scratch (Fig. 5). Cells transfected with CrkI/II siRNA or CrkL siRNA were able to migrate only 15μm in the same period of time (Fig. 5). These results demonstrate that functional Crk adaptor proteins are required for efficient fill in of the scratch.

Furthermore, HGF induced signaling pathways are unable to overcome the absence of Crk mediated signaling in the promotion of wound repair.

Figure 5


MDA-231 - Control
MDA-231 - CrkI/II siRNA
MDA-231 - CrkL siRNA

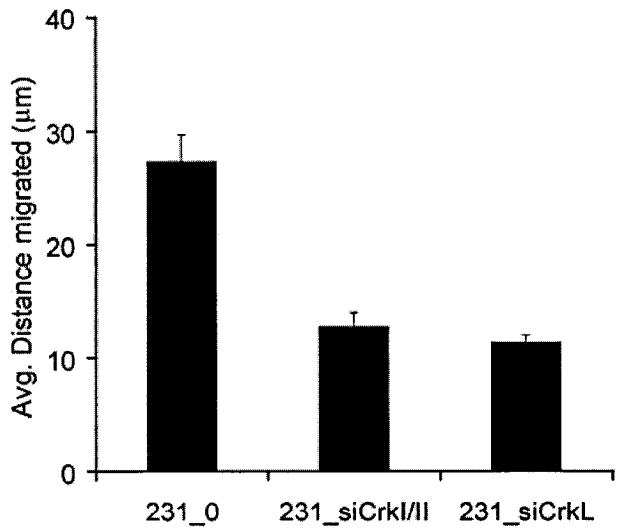

Avg. Distance migrated (µm)
40
30
20
10
0
231_0
231_siCrkI/II
231_siCrkL

Figure 5: MDA-231 and Hela cells were transfected as previously described and once grown to confluency epithelial monolayers were scratched using a p100 tip. Cells were left to fill in the wound scratch for 18 hours and images were obtained using a Retiga 1300 digital camera (QIMAGING, Burnaby, BC, Canada) and a Zeiss AxioVert 135 microscope (Carl Zeiss Canada Ltd., Toronto, ON, Canada).

4.6 Crk adaptor proteins are required during cell migration and invasion of a panel of uncharacterized human tumour cell lines

In an attempt to assess whether Crk adaptor proteins are required for cell migration and cell invasion signals in multiple human tumour cell lines we transfected a panel of cancer cell lines with CrkI/II siRNA alone, with CrkL siRNA alone and with both. Protein knockdown was assessed by western blotting as previously described. Figure 6 confirms CrkI and CrkII knockdown in our panel of cancer cell lines (Figure 6A). Although the knockdown varies according to the cell line it is clear that the siRNA efficiently downregulates CrkI and CrkII protein expression in all cell lines. Next we performed migration and invasion assays on H1299, KB and MDA-435s cells using the same four transfection conditions. In all of these cell lines CrkI/II or CrkL knockdown or both was sufficient to impair cell migration and cell invasion to different extents (figures 6B-6G). All of these experiments were performed for a minimum of 3 times per cell line, where all showed comparable results and p-values<0.05. Error bars represent the standard deviation when capturing 5 separate fields of each condition in the representative experiment as previously described. These results support our hypothesis that Crk adaptor proteins may act as a node in cell migration signaling, in which independently of upstream signaling regulators Crk function is required during cell migration and highlight a role for Crk proteins in invasive tumour spread.

Figure 6A

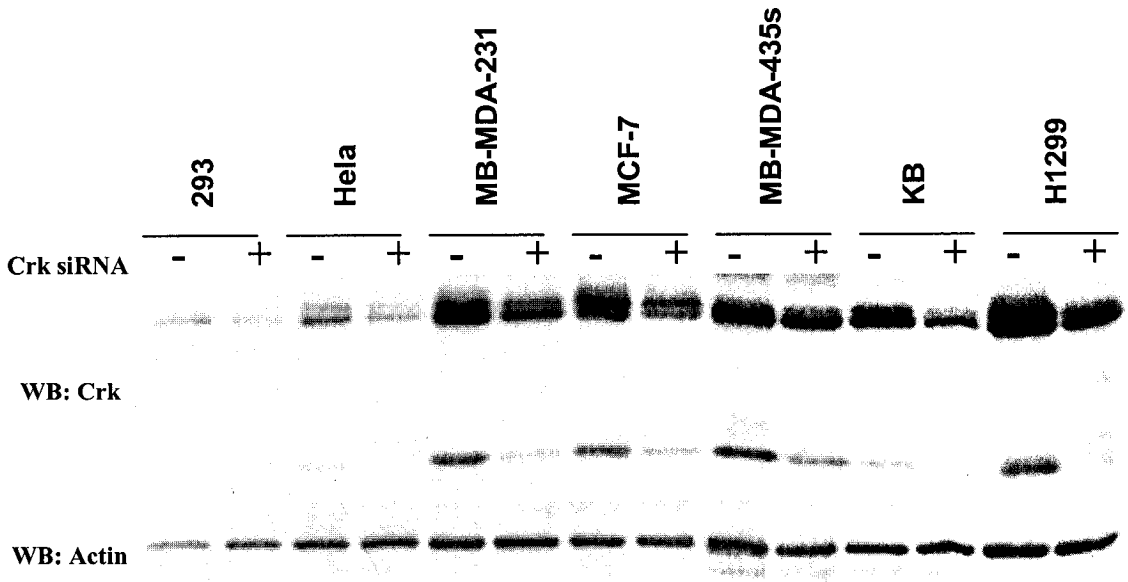


Figure 6

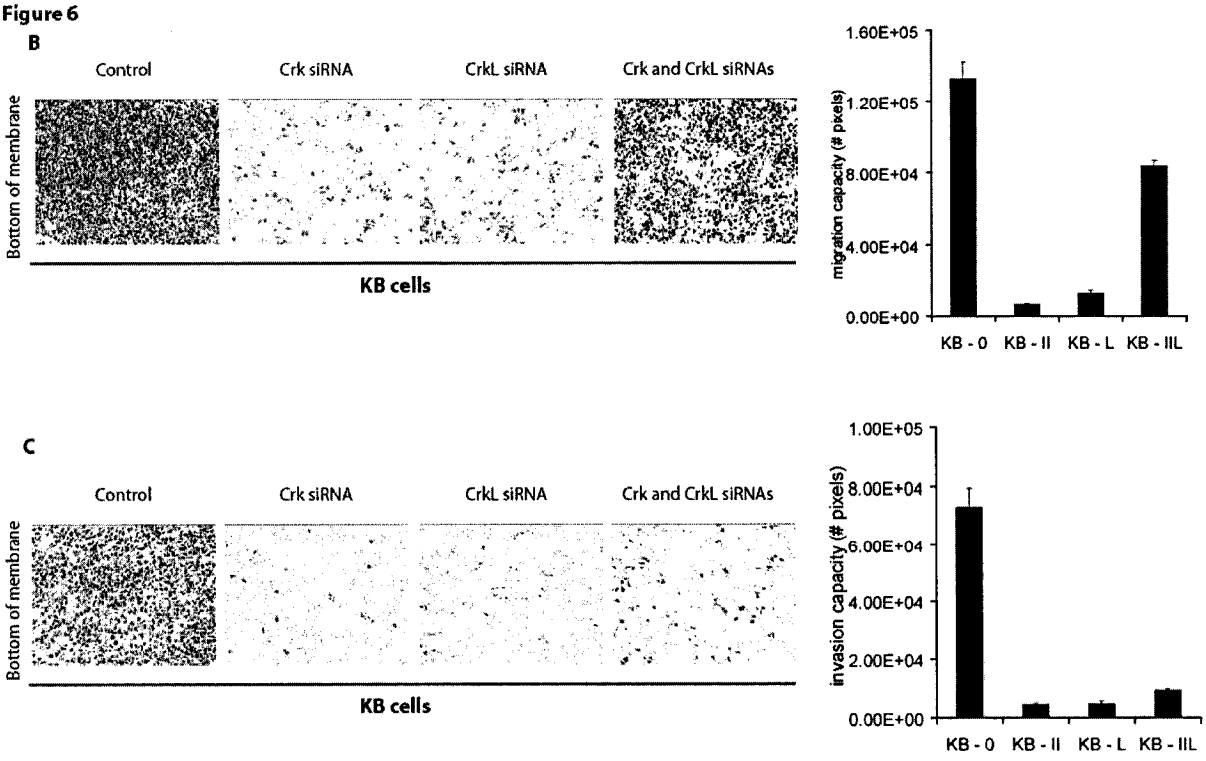


Figure 6

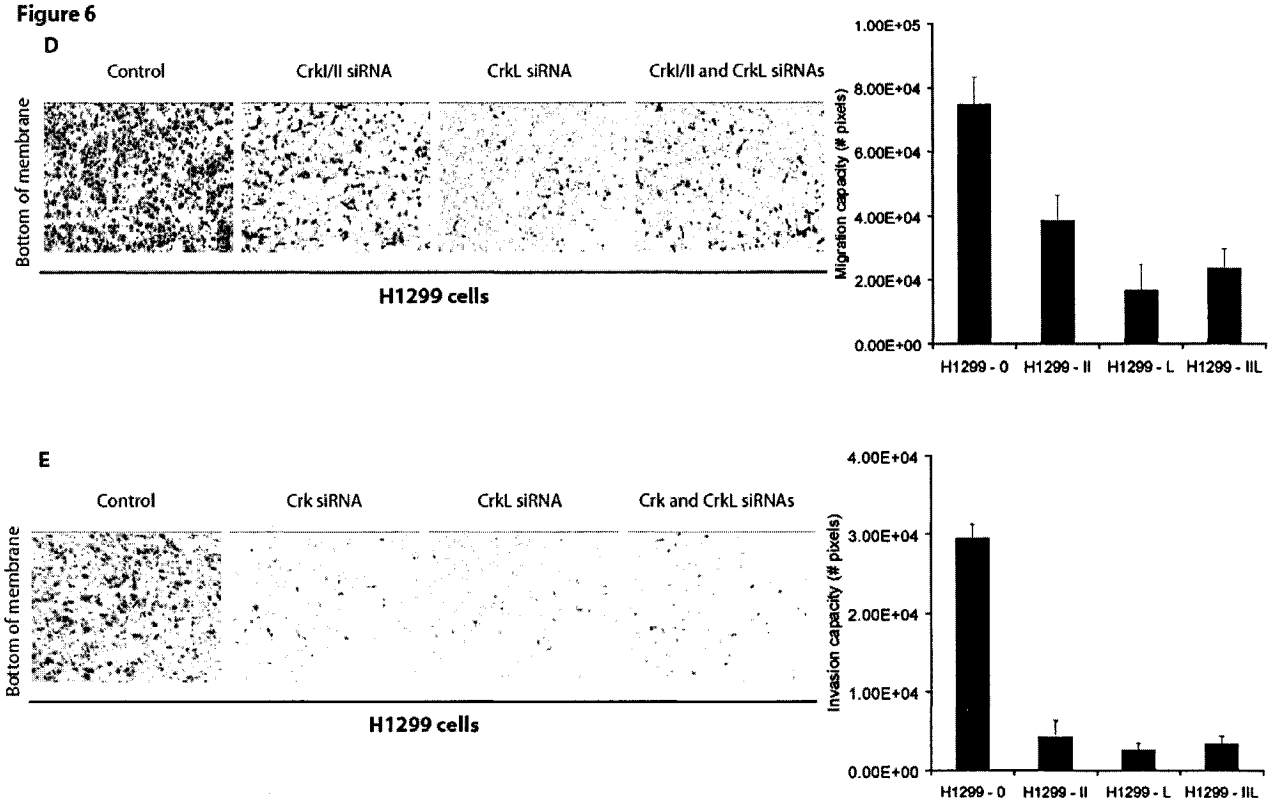
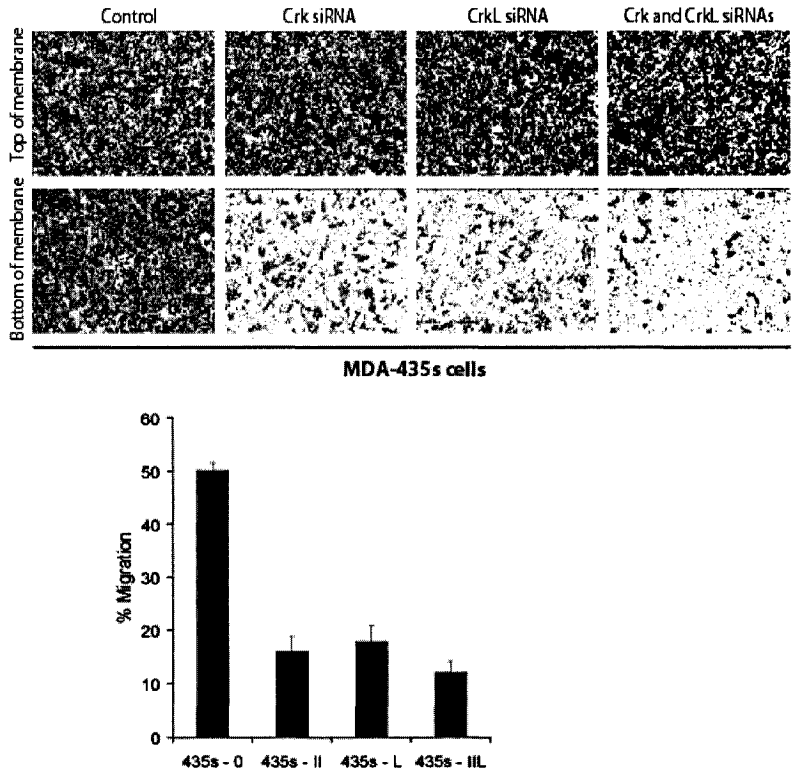

D
Control
CrkI/II siRNA
CrkL siRNA
CrkI/II and CrkL siRNAs
Bottom of membrane
H1299 cells
Migration capacity (# pixels)
1.00E+05
8.00E+04
6.00E+04
4.00E+04
2.00E+04
0.00E+00
H1299 - 0
H1299 - II
H1299 - L
H1299 - IIL
E
Control
Crk siRNA
CrkL siRNA
Crk and CrkL siRNAs
Bottom of membrane
H1299 cells
Invasion capacity (# pixels)
4.00E+04
3.00E+04
2.00E+04
1.00E+04
0.00E+00
H1299 - 0
H1299 - II
H1299 - L
H1299 - IIL

Figure 6

F


Control
Crk siRNA
CrkL siRNA
Crk and CrkL siRNAs
Top of membrane
Bottom of membrane
MDA-435s cells
% Migration
60
50
40
30
20
10
0
435s - 0
435s - II
435s - L
435s - IIL

G

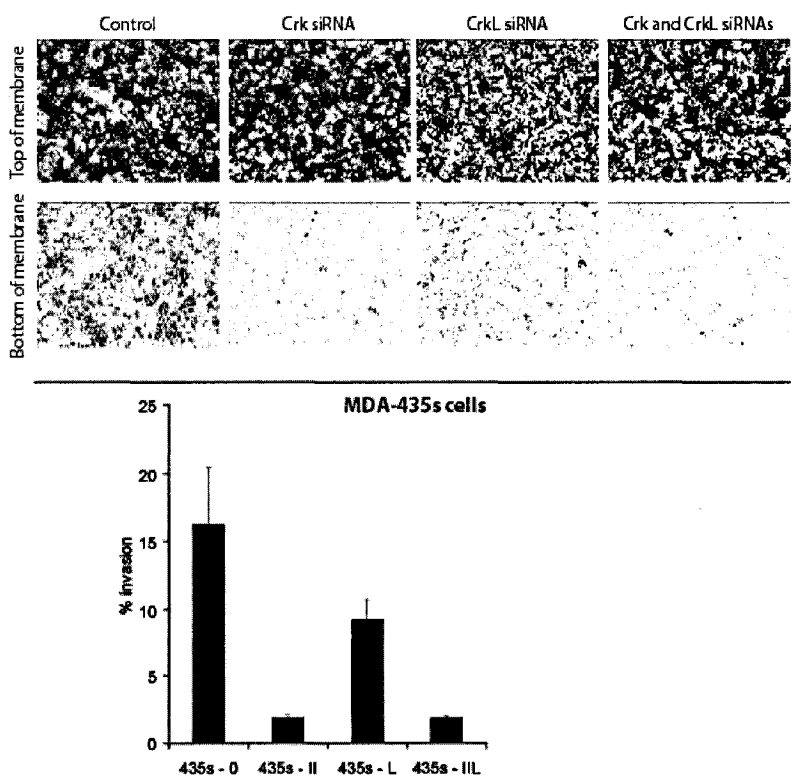

Control
Crk siRNA
CrkL siRNA
Crk and CrkL siRNAs
Top of membrane
Bottom of membrane
MDA-435s cells
% Invasion
25
20
15
10
5
0
435s - 0
435s - II
435s - L
435s - IIL

Figure 6A: A panel of human cancer cell lines was transfected with Crl/II siRNA and protein lysates were western blotted for analysis of CrkI/II protein levels. Actin protein levels were used as loading control.

Figure 6B – 6G: migration and invasion assays of a panel of human tumour cell lines. KB, H1299 and MDA-435s cells were transfected with CrkI/II and CrkL siRNAs and assayed for migration and invasion 48 hours post-transfection. Image analysis of these assays was carried out using Northern Eclipse version 6.0 (Empix Imaging, Mississauga, ON, Canada) and Scion Image for Windows 2000 based on NIH image for MacIntosh of NIH, USA. A minimum of three experiments was performed for each cell line and each condition was imaged in five separate fields.

4.7 CrkI/II or CrkL "knockdown" inhibits paxillin relocalization to focal adhesions in MDA-231 breast cancer cells

Crk-p130Cas and Crk-paxillin complexes assemble at focal adhesions and appear to be required for the activation of small RhoGTPase Rac1 that mediates membrane protrusions during cell migration [8, 10, 11, 23]. Since Crk knockdown elicited a similar phenotype as the paxillin null fibroblasts [19] and since our lab had previously proposed that Crk adaptor proteins recruit a paxillin/git2/pix complex into focal adhesions in MDCK cells overexpressing CrkII [10], we proceeded to verify whether paxillin localization was affected by the knockdown of Crk protein expression levels. MDA-231 cells transiently transfected with siRNA were trypsinized and replated onto fibronectin-coated coverslips 48 hours post-transfection. Cells were left to adhere onto the coverslips for 2 hours or overnight and subsequently stained with antibodies to paxillin and phalloidin, which stains polymerized actin. MDA-231 cells transfected with Crk siRNA presented a roundish morphology and showed fewer and less bundled stress fibers than mock transfected cells (Fig. 7). Most importantly, there was a decrease in paxillin localization at focal adhesions in CrkI/II siRNA transfected cells. Similar results where observed in CrkL siRNA transfected MDA-231, although their morphology was more fibroblastic. Differences in morphology may be indicative of the different signals, and possibly different binding partners that may be downstream of CrkI/II proteins and CrkL despite their homology. These results are also suggestive that Crk

adaptor proteins may be required for the recruitment or the stabilization of paxillin complexes at focal adhesions.

However, the question still remained as to whether Crk knockdown was affecting structural integrity of focal adhesions or whether it directly affected paxillin recruitment to these subcellular compartments. To address this question MDA-231 siRNA transfected cells were stained with vinculin and phalloidin. Vinculin was mostly cytoplasmic in CrkI/II siRNA or CrkL siRNA transfected cells. These results lead us to believe that CrkI/II and CrkL are required for the recruitment and stability of paxillin complexes at focal adhesions. Furthermore, paxillin localization at these sites is concomitant with the ability to form strong or stable focal adhesions required during cell migration and adhesion. Also, these results highlight a role for Crk proteins in maintenance of focal adhesion integrity.

Figure 7

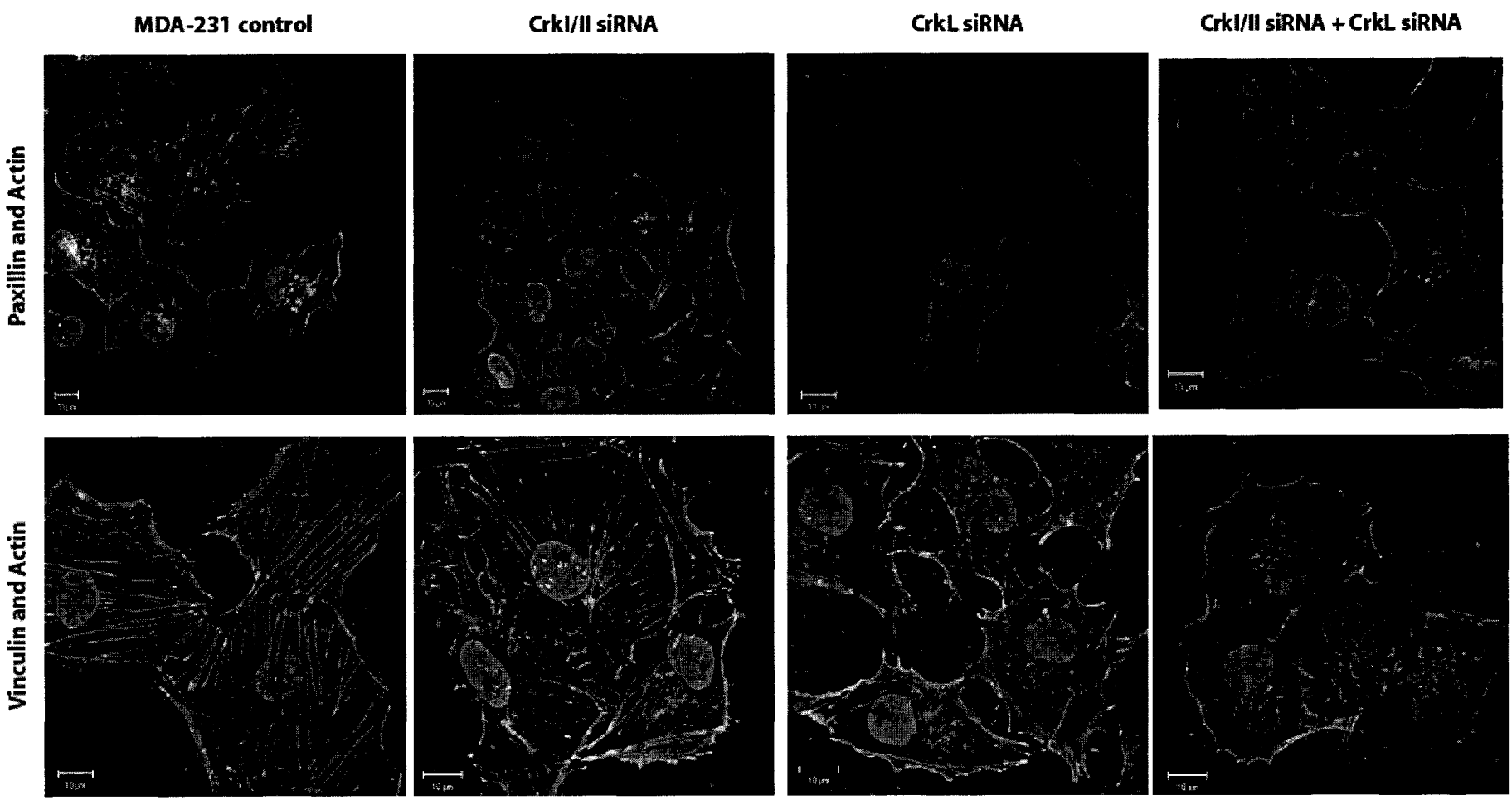


Figure 7: MDA-231 cells were transfected and replated on to fibronectin coated coverslips 48 hours post transfection. Once fixed using 4% paraformaldehyde cells were co-stained with α-Paxillin/Alexa Fluor 555 goat anti-mouse and Phalloidin-488 for the F-actin staining or α-Vinculin/Alexa Fluor 555 goat anti-mouse and Phalloidin-488.

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Chapter 5 Discussion

A role for Crk adaptor proteins in cell migration has been extensively studied in mammalian cells, as well as model organisms such as *c. elegans* and *drosophila*. Serum starved COS cells expressing a Crk SH3 or SH2 mutant failed to migrate through a vitronectin coated membrane while WT Crk overexpression enhanced their migration capacity [1]. Similarly, overexpression of CrkL but not of a CrkLΔSH2 or CrkLΔSH3 mutant enhanced cell adhesion and migration across a fibronectin-coated membrane in haematopoetic cells both spontaneously or in response to chemokine stimulation [2]. Such a phenotype is a result of the function of Crk as mediator of signalling cascades. Crk acts as an adaptor protein where its SH2 domain links phosphotyrosine containing proteins with downstream signals that interact with Crk's SH3 domain. Examples of the importance of Crk in mediating signals involved in cell migration include its interaction with Tyr31 and Tyr118 in Paxillin. Mutations in both of these tyrosines lead to the inhibition of Crk-Paxillin complexes and impaired cell motility [3]. Moreover, overexpression of CrkII or CrkL in MDCK cells promotes the targeting of Paxillin to focal adhesions while Crk SH2 or SH3 domain mutants failed to do so [4]. We had previously shown that stable cell lines overexpressing CrkII exhibited enhanced formation of lamellipodia and cell spreading which correlated with an increased association of CrkII with a paxillin, Git-2, β-PIX complex and higher levels of Rac1 activity [4] (Chapter 3, figure 1). The association of CrkL with the Rap exchange factor C3G has also been postulated as a crucial modulator of integrin mediated cell adhesion [5]. CrkL

SH3 or C3G guanine exchange factor mutants, both abrogated CrkL-induced increased cell adhesion [5]. A pivotal role for Crk in ephrin-stimulated cell ruffling was recently confirmed by overexpression of Crk SH2/SH3 mutants and by targeting it using siRNA duplexes[6].

Despite the accumulation of evidence for a function for Crk adaptor proteins in cell adhesion and cell migration/invasion downstream of various stimuli, and the increasing understanding of the function of its protein complexes, the exact mechanisms by which Crk promotes cell migration and its significance in inherently motile tumour cells still remains elusive. The goal of our study was to validate the function of Crk as a central node involved in cell migration and to establish a possible mechanism through which Crk proteins regulate these biological responses using RNA interference. Our results demonstrate that Crk adaptor proteins are required for migration and invasion of highly invasive cancer cell lines (Chapter 4, Fig. 3, 5 and 6). Moreover, these results suggest that Crk adaptor proteins play an important role in the promotion of cell migration signals irrespectively of upstream regulators in tumour cell lines (Chapter 4, Fig. 6). Crk participates in the establishment of cell-matrix contacts and their turnover for proper actin bundling and formation of stress fibers during cell migration as shown by adhesion and spreading assays (Chapter 4, figure 4). Consistent with our previously published data in which Crk overexpression promoted the redistribution of Paxillin to focal complexes [4], Crk knockdown disrupted Paxillin targeting to focal adhesion sites (Chapter 4, Fig. 7). Furthermore, Crk knockdown inhibited focal adhesion complex formation and

actin polymerization as evidenced by the decrease in bundled actin stress fibers and partial-disruption of the localization of Vinculin in Crk siRNA transfected cells (Chapter 4, figure 7).

5.1 Crk adaptor proteins are critical modulators of cell migration and invasion

To examine the requirements for Crk adaptor proteins in cell migration and invasion we adopted an RNA interference approach and established the conditions required for a consistent knockdown of these proteins in various cell lines (Chapter 4, figure 1 and figure 6). The transfection of CrkI/II siRNA at various concentrations demonstrated that 100pM were most effective at knocking down endogenous levels of CrkI and CrkII (Chapter 4, figure 1B and 1D). Time point studies showed that the most dramatic knockdown of these proteins was detected between 48 and 72 hours post transfection (data not shown). The specificity of the knockdown was further examined by co-transfection with CrkII and CrkL cDNA respectively (Chapter 4, figure 1C), by RT_PCR analysis of mRNA stability (data not shown) and by transfection of an siRNA targeted to a separate protein (Caspase 8 siRNA, Chapter 4, figure 1D). CrkI/II and CrkL siRNA constructs specifically inhibited the expression of their counterpart co-transfected cDNA (Chapter 4, figure 1C). The expression of an siRNA targeted against Caspase 8 did not affect Crk adaptor protein levels (Chapter 4, figure 1D), although the expression of two other siRNA constructs targeted to separate regions in the Crk or CrkL mRNA effectively decreased

CrkI/II and CrkL protein levels respectively. These results suggest that CrkI/II/L protein expression was specifically decreased by constructs targeted to their mRNA and not by non-specific RNA interference. Although Crk protein levels were reduced, we failed to observe complete loss of Crk proteins; even though labelled siRNAs demonstrated that close to 100% of the cells were taking up the siRNAs (Chapter 4, figure 1F). These results may be reflective of the high endogenous expression levels of Crk adaptor proteins.

The migration and invasion of single cells was evaluated using boyden chamber transwell assays. CrkI/II or CrkL siRNA "knockdown" significantly decreased the migration and invasion capacity of various highly invasive tumour cell lines (Chapter 4, figures 3 and 6). Although the signals that promote the invasive phenotype of these cell lines are not completely characterized, the data presented in this thesis supports a critical role for Crk adaptor proteins in tumour cell migration and invasion. These results are consistent with the knowledge that Crk functions as a modulator of intracellular signals downstream from various growth factor receptors, such as the Met receptor [7, 8], EGFR [9, 10], VEGFR [11] and ErbB2/Neu receptor [12]. In an attempt to confirm that the decreased migration and invasion of tumolur cells was a result of knockdown of Crk proteins we established that transfection of Crk targeted siRNAs failed to induce apoptosis or significantly affect cell proliferation. Although Crk adaptor proteins have previously been reported to regulate apoptosis [13, 14], Caspase 3 and annexinV assays confirmed that Crk knockdown did not induce cell death in our assays (Chapter 4, figure 2).

Although the migration of single cells versus the migration of epithelium sheets is two independent events, most of the signalling pathways involved in both cases are very similar. The dispersal of epithelial sheets also involves the extension of membrane protrusions such as lamellipodia and filopodia, the reorientation of organelles towards the direction of migration and the establishment of focal contacts with the substrate at the leading edge of the colony [15]. The Rho-family small G-proteins tightly regulate all of these cytoskeletal changes.

Stress in a confluent epithelium caused by a wound is sufficient to trigger a migratory response in a directional manner. MDA-231 breast cancer cells transfected with CrkI/II siRNA or CrkL siRNA, or both showed an impaired ability of wound closure compared to mock cells (data not shown). Moreover, cells in which Crk protein levels are reduced migrated at about half the speed of control cells (Chapter 4, Fig. 5). Interestingly, although wound closure occurred faster when cells were treated with HGF, this was not sufficient to overcome the migration deficit in cells where Crk proteins were decreased. Cells transfected with siRNA to CrkI/II/L still exhibited a two-fold decrease in wound closure capacity when compared to control cells (Chapter 4, figure 5). These results suggest that Crk signalling is essential for cell motility independently of the stimulus. It is also important to point out that alternative signals HGF induced by are not able to overcome the consequence of decreased Crk signalling for cell migration.

These results raise the question as to which remodelling events are regulated by Crk during cell migration. For example, does Crk "knockdown" affect the re-polarization of organelles and cell body, does it affect cell membrane extension at the leading edge, or does it affect cell adhesion to the substrate required for cell propulsion? Evidence from the literature point to a function for Crk in the promotion and regulation of cell-matrix adhesion contacts as a hypothesis for the anomalous migration in Crk knockdown cells [16-19].

5.2 The "knockdown" of CrkI/II/L affects cell migration by disrupting adhesion and spreading

Cell motility is a complex event that is dependent on the coordinated remodelling of the actin cytoskeleton and the regulated assembly and turnover of focal adhesions (for review see [20]). Initially cell membrane protrusions extend in the direction of migration as a result of Rac1 targeting to lamellipodia and its activation [21]. Membrane extension is driven by actin polymerization. Adhesion to the substrate provides anchoring required for the stabilization of membrane protrusions and the clustering of regulatory signalling and structural complexes that will further promote actin bundling [22]. Adhesion to extracellular matrix is mediated at focal adhesion sites through clustering of integrins and cytoskeletal linking proteins such as Vinculin, Filamin, α-actinin, actopaxin/parvin, paxillin (reviewed in [23]). The Arp2/3 complex when recruited to focal adhesion sites initiates polymerization of the actin stress fibers by adding monomeric actin to the newly nucleated filaments. This mechanism has

been suggested to be the driving force pushing the membrane outwardly generating membrane protrusions [24, 25]. Integrin recruitment and clustering at membrane protrusions activates Rac1 [26]. Rac1 activity mediates further activation of the Arp2/3 complex through Scar/WAVE activation or through activation of p21-activated kinase protein family. We have shown that the overexpression of CrkII in MDCK cells leads to an increased activity of Rac1 consistent with an increase in lamellipodia formation [9]. Furthermore, microinjection of vectors expressing Crk SH2 and SH3 mutant proteins blocks HGF-induced membrane protrusions [8]. Cell spreading and lamellipodia formation in Crk-overpressing cells is abrogated by the microinjection of a dominant negative Rac1 construct [4, 8]. Together, these data provide evidence for Crk involvement in the stabilization of membrane spreading and cell-matrix adhesion through a Rac dependent mechanism.

We observed a decrease in the ability of MDA-231 and Hela cells transfected with CrkI/II or CrkL siRNA or CrkI/II + CrkL siRNAs to attach to fibronectin when compared to control cells (Chapter 4, figure 4). Most importantly we observed the decreased ability of these cells to spread to the same extent as control cells (Chapter 4, figure 4). Time-lapse videomicroscopy further demonstrated that Crk siRNA "knockdown" cells, although still appeared to adhere to the matrix, were unable to spread and some cells lost their initial adhesions (Chapter 4, figure 4D). The analysis of this data supports a role for Crk for the stability and maintenance of cell matrix adhesions. It is possible that Crk siRNA "knockdown" prevents further Rac1 activity that is required for actin

polymerization during membrane extension. Another possibility might be that Crk recruitment of focal adhesion signalling complexes is essential for the formation and maintenance of focal adhesion contacts. Since Crk has also been shown to be a key activator of Rap1 through C3G activity, and Rap1 activity mediates integrin clustering at the leading edge during cell migration [18, 27, 28], it may be that a certain pool of Crk adaptor proteins are involved in the targeting or stability of integrins at cell-matrix contacts [18]. Yet another mechanism may be through Crk dependent localization of paxillin complexes to focal adhesions [4]. Since paxillin knockout mutants show a similar decrease in cell migration and adhesion and similar cell morphology to our Crk siRNA "knockdown" cells this raised a possibility that Crk was important for correct Paxillin subcellular localization.

5.3 Crk knockdown affects cytoskeletal-remodelling: including relocalization of Paxillin, focal adhesion assembly and actin bundling

The turnover of cell-matrix contacts and the ability to establish new contacts is one of the integral events of cell motility. Focal complexes depend on Rac activation and the targeting of focal adhesion multi-protein assemblies. These are the initial sensory contacts to be established with the substrate upon membrane protrusions. Focal complexes can mature into focal adhesions and provide anchorage for the cell body propulsion through further actin polymerization or dissociate if a proper integrin-extracellular matrix association is not established [29]. Focal adhesion maturation is dependent on RhoA

activity and the activation of its downstream effector ROCK [30]. The association of Crk adaptor proteins with p130Cas or Paxillin complexes had been previously established as potent mediators of Rac dependent membrane protrusions [4, 31]. Moreover, Crk overexpression enhanced Paxillin/Git2/β-Pix complex assembly in MDCK cells and their recruitment to focal adhesions was enhanced in cells microinjected with CrkL [4]. Although PAK interaction with such complexes was not established, previous studies demonstrated that β-Pix interaction with Pak is important for PAK mediated Rac and Cdc42 activation and PAK dependent actin remodelling [32-34].

Immunofluorescence analysis of paxillin localization in MDA-231 cells plated on fibronectin coated coverslips revealed that paxillin is predominantly found at focal adhesions at the end of stress fibers (Chapter 4, figure 7). When levels of Crk adaptor proteins were decreased by RNAi, cells exhibited changes in morphology, showed reduced actin bundling, and paxillin was re-localized to the cytoplasm (Chapter 4, figure 7). These results suggested that either Crk knockdown affected the stability of focal adhesions and paxillin-complexes at these sites, decreasing anchored and bundled actin or Crk protein "knockdown" prevented the formation of new focal complexes and their maturation into focal adhesions. When cells were plated on fibronectin post-transfection with siRNAs we observed an even more dramatic decrease in paxillin localization to focal adhesions (Chapter 4, figure 7) when compared with transfection of cells that had already established adhesions to fibronectin coated coverslips (data not shown). However the fact that these cells where still able to adhere to the

substrate would suggest that Crk is not required for the establishment of initial contacts with the matrix but instead for the proper maintenance of these contacts and the promotion of cell spreading. Such results would also suggest that the "knockdown" of Crk adaptor proteins affects the ability to form new focal contacts with the matrix that arise from cell spreading. It remains to be determined whether Rac activity is affected by Crk knockdown.

The polymerization of actin stress fibers is dependent initially on the proper assembly of focal complexes followed by activation of Rho, which is required for the maturation and elongation of the actin filaments. MDA-231 breast cancer cells treated with a Rho Kinase inhibitor show complete dissociation of actin stress fibers but Paxillin localization remained at the membrane edge in smaller focal complexes (data not shown). Crk protein "knockdown" in MDA-231 cells did not completely abrogate formation of stress fibers, although these appeared to contain less bundled actin (Chapter 4, figure 7). Consistent with this, Vinculin was present at the end of stress fibers in both mock and Crk siRNA transfected cells, although decreased Vinculin containing adhesions were observed in Crk siRNA cells. Recently Vinculin was proposed to be a mediator of the Arp2/3 complex targeting to focal adhesions, but not required for its activation [25]. It is possible, that although vinculin localization may not be dependent on Crk adaptor proteins in a similar manner to Paxillin, but that Crk-dependent Rac activity at these sites is required for Arp2/3 complex activation, thereby inhibiting proper actin polymerization. Crk protein "knockdown" may also affect the stability of paxillin complexes at focal adhesion

sites. These results would also suggest the possibility for a model where a Crk/Paxillin/Git2/Pix complex may be involved in the increased activation of Rac required for cell spreading during migration. The failure in targeting these complexes to focal adhesions may also inhibit Pak-dependent actin remodelling (figure 1).

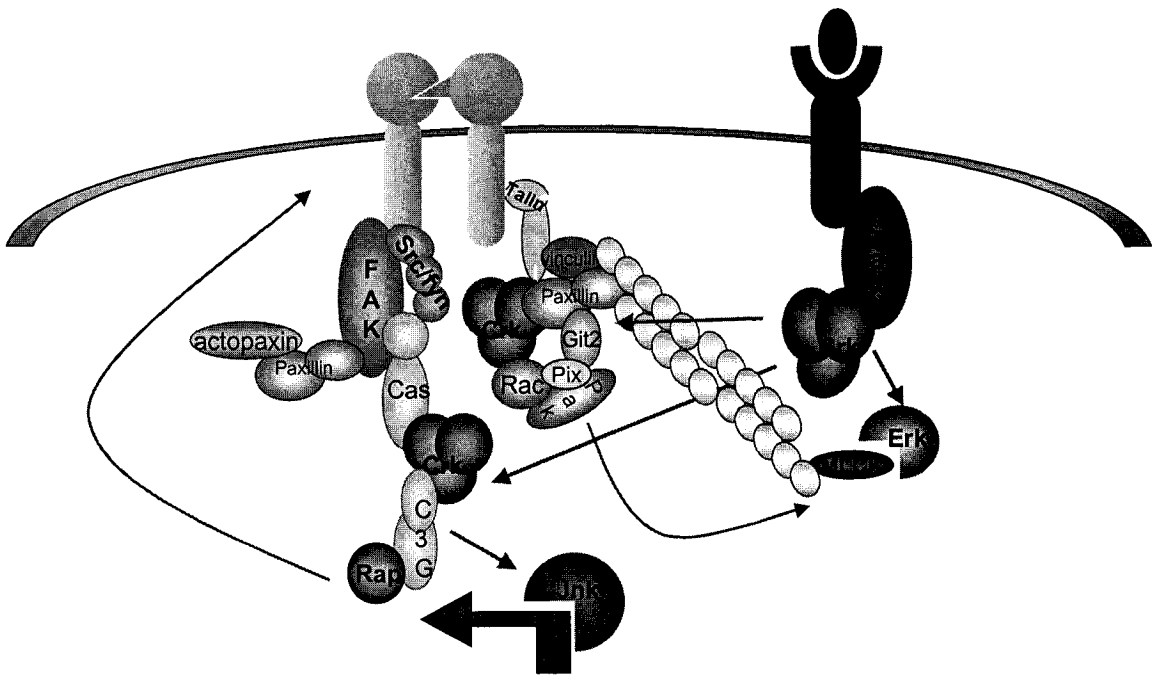

Figure 1: Proposed model

In conclusion our results have provided evidence for a role for Crk adaptor proteins in the maintenance and remodelling of the actin cytoskeletal changes that take place during migration of tumour cells. Furthermore, the function of Crk during cell migration is required irrespectively of upstream

signals as established by the inhibition of cell migration of various highly motile human cancer cell lines. The ability to inhibit the function of Crk using RNA interference approaches may become useful in the generation of novel anti-metastatic therapeutics.

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Crk synergizes with epidermal growth factor for epithelial invasion and morphogenesis and is required for the met morphogenic program.
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Crk Synergizes with Epidermal Growth Factor for Epithelial Invasion and Morphogenesis and Is Required for the Met Morphogenic Program*

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Activation of the Met receptor tyrosine kinase through its ligand, hepatocyte growth factor, stimulates cell spreading, cell dispersal, and the inherent morphogenic program of various epithelial cell lines. Although both hepatocyte growth factor and epidermal growth factor (EGF) can activate downstream signaling pathways in Madin-Darby canine kidney epithelial cells, EGF fails to promote the breakdown of cell-cell junctional complexes and initiate an invasive morphogenic program. We have undertaken a strategy to identify signals that synergize with EGF in this process. We provide evidence that the overexpression of the CrkII adapter protein complements EGF-stimulated pathways to induce cell dispersal in two-dimensional cultures and cell invasion and branching morphogenesis in three-dimensional collagen gels. This finding correlates with the ability of CrkII to promote the breakdown of adherens junctions in stable cell lines and the ability of EGF to stimulate enhanced Rac activity in cells overexpressing CrkII. We have previously shown that the Gab1-docking protein is required for branching morphogenesis downstream of the Met receptor. Consistent with a role for CrkII in promoting EGF-dependent branching morphogenesis, the binding of Gab1 to CrkII is required for the branching morphogenic program downstream of Met. Together, our data support a role for the CrkII adapter protein in epithelial invasion and morphogenesis and underscores the importance of considering the synergistic actions of signaling pathways in cancer progression.

Epithelial morphogenesis is essential for normal embryonic development and involves proliferation, migration, cellular invasion, turnover of surrounding extracellular matrix, and the deposition of newly synthesized extracellular matrix (1). Several growth factors stimulate the morphogenic program of epithelial cells. One of the most potent inducers of a morphogenic program in epithelial cells is hepatocyte growth factor (HGF)[1] (2). HGF is a mesenchymal derived growth factor that promotes several distinct biological responses through activation of the Met receptor tyrosine kinase (3). HGF was originally identified as a potent mitogen for primary rat hepatocytes (4), and HGF serum levels increase following damage to the liver, kidney, stomach, or lung (5). HGF was independently isolated as "scatter factor" as it stimulates epithelial cell dissociation and migration (6). HGF is also a potent morphogen for Madin-Darby canine kidney (MDCK) cells (7) and promotes the inherent morphogenic program of kidney, breast, and lung epithelium grown in matrix cultures (2, 9). Importantly, HGF and Met are deregulated in several human tumors (10) and can promote tumor metastasis and angiogenesis (11, 12).

Epidermal growth factor (EGF) is an important regulator of embryonic development and cell growth. In addition, EGF receptor knock-out mice exhibit impaired ductal growth and branching morphogenesis (13), implicating EGF or other EGF receptor ligands in the morphogenic process. EGF can stimulate branching morphogenesis in some mammary and kidney epithelial cells (13–15) and promotes cell dispersal and invasion in several carcinoma cell lines (17–20). However, the EGF-dependent signals required for these processes are poorly understood.

Whereas HGF promotes a branching morphogenic program in MDCK and primary renal proximal tubular epithelial cells, EGF and other growth factors fail to do so (2, 7, 21). However, treatment of primary renal proximal tubular epithelial cells with a combination of growth factors promotes a similar morphogenic response as HGF (21), suggesting that the co-coordinated activation of multiple signaling pathways must be achieved to undergo an invasive morphogenic program. Hence, MDCK cells provide an experimental system to examine the signals that cooperate with EGF to promote epithelial cell dispersal and morphogenesis.

Using chimeric Met receptors, we have undertaken structure function studies to define Met-dependent signals required for the morphogenic program. These demonstrated that a single tyrosine residue (Tyr-1356) and, in particular, the recruitment of the Grb2 adapter protein to this tyrosine is critical for the morphogenic process (22, 23). Tyrosine 1356 forms a multisubstrate binding site, coupling the Met receptor directly with the Grb2 and Shc adapter proteins and indirectly with Gab1-docking protein (23–28). The morphogenic program of Met receptor mutants is rescued following overexpression of the Gab1-docking protein (29). This identifies Gab1 as a critical modulator of

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[1] The abbreviations used are: HGF, hepatocyte growth factor; MDCK, Madin-Darby canine kidney; PH, pleckstrin homology; EGF, epidermal growth factor; SH2 or SH3, Src homology 2 or 3, respectively; FBS, fetal bovine serum; PI3K, phosphatidylinositol 3′-kinase; HA, hemagglutinin; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; CSF, colony-stimulating factor; CRIB, Cdc42/Rac interactive binding.

the morphogenic response downstream from the Met receptor and allows a structure function approach to define the Gab1-dependent signals required.

Gab1 is a member of a family of docking proteins: Gab1, Gab2, and Gab3, which contain a conserved pleckstrin homology (PH) domain and multiple tyrosine residues that provide binding sites for Src homology 2 (SH2) domain containing proteins (30–34). Gab1 acts to integrate signals downstream from the Met receptor. Following tyrosine phosphorylation, Gab1 associates with multiple signaling proteins including the p85 subunit of PI3K, phospholipase Cγ, CrkII/L, and the SHP-2 tyrosine phosphatase (29, 35–40). The Gab1 PH domain has specificity for phosphatidylinositol 3,4,5-trisphosphate and is required for the morphogenic response downstream from the Met receptor (29, 41) as is the recruitment of the SHP-2 phosphatase to Gab1 (39).

CrkII and CrkL are composed of a single SH2 and two SH3 domains (SH2-SH3-SH3) (43, 44). The Crk SH2 domain binds a number of tyrosine-phosphorylated proteins including p130Cas, paxillin, Cbl, and Gab1, whereas the amino-terminal SH3 domain binds C3G, DOCK180, and Abl (45). The overexpression of CrkII or CrkL enhances cell migration (46–50). However, the role of Crk adapter proteins in epithelial morphogenesis has not been addressed. We demonstrate that the coupling of Crk with Gab1 is required for the invasive morphogenic program downstream from the Met receptor. Moreover, the overexpression of CrkII in MDCK cells synergizes with EGF-stimulated signaling pathways to promote the dispersal of colonies of MDCK epithelial cells, invasion, and branching morphogenesis, whereas each alone is insufficient.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—Dr. George Vande Woude (Van Andel Research Institute, Grand Rapids, MI) provided HGF, and CSF-1 was provided by the Genetics Institute (Boston, MA). EGF was purchased from Roche Diagnostics (Laval, Quebec, Canada). CrkII and Rac antibodies were purchased from BD Transduction Laboratories (Mississauga, Ontario, Canada). HA.11 antibodies were obtained from Berkley Antibody Company (Berkley, CA). Antibodies recognizing the phosphorylated form of ERK1/2 were purchased from New England BioLabs (Mississauga, Ontario, Canada). Dr. John Blenis (Harvard Medical School, Boston, MA) provided an ERK1/2 antibody (C2) that recognizes total ERK1/2. Met (144) antibodies were described previously (51). pcDNA1.1-Gab1ΔCrk expression plasmids were described previously (38).

Cell Culture—MDCK cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and gentamicin (Invitrogen). The generation of stable cell lines overexpressing CrkII was described previously (52). For the generation of stable cell lines expressing Gab1ΔCrk, MDCK cells expressing CSF-Met N1358H (CSF-MetΔGrb2) (23) were co-transfected with pcDNA1.1-Gab1ΔCrk and pLXSH, which confers resistance to Hygromycin B, using GenePorter (Gene Therapy Systems, San Diego, CA). Cell lines were selected in Hygromycin B (300 ng/ml, Roche Diagnostics) for 10–14 days, and stable clones were isolated and screened by Western blotting.

Indirect Immunofluorescence—Cells were fixed in 3.7% formaldehyde diluted in phosphate-buffered saline and processed for indirect immunofluorescence as described previously (52).

Collagen Assays—The ability of MDCK cells to form branching tubules was assayed as described previously (22). 5×10^3 cells were resuspended in 500 μl of a collagen solution composed of 95–98% Type I collagen with the remainder composed of Type III collagen (Cohesion Technologies, Inc., Palo Alto, CA) and layered over 350 μl of the same collagen solution in a 24-well plate. The cells were maintained in Liebowitz medium containing 5% FBS and allowed to form cysts for 5–7 days. For stimulations, HGF (15 units/ml), CSF-1 (5 units/ml), or EGF (20 or 100 ng/ml) was added to Liebowitz medium containing 3% FBS. Fresh growth factor and medium were added every 5–6 days. The tubules were photographed 10–14 days later using a Retiga 1300 digital camera (QIMAGING, Burnaby, British Columbia, Canada) and a Zeiss Axiovert 135 microscope with a ×10 or 32 objective (Carl Zeiss Canada Ltd., Toronto, Ontario, Canada). Image analysis was carried out using Northern Eclipse version 6.0 (Empix Imaging, Missisauga, Ontario, Canada). Each assay was quantitated by counting the number of cysts and branched tubules in 4–6 independent fields for each cell line using a dissecting microscope. The results from 4–5 independent experiments were pooled and are represented graphically. The invasion assays were carried out in the same fashion as described above with the exception that 10^4 cells were seeded and allowed to form small colonies for 2 days prior to stimulation with growth factor. Cells were photographed 2 days later using a ×32 objective.

Growth Factor Stimulations—MDCK and MDCK cells overexpressing CrkII were plated at 6×10^5/100-mm dish and were serum-starved the next day for 20 h in Dulbecco's modified Eagle's medium containing 0.02% FBS. Cells were stimulated with 70 units/ml HGF or 70 ng/ml EGF for 5 and 180 min, respectively. Cells were 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 phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 50 mM NaF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. 30 μg of cell lysate was used for Western blotting with antibodies recognizing the phosphorylated forms of ERK1/2. The membranes were stripped and reprobed with ERK1/2 antibodies.

Rac Pulldown Assays—MDCK and MDCK cells overexpressing CrkII were grown for 2 days in Dulbecco's modified Eagle's medium containing 10% FBS and serum-starved for 4 h in Dulbecco's modified Eagle's medium containing 0.02% FBS. Cells were then stimulated with 70 units/ml HGF or 70 ng/ml EGF for the indicated times and lysed in Rac lysis buffer (53). 700 μg of cell lysate was used for pull down assays with the CRIB domain of PAK1 fused to glutathione *S*-transferase as described previously (53).

RESULTS

Overexpression of CrkII Promotes EGF-dependent Cell Dispersal and Invasion—HGF induces the dispersal and morphogenesis of MDCK cells. Other growth factors, such as EGF, fail to induce this response (29, 54), even though MDCK cells express the EGF receptor and downstream signaling pathways including Gab1 are activated following EGF stimulation (29). Thus, MDCK cells provide an experimental system to examine the signals that cooperate with EGF to promote cell dispersal and morphogenesis. Cell dispersal in response to HGF (Fig. 1*A*, *d–f*) occurs in a stepwise process, whereby cells in the colony spread initially lose their cell-cell adherens junctions and then adopt a fibroblastic cell morphology and disperse (53, 55–57). To examine why EGF fails to induce the dispersal of colonies of MDCK cells, we examined the response of MDCK cells to EGF. Although colonies of MDCK cells show some morphological changes in response to EGF (20 and 100 ng/ml), they failed to disperse (Fig. 1*A*, *g–l*). Moreover, in response to EGF, cells retain adherens junctions and tight junctions as indicated by the presence of β-catenin and ZO-1 at cell-cell junctions (Fig. 1*A*, *g–l*). In contrast, in response to HGF, adherens-based cell-cell junctions are decreased in spread cells and lost in dispersed cells as demonstrated by the loss of β-catenin and ZO-1 at the cell membrane (Fig. 1*A*, *d–f*) (55, 57).

Cell spreading and loss of adherens junctions in response to HGF requires activation of PI3K, MEK1, and the small GTPase Rac (53, 55–57). In a search for other HGF-dependent signals that play a role in this process, we have established that overexpression of the CrkII adapter protein in MDCK cells promotes cell spreading and loss of adherens junctions in the absence of HGF (52). CrkII overexpression mimics the early stages following HGF stimulation, and the cells remain as colonies (Fig. 1*B*, *f*). Consistent with this finding, MDCK cells overexpressing CrkII dispersed in response to suboptimal levels of HGF (Fig. 1*B*, *b* and *g*, *0.5 units/ml*), demonstrating that CrkII can synergize with HGF for epithelial cell dispersal.

These results prompted us to examine whether CrkII would synergize with a growth factor such as EGF that fails to promote the dispersal and invasion of MDCK cells. Unlike parental MDCK cells (Fig. 1*B*, *d* and *e*), MDCK cells overexpressing CrkII dispersed in response to EGF (Fig. 1*B*, *i* and *j*, *20* or *100 ng/ml*). Hence, CrkII overexpression synergizes with EGF to promote the dispersal of colonies of MDCK cells, suggesting

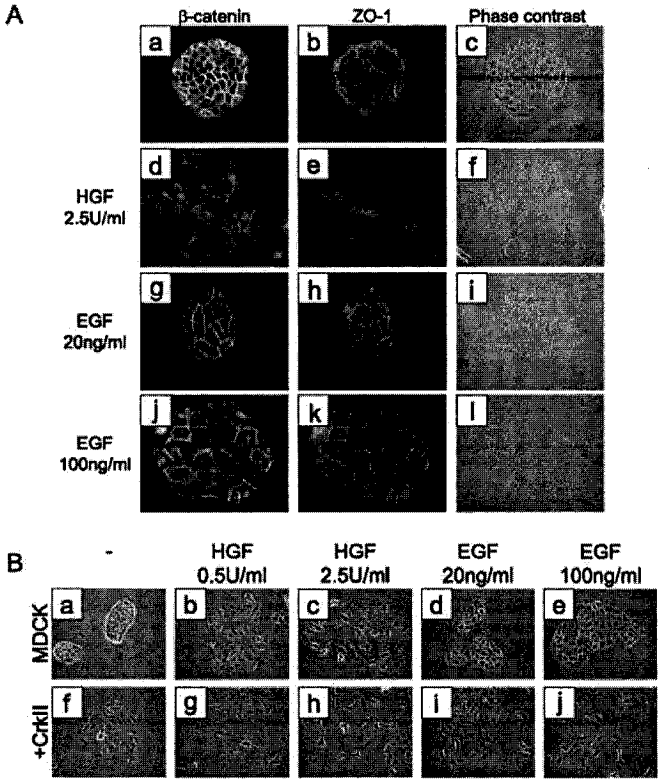

FIG. 1. **The inability of EGF to promote cell dispersal correlates with its inability to promote the breakdown of adherens junctions and tight junctions.** *A*, MDCK cells (*a–c*) were stimulated for 24 h with 2.5 units/ml HGF (*d–f*), 20 ng/ml EGF (*g–i*), or 100 ng/ml EGF (*j–l*) and fixed. Cells were stained with β-catenin/α-mouse-CY3 antibodies (*a*, *d*, *g*, and *j*) and ZO-1/α-rabbit-Alexa488 antibodies (*b*, *e*, *h*, and *k*). Corresponding phase-contrast images are shown (*c*, *f*, *i*, and *l*). *B*, MDCK (*a–e*) and MDCK cells overexpressing CrkII (*f–j*) were left unstimulated (*a* and *f*) or stimulated for 24 h with 0.5 units/ml HGF (*b* and *g*), 2.5 units/ml HGF (*c* and *h*), 20 ng/ml EGF (*d* and *i*), or 100 ng/ml EGF (*e* and *j*), fixed, and photographed.

that CrkII may synergize with EGF to promote an invasive and morphogenic response. Cell invasiveness in response to EGF or HGF was examined by stimulating MDCK and MDCK cells overexpressing CrkII seeded in three-dimensional collagen gels (Fig. 2). In the absence of growth factor stimulation, both MDCK and MDCK cells overexpressing CrkII formed small spherical colonies (Fig. 2, *a* and *e*). HGF stimulation of MDCK and MDCK cells overexpressing CrkII promoted the dispersal and invasion of cells (Fig. 2, *b* and *f*). In contrast, EGF stimulation of MDCK cells promoted the formation of small cellular extensions, but the cells failed to detach and invade the collagen gel (Fig. 2, *c* and *d*). In contrast, in the presence of EGF, MDCK cells overexpressing CrkII dispersed and invaded the collagen gel where 80–90% of the colonies underwent invasion in response to EGF (Fig. 2, *g* and *h*). 80–100 colonies were scored for each condition. The invasive response to EGF in CrkII-overexpressing cells (Fig. 2, *g* and *h*) was similar to cells stimulated with HGF (Fig. 2, *b* and *f*). Similar responses were observed in several independent clones of MDCK cells overexpressing CrkII (data not shown). Hence, although the overexpression of CrkII or the activation of EGF-dependent signaling pathways is not sufficient for the dispersal of epithelial colonies or the invasion of MDCK cells plated in three-dimensional collagen gels, together they cooperate to promote both cell dispersal and invasion.

MEK1-dependent Signals Synergize with CrkII to Promote the Loss of Tight Junctions and Cell Dispersal—The ability of EGF to promote cell dispersal and invasion in MDCK cells overexpressing CrkII but not parental MDCK cells suggested that CrkII-dependent signals synergize with EGF. In response to HGF, the loss of adherens junctions is blocked by pharmacological inhibitors of MEK1 and PI3K (55, 57). To investigate the EGF-dependent signals required for the dispersal of CrkII-overexpressing cells, cells were pretreated with pharmacological inhibitors of MEK1 (U0126) and PI3K (LY294002). Whereas LY294002 inhibited HGF-stimulated dispersal of MDCK cells and loss of the tight junction marker, ZO-1 (Fig. 3*A*, *c* and *d*), LY294002 pretreatment failed to inhibit EGF- or HGF-induced dispersal of CrkII-overexpressing cells (Fig. 3*B*, *e*, *f*, *k*, and *l*). In contrast, the pretreatment of CrkII-overexpressing cells with UO126 blocked both the HGF- and EGF-dependent loss of ZO-1 from cell-cell junctions and cell scatter (Fig. 3*B*, *g*, *h*, *m*, and *n*), indicating that MEK1-dependent pathways are absolutely required.

Sustained ERK1/2 activation in response to HGF correlates with a Met-dependent morphogenic response (39), and the pharmacological inhibition of MEK1 blocked both cell dispersal (57) and the morphogenic program (54) in response to HGF. Hence, the inability of EGF to stimulate cell dispersal and branching morphogenesis in MDCK cells may reflect the ability of HGF but not EGF to promote sustained ERK1/2 activation. To examine this possibility, we established whether the overexpression of CrkII altered the temporal activation of ERK1/2 in response to EGF. Lysates were prepared from MDCK and MDCK cells overexpressing CrkII stimulated with HGF or EGF for 5 and 180 min and immunoblotted with a phosphorylation-specific ERK1/2 antibody raised against the active site. EGF- and HGF-stimulated ERK1/2 phosphorylation was increased and similar in MDCK cells stimulated for 5 min (Fig. 4*A*, *upper panel*). Although HGF stimulation promoted sustained ERK1/2 phosphorylation, up to 180 min, ERK1/2 phosphorylation returned to basal levels in both CrkII-overexpressing and in MDCK cells stimulated with EGF (Fig. 4*A*, *upper panel*). All samples contained similar levels of ERK1/2 (Fig. 4*A*, *lower panel*). Although the overexpression of CrkII did not promote sustained ERK1/2 phosphorylation in response to EGF, the ability of EGF to promote dispersal in MDCK cells overexpressing CrkII is MEK1-dependent.

CrkII-overexpressing Cells Exhibit Elevated Rac Activation—HGF-dependent breakdown of cell-cell junctions and cell spreading requires the activity of members of the Rho GTPase family, Rac and Cdc42 (53, 56). In MDCK cells, HGF stimulation leads to the activation of Rac and Cdc42 (53), and HGF-induced cell spreading is inhibited by the expression of dominant negative mutants of Rac1 (N17Rac1) that fail to bind GTP (53, 56). Similarly, CrkII fails to promote cell spreading when microinjected together with N17Rac1 (52). To establish whether the ability of EGF to stimulate the spreading of colonies of MDCK cells overexpressing CrkII reflects elevated Rac activity. MDCK and MDCK cells overexpressing CrkII were stimulated or not with HGF or EGF, and GTP-bound Rac levels were assayed *in vitro* using a glutathione *S*-transferase fusion protein containing the PAK1 CRIB domain. As shown previously (53), the stimulation of MDCK cells with HGF induced a modest activation of Rac (Fig. 4*B*, *upper panel*). EGF stimulation of MDCK cells also induced the activation of Rac, but Rac activation was consistently lower than that observed following HGF stimulation (Fig. 4*B*, *upper panel*). In contrast, MDCK cells overexpressing CrkII showed elevated levels of GTP-bound Rac (Fig. 4*B*, *upper panel*) (52), and Rac activation was greatly enhanced following stimulation with HGF or EGF when compared with parental MDCK cells (Fig. 4*B*, *upper panel*). Similar levels of Rac were detected in whole cell lysates (Fig. 4*B*, *lower panel*).

CrkII Synergizes with EGF to Promote a Morphogenic Program—As EGF does not promote invasion (Fig. 2) or branching

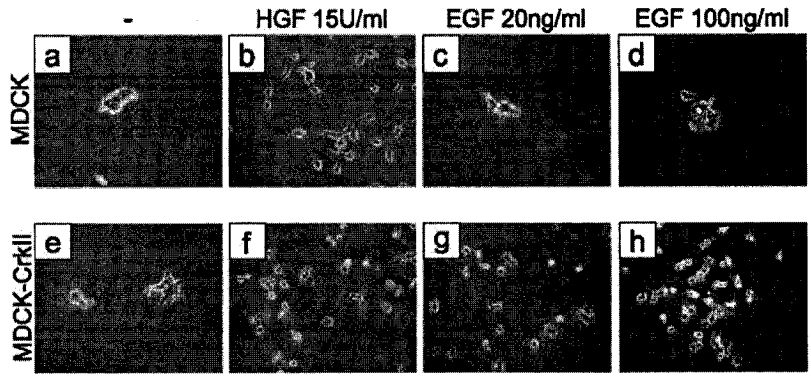

FIG. 2. **CrkII overexpression in MDCK cells promotes invasion following EGF stimulation.** MDCK (*a–d*) and MDCK cells overexpressing CrkII (*e–h*) were plated in three-dimensional collagen gels. 48 h later, cells were left untreated (*a* and *e*) or treated with 15 units/ml HGF (*b* and *f*), 20 ng/ml EGF (*c* and *g*), or 100 ng/ml EGF (*d* and *h*). Cells were photographed 48 h later.

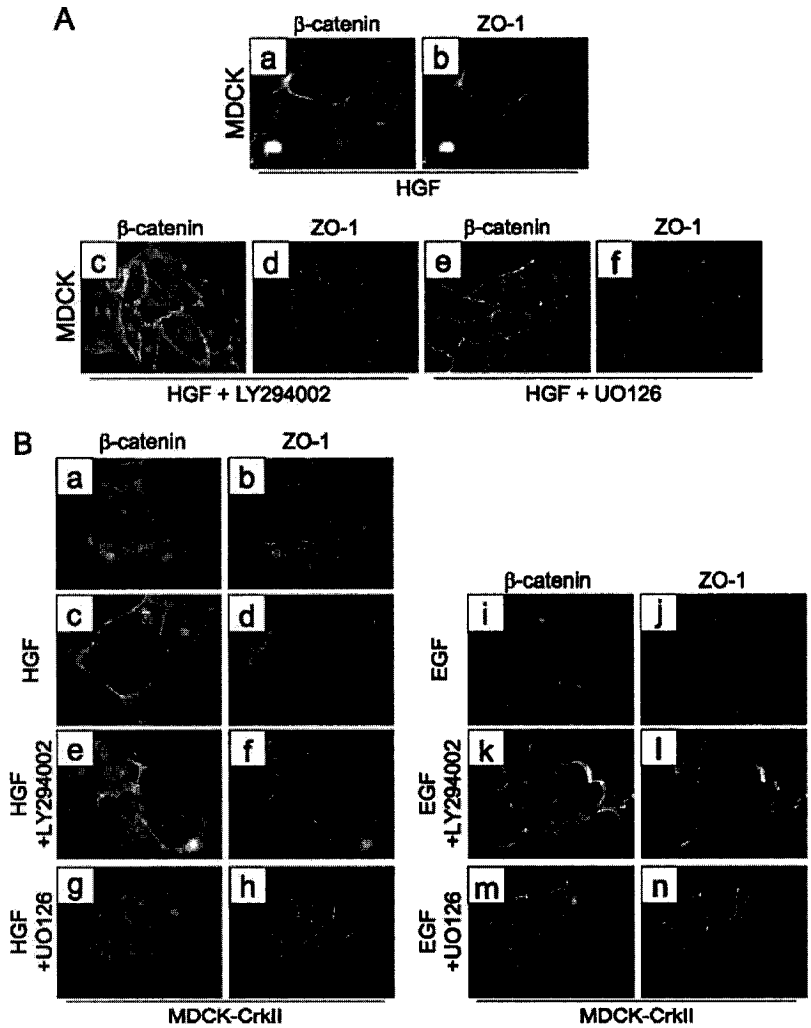

FIG. 3. **PI3K but not MEK1 activity is dispensable for HGF- and EGF-stimulated cell dispersal in MDCK cells overexpressing CrkII.** *A*, MDCK cells were pretreated with Me_2SO (*a* and *b*), 25 μM LY294002 (*c* and *d*), or 5 μM UO126 (*e* and *f*) for 60 min prior to stimulation with 10 units/ml HGF for 24 h. Cells were fixed and co-stained with β-catenin/α-mouse-CY3 antibodies (*a*, *c*, and *e*) and ZO-1/α-rabbit-Alexa488 antibodies (*b*, *d*, and *f*). *B*, MDCK cells overexpressing CrkII were pretreated with Me_2SO (*a–d*, *i*, and *j*), 25 μM LY294002 (*e*, *f*, *k*, and *l*), or 5 μM UO126 (*g*, *h*, *m*, and *n*) for 60 min prior to stimulation with 10 units/ml HGF (*c–h*) or 20 ng/ml EGF (*i–n*) for 24 h. Cells were fixed and co-stained with β-catenin/α-mouse-CY3 antibodies (*a*, *c*, *e*, *g*, *i*, *k*, and *m*) and ZO-1/α-rabbit-Alexa488 antibodies (*b*, *d*, *f*, *h*, *j*, *l*, and *n*).

morphogenesis of MDCK cells (29) but promotes the invasion of cells overexpressing CrkII (Fig. 2), we determined whether EGF could also promote a morphogenic program in CrkII-overexpressing cells. Cells were seeded in three-dimensional collagen gels and allowed to form cysts (a hollow sphere of polarized epithelia) for 5 days. Cysts were then stimulated with HGF or EGF, and the appearance of branching tubules was monitored over the course of 10–14 days. As described previously (2, 23), HGF stimulation of MDCK results in the formation of branched tubules (Fig. 5*A*, *b*), structures whose length is five times greater than their width. The overexpression of CrkII (Fig. 5*A*, *e*) or stimulation of MDCK cells with EGF (Fig. 5*A*, *c* and *d*) failed to promote branching morphogenesis. Rather, CrkII overexpression or EGF stimulation each promoted cell growth as displayed by the larger size of the cysts (Fig. 5*A*, *c–e*) when compared with unstimulated MDCK cells (Fig. 5*A*, *a*). Consistent with the ability of EGF stimulation to promote the invasion of MDCK cells overexpressing CrkII in three-dimensional collagen gels (Fig. 2), EGF promoted the formation of branching tubules in MDCK cells overexpressing CrkII (Fig. 5*A*, *g* and *h*). For each cell line, the results from four independent experiments were quantified and pooled to represent the percentage of cells that form branching tubules (Fig. 5*B*). Whereas vector-transfected MDCK cells remained as cysts

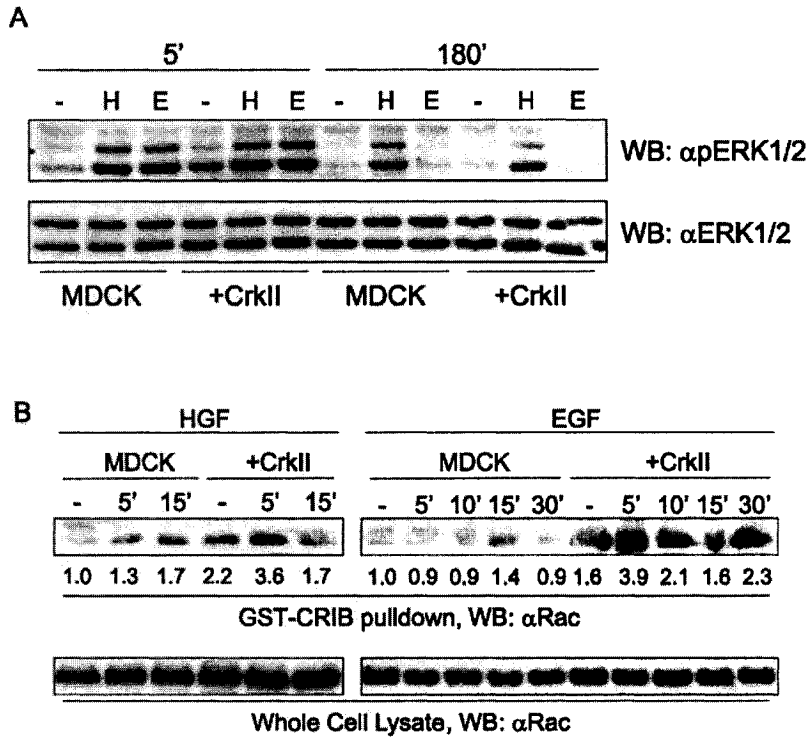

FIG. 4. **Overexpression of CrkII promotes elevated Rac activation but fails to promote sustained ERK1/2 activation in response to EGF.** *A*, MDCK and MDCK cells overexpressing CrkII were stimulated with 70 units/ml HGF (*H*) or 70 ng/ml EGF (*E*) for the indicated times. 30 μg of whole cell lysate was subjected to SDS-polyacrylamide gel electrophoresis, and proteins on the gel were transferred to nitrocellulose membranes. Western blotting was performed with αpERK1/2 (*upper panel*), and the membranes were stripped and reprobed with αERK1/2 (*lower panel*). *B*, MDCK or MDCK cells overexpressing CrkII was stimulated with 70 units/ml HGF or 70 ng/ml EGF for the indicated times. Cells were lysed, and 700 μg of protein lysate was incubated for 60 min with glutathione *S*-transferase-CRIB fusion proteins bound to glutathione-Sepharose beads. The beads were washed extensively, and bound proteins together with 20 μg 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 αRac. Fold induction is expressed relative to unstimulated MDCK cells. *WB*, Western blotting.

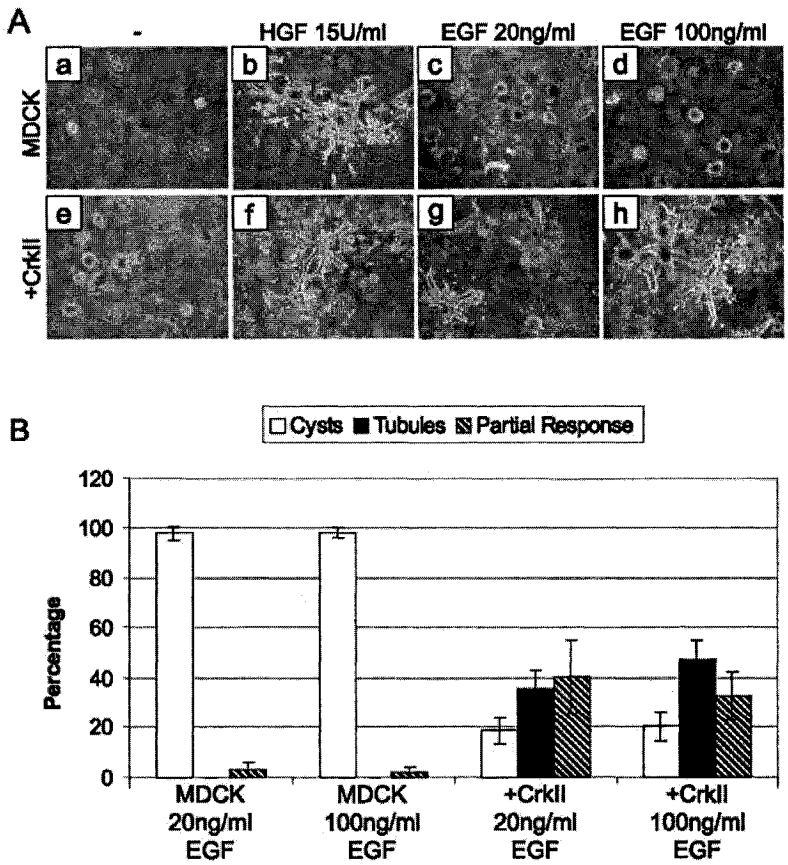

FIG. 5. **MDCK cells overexpressing CrkII form branching tubules in response to EGF, whereas control cells form large cysts.** *A*, MDCK (*a–d*) and MDCK cells overexpressing CrkII (*e–h*) were plated in three-dimensional collagen gels and allowed to form cysts for 5–7 days. Cells were then left untreated (*a* and *e*) or treated with 15 units/ml HGF (*b* and *f*), 20 ng/ml EGF (*c* and *g*), or 100 ng/ml EGF (*d* and *h*). Branched tubules appeared 10–14 days later and were photographed. *B*, quantitation of the morphogenic response following stimulation with EGF (20 and 100 ng/ml) was performed as described under "Experimental Procedures." Results from four independent experiments were pooled and plotted as the percentage of cysts that have undergone branching morphogenesis.

in response to 20 or 100 ng/ml EGF (98%), CrkII-overexpressing cell lines responded to 20 and 100 ng/ml EGF with only 18–20% of the original cysts showing no response (Fig. 5*B*). EGF stimulation of MDCK cells overexpressing CrkII generated branched tubules (35–47%) as well as structures too short to be considered tubules (32–40%, referred to as *Partial Response* in Fig. 5*B*). Interestingly, the tubules obtained following HGF stimulation of MDCK cells overexpressing CrkII appeared more branched (Fig. 5*A*, *b* and *f*), further supporting a role for CrkII in branching morphogenesis.

A Gab1-Crk Complex Is Required for a Met-dependent Morphogenic Program—In a search for signals required for the Met-dependent morphogenic program, we had previously generated a chimeric CSF-Met receptor, allowing a structure func-

tion analysis of Met-dependent signals in response to CSF in MDCK cells that express endogenous Met receptors. The Gab1-docking protein was found to rescue the morphogenic defect of a Met receptor mutant that fails to recruit the Grb2 adapter protein and has a reduced ability to recruit Gab1 (CSF-MetΔGrb2) (29, 39, 41, 58). Following activation of the Met receptor, Gab1 is tyrosine-phosphorylated and recruits multiple signaling proteins including the Crk adapter proteins (35–38). We determined whether the binding of Crk to Gab1 was required for HGF-dependent branching morphogenesis in MDCK cells as Gab1 binds to CrkII in MDCK cells stimulated with HGF (52). We have previously shown that a Gab1 mutant in which five tyrosine residues contained within a YXXP motif were substituted with phenylalanine (Gab1ΔCrk) failed to bind CrkII (38). This mutant was overexpressed in MDCK cells expressing CSF-MetΔGrb2, and several independent clones expressing HA-tagged Gab1ΔCrk at equivalent levels or greater than HA-tagged wild type Gab1 were selected (Fig. 6*A*). Similar levels of CSF-MetΔGrb2 were expressed in each cell line (Fig. 6*B*). All cell lines formed cysts when plated in collagen gels (Fig. 6*C*, *a*, *d*, and *g*). As reported previously, MDCK cells expressing a chimeric CSF-MetΔGrb2 receptor failed to undergo branching morphogenesis (Fig. 6*C*, *c*) (23, 29), whereas the overexpression of wild type Gab1 in these cells promoted a morphogenic program in response to CSF (Fig. 6*C*, *f*) (29). In contrast, the overexpression of Gab1ΔCrk mutant did not efficiently rescue the branching morphogenesis phenotype of MDCK cells expressing a chimeric CSF-MetΔGrb2 (Fig. 6*C*, *i*). The majority of cells expressing Gab1ΔCrk remained as cysts or formed stunted tubule-like structures, which failed to branch in response to CSF (Fig. 6*C*, *i*). The results from six independent experiments were quantified and pooled together (Fig. 6*D*). Stunted tubule-like structures that failed to branch in cells expressing Gab1ΔCrk were scored as a "partial response." Whereas 56% of cells overexpressing a wild type Gab1 protein efficiently formed branching tubules in response to CSF-Met activation, <6% of all cells overexpressing Gab1ΔCrk formed branching tubules (Fig. 6*D*). In contrast, 50% of cells expressing Gab1ΔCrk formed unbranched tubule-like structures in response to CSF stimulation (Fig. 6*D*). Importantly, 70% of all cells expressing vector, Gab1, or Gab1ΔCrk formed branching tubules in response to HGF activation of the endogenous Met receptor, indicating that the morphogenic program was not impaired (Fig. 6*C*, *b*, *e*, and *h* and data not shown). Hence, the recruitment of Crk to the Gab1-docking protein is critical for the epithelial branching morphogenic program induced by the Met receptor.

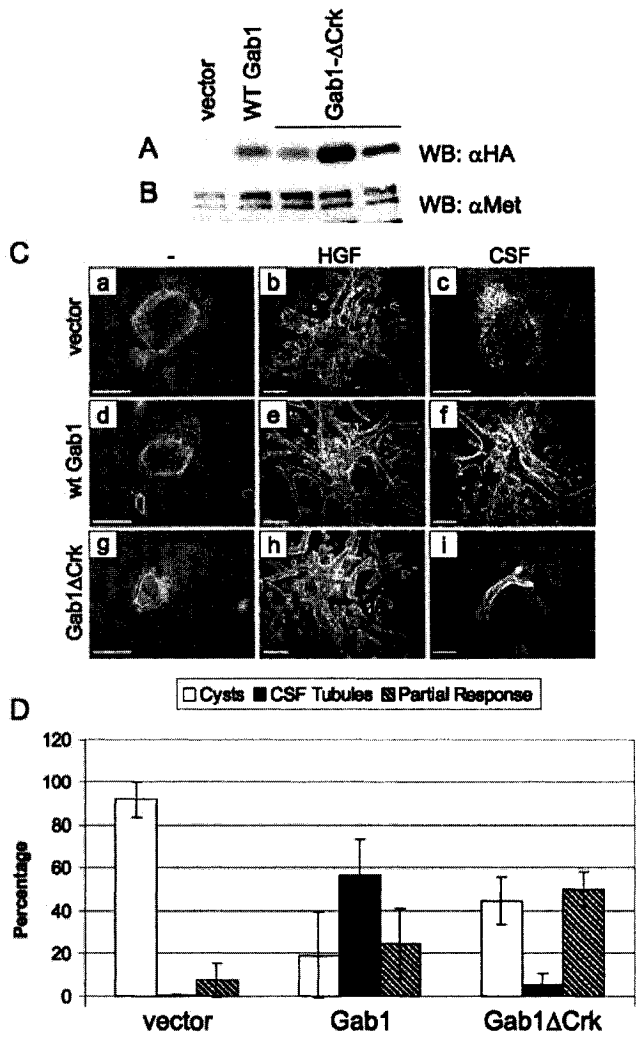

FIG. 6. **The expression of Gab1ΔCrk fails to restore branching morphogenesis in MDCK cells expressing CSF-MetΔGrb2.** *A* and *B*, proteins from lysates of MDCK cells expressing CSF-MetΔGrb2 and vector, HA-Gab1 or HA-Gab1ΔCrk, were subjected to Western blotting with α-HA (*A*) or α-Met (*B*). *C*, MDCK cells expressing CSF-MetΔGrb2 and vector, Gab1 or Gab1ΔCrk, were seeded in collagen and allowed to form cysts for 7 days. Cysts were left unstimulated (*a*, *d*, and *g*) or stimulated with 15 units/ml HGF (*b*, *e*, and *h*) or 5 units/ml CSF-1 (*c*, *f*, and *i*). Branching tubule formation was visualized 10–14 days later, and structures were photographed. The *large bar* represents 50 μm, and the *small bar* represents 100 μm. *D*, quantitation of the morphogenic response following stimulation with HGF or CSF-1 was performed as described under "Experimental Procedures." Results from six independent experiments were pooled together and plotted as the percentage of cysts that have undergone branching morphogenesis. Partial responses represent tubule-like structures, which failed to branch.

DISCUSSION

HGF but not EGF promotes cell dispersal and branching morphogenesis in MDCK cells. This provides an experimental system to identify HGF-dependent signals and to dissect signals that synergize with EGF in mediating the dispersal of epithelial sheets, epithelial remodeling, invasion, and morphogenesis. We show that the recruitment of the Crk adapter protein to the Gab1-docking protein is required for the morphogenic response downstream from Met. Moreover, the overexpression of the CrkII adapter protein converts an EGF-dependent signal in MDCK cells from noninvasive to invasive, promoting the dispersal of epithelial cell sheets, invasion, and branching morphogenesis. EGF-dependent MEK1 activity is required for the breakdown of tight junctions and dispersal of CrkII-overexpressing cells. Although, CrkII overexpression does not alter EGF-dependent ERK1/2 activation, CrkII overexpression synergizes with EGF to promote a robust activation of Rac. Together, our data support a role for the CrkII adapter protein in the integration of upstream signals to promote epithelial cell dispersal, invasion, and morphogenesis.

The dispersal of epithelial cells in response to HGF occurs in a stepwise progression, which involves cell spreading and the breakdown of epithelial adherens junctions and tight junctions. The inability of EGF to stimulate cell dispersal and invasion correlates with the inability of a EGF-dependent signal to stimulate the breakdown of adherens and tight junctions in MDCK cells (Fig. 1*A*). In a search for signals that could synergize with EGF to induce an invasive response in MDCK cells, we established that the overexpression of CrkII promoted the early stages of an HGF response including cell spreading and breakdown of adherens junctions (52). However, in the absence of HGF stimulation, MDCK cells overexpressing CrkII failed to scatter or invade collagen gels (Fig. 2), consistent with the

inability of these cells to breakdown ZO-1 containing tight junctions and disperse in two-dimensional cultures (Fig. 1*B*) (52). The addition of EGF to cells overexpressing CrkII promoted the dispersal of epithelial colonies in two-dimensional cultures and the invasion of cells in three-dimensional collagen gels (Figs. 1*B* and 2). This finding suggests that although the activation of EGF-stimulated or CrkII-dependent signaling pathways alone is not sufficient, together they can cooperate to activate signals required for cell dispersal and invasion.

Our work and the work of others (55, 57) have identified several of the signals required for cell dispersal in response to HGF. HGF-dependent cell spreading and loss of adherens junctions are blocked by inhibitors of MEK1 and PI3K (55, 57) and require the activation of the Rho GTPases, Rac and Cdc42 (53, 56). Consistent with the synergy between EGF and CrkII signals, MDCK cells overexpressing CrkII displayed a robust activation of Rac in response to HGF or EGF compared with MDCK cells stimulated with HGF or EGF (Fig. 4*B*). Hence CrkII overexpression synergizes with HGF and EGF in promoting the activation of Rac. The involvement of Rac in cell invasion has been established in several experimental systems (59–62). The overexpression of activated Rac1 promotes the invasion of some carcinoma cell lines in collagen (59, 63), whereas the dominant negative forms of Rac1 inhibit leptin-stimulated cell invasion in collagen gels (60), implicating Rac1-dependent pathways in epithelial cell invasion. However, activated Rac also promotes enhanced cell-cell junction assembly in MDCK cells (64), inhibiting their dispersal in response to HGF (65, 66). The differences observed with activated Rac and CrkII overexpression in MDCK cells may reflect the ability of Rac activity to turn over in MDCK cells overexpressing CrkII, in contrast with cells expressing activated Rac where Rac is constantly GTP-bound. Whereas MDCK cells overexpressing wild type Rac1 are more polarized than vector-transfected MDCK cells, they are able to disperse in response to HGF (data not shown). Moreover, the overexpression of wild type Rac1 in MDCK cells was not sufficient to promote an invasive response to EGF (data not shown), implicating additional Crk-dependent signals in the invasive response.

EGF-induced loss of ZO-1 at tight junctions and dispersal of CrkII-overexpressing cells were blocked by pharmacological inhibitors of MEK1 but not PI3K (Fig. 3*B*). This finding is consistent with a requirement for MEK1 activity in the loss of ZO-1 from tight junctions and for the maintenance of the dispersed phenotype in epithelial cells expressing activated Ha-Ras or Raf-1 (67, 68). This finding also supports previous data indicating a requirement for PI3K activity in the breakdown of adherens junctions (55, 57). Because CrkII overexpressing cells show decreased adherens junctions as visualized through reduced β-catenin staining at cell-cell junctions (Fig. 3*B*), CrkII overexpression may have overcome the requirement for PI3K in the breakdown of adherens junctions.

As an adapter protein, Crk contains an SH2 domain that binds tyrosine-phosphorylated proteins (45) and an SH3 domain that binds proteins containing PXLPXK motifs commonly found in exchange factors for members of the Ras superfamily of GTP-binding proteins (69). The mechanism through which CrkII promotes elevated basal Rac activity and elevated Rac activity in response to HGF and EGF is currently unknown. CrkII can activate Rac through an interaction with DOCK180, an exchange factor for Rac1 (70, 71). However, an association of CrkII with DOCK180 was not observed in CrkII-overexpressing MDCK cells, although DOCK180 is expressed in these cells (data not shown), suggesting that other Rac exchange factors may be involved. Alternatively, CrkII may activate Rac indirectly through an alternate mechanism.

The observation that CrkII overexpression did not alter EGF-dependent ERK1/2 activation is in contrast to previous data where the overexpression of v-Crk in PC12 cells promoted sustained ERK1/2 activity and neurite outgrowth in response to EGF (72). This may represent differences in v-Crk *versus* CrkII in addition to differences in cellular context and the possible presence of different Crk-binding proteins in these two cell types. Nevertheless, the observation that a loss of tight junctions requires EGF-dependent MEK1 activation supports a synergistic interaction between an EGF signal and CrkII for cell dispersal and invasion. This finding is consistent with the ability of EGF to stimulate the dispersal and invasion of several carcinoma cell lines (17–20), each of which may have undergone several genetic changes such as the loss of adherens junctions, the consequence of which is similar to MDCK cells overexpressing CrkII.

Epithelial morphogenesis requires cell invasion as well as the ability to reorganize and reform cellular junctions (42). The formation of a cyst of polarized MDCK epithelial cells requires 5 days in collagen. Once polarized, cells are subjected to additional signals from surrounding matrix that act to promote epithelial organization (16, 42). We have previously demonstrated that the docking protein Gab1 is required for the morphogenic program in response to HGF stimulation (29). In the absence of any catalytic activity, Gab1 functions as a docking protein that when phosphorylated by Met or other receptors recruits multiple signaling proteins including PI3K and SHP-2 (29, 36, 39) as well as CrkII and CrkL (35–38). The significance of Gab1/Crk coupling in branching morphogenesis had not been previously addressed. We provide evidence supporting a role for Gab1/Crk coupling in branching morphogenesis downstream from a Met receptor tyrosine kinase unable to bind Grb2 (Fig. 6*C*). Although the precise role for Crk in this process is unknown, CrkII overexpression enhances HGF-dependent activation of Rac (Fig. 4*B*) and Rac1 is involved in epithelial remodeling (8). Furthermore, this finding is consistent with the observation that overexpression of CrkII in MDCK cells synergizes with EGF to promote epithelial remodeling and branching morphogenesis (Fig. 5). In conclusion, we have demonstrated that the coupling of Gab1 with Crk is required for branching morphogenesis following activation of the Met receptor tyrosine kinase. These results emphasize the potential importance of Crk-dependent signaling pathways in epithelial morphogenesis and invasion. The ability of CrkII overexpression to switch an EGF signal in MDCK cells from non-invasive to invasive underscores the importance of considering the synergistic actions of signaling pathways in cancer progression.

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Crk Associates with a Multimolecular Paxillin/GIT2/β-PIX Complex and Promotes Rac-dependent Relocalization of Paxillin to Focal Contacts

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We have previously demonstrated that the CrkII and CrkL adapter proteins are required for the spreading of epithelial colonies and the breakdown of adherens junctions in response to hepatocyte growth factor. When overexpressed, CrkII and CrkL promote lamellipodia formation, cell spreading, and the loss of epithelial adherens junctions in the absence of hepatocyte growth factor. The exact mechanism by which Crk proteins elicit these changes is unclear. We show that the overexpression of CrkII or CrkL, but not Src homology 2 or amino-terminal Src homology 3 domain mutant Crk proteins, promotes the relocalization of Paxillin to focal contacts throughout the cell and within lamellipodia in a Rac-dependent manner. In stable cell lines overexpressing CrkII, enhanced lamellipodia formation and cell spreading correlate with an increased association of CrkII with Paxillin, GIT2 (an ARF-GAP) and β-PIX (a Rac1 exchange factor). Mutants of Paxillin that fail to associate with Crk or GIT2, or do not target to focal adhesions inhibit Crk-dependent cell spreading and lamellipodia formation. We conclude from these studies that the association of Crk with Paxillin is important for the spreading of epithelial colonies, by influencing the recruitment of Paxillin to focal complexes and promoting the enhanced assembly of Paxillin/GIT2/β-PIX complexes.

INTRODUCTION

Epithelial-mesenchymal (EM) transitions are characterized by the loss of epithelial cell-cell junctions and cell polarity and the acquisition of a motile mesenchymal phenotype (Boyer *et al.*, 2000). The dispersal of epithelial colonies is a dynamic process initiated by the reorganization of the actin cytoskeleton and the formation of membrane protrusions within cells at the edge of the colony (Lauffenburger and Horwitz, 1996). As cells spread, new focal contacts are formed at the leading edge of the colony, whereas existing ones are remodeled (Webb *et al.*, 2002). On loss of cell-cell junctions, this process is complete and dispersed cells acquire a fibroblastic morphology with enhanced cell motility (Lauffenburger and Horwitz, 1996).

EM transitions and epithelial dispersal are tightly regulated and require the coordinated activation and targeting of structural and signaling complexes that modulate the remodeling of the actin and microtubule network required for cell migration (Sastry and Burridge, 2000; Wittmann and Waterman-Storer, 2001; Webb *et al.*, 2002). Hepatocyte growth factor (HGF) is a potent modulator of EM transitions in vitro (Weidner *et al.*, 1993; Zhu *et al.*, 1994) and in vivo (Birchmeier and Gherardi, 1998). HGF stimulates the breakdown of cell-cell junctions and the dispersal of sheets of epithelial cells, increasing their invasiveness (Stoker *et al.*, 1987; Weidner *et al.*, 1990). In a search for signals downstream from the HGF/Met receptor tyrosine kinase involved in the dispersal of epithelial sheets, we recently demonstrated that Crk adapter proteins are required for HGF-induced lamellipodia formation and cell spreading (Lamorte *et al.*, 2002b). Moreover, overexpression of the CrkII or CrkL adapter protein promotes lamellipodia formation, cell spreading, and loss of adherens junctions independently of

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Abbreviations used: ARF, ADP-ribosylation factor; EM, epithelial-mesenchymal; FBS, fetal bovine serum; GAP, GTPase activating protein; GTPase, guanosine triphosphatase; HGF, hepatocyte growth factor; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; SH2, Src homology 2; SH3, Src homology 3.

HGF (Lamorte *et al.*, 2002b). CrkII and CrkL are composed of a single Src homology 2 (SH2) and two Src homology 3 (SH3) domains (SH2-SH3-SH3) (Reichman *et al.*, 1992; ten Hoeve *et al.*, 1993). Crk proteins function as adapter proteins to assemble signaling complexes. The Crk SH2 domain binds tyrosine phosphorylated proteins involved in cell spreading, actin reorganization, and cell migration, including p130Cas and Paxillin (Feller, 2001), as well as Gab1, a docking protein involved in epithelial morphogenesis (Maroun *et al.*, 1999; Lamorte *et al.*, 2002a). Through its amino terminal SH3 domain Crk interacts constitutively with proline rich motifs present within several protein, including C3G, an exchange factor for Rap1 (Gotoh *et al.*, 1995) and DOCK180, an exchange factor for Rac1 (Kiyokawa *et al.*, 1998a; Nolan *et al.*, 1998) as well as the Abl tyrosine kinase (Feller *et al.*, 1994). Genetic studies in *Caenorhabditis elegans* have demonstrated a role for CrkII and DOCK180 in phagocytosis and polarized cell migration required for normal pathfinding of the distal tip cells of the developing gonad (Reddien and Horvitz, 2000). In tissue culture, the overexpression of CrkII or CrkL enhances the migration of mammalian cells when assayed as single cells in Boyden chambers (Klemke *et al.*, 1998; Uemura and Griffin, 1999; Cho and Klemke, 2000; Spencer *et al.*, 2000; Hemmeryckx *et al.*, 2001) or on collagen matrices (Petit *et al.*, 2000). However, the mechanism through which Crk proteins promote the spreading and motility of epithelial colonies is not completely understood.

The role of the Rho family of small GTPases in regulating actin cytoskeletal dynamics is well established (Hall, 1998). The activation of Rac1 is required for lamellipodia formation, Cdc42 for filopodial extensions, and RhoA for the bundling of actin stress fibers and the formation of mature focal adhesions. More recently, members of the ADP-ribosylation factor (ARF) family of GTPases have been implicated in the remodeling of the actin cytoskeleton. ARF proteins have been characterized primarily based on their role in the regulation of membrane traffic (Chavrier and Goud, 1999). Moreover, ARF6 activity regulates the targeting of Rac1 to the membrane and is required for Rac1-induced lamellipodia formation (Radhakrishna *et al.*, 1999). In addition, ARF6 activity is involved in the breakdown of epithelial cell-cell junctions through the internalization of E-cadherin/β-catenin complexes in response to HGF (Palacios *et al.*, 2001). In further support of the regulation of actin reorganization and cell migration by ARF GTPases, ARF guanine nucleotide exchange factors and ARF-GTPase activating proteins (ARF-GAP) regulate these processes as well (Franco *et al.*, 1999; Turner *et al.*, 1999; Di Cesare *et al.*, 2000; Jackson *et al.*, 2000; Kondo *et al.*, 2000; Randazzo *et al.*, 2000; Mazaki *et al.*, 2001; Santy and Casanova, 2001; Uchida *et al.*, 2001; West *et al.*, 2001; Brown *et al.*, 2002; Liu *et al.*, 2002a; Manabe Ri *et al.*, 2002). For example, the overexpression of various ARF-GAP proteins modulates the formation and/or turnover of focal adhesions (Di Cesare *et al.*, 2000; Jackson *et al.*, 2000; Kondo *et al.*, 2000; Randazzo *et al.*, 2000; Mazaki *et al.*, 2001; Liu *et al.*, 2002a) and the overexpression of an ARF guanine nucleotide exchange factor, ARNO, enhances the spreading and dispersal of epithelial cells (Santy and Casanova, 2001). In addition to their role as GAPs, ARF-GAP proteins may also influence signaling pathways through additional protein–protein interactions. GIT2/PKL is a Paxillin binding protein with an ARF-GAP domain (Turner *et al.*, 1999; Premont *et al.*, 2000) that localizes to focal adhesions (Brown *et al.*, 2002) and links Paxillin to an exchange factor for Rac1, β-PIX/Cool (Bagrodia *et al.*, 1998; Manser *et al.*, 1998).

Focal adhesions are multiprotein complexes, containing integrins, focal adhesion kinase (FAK), Paxillin, and other molecules that serve to anchor the actin cytoskeleton to the plasma membrane and provide attachments with the extracellular matrix (Geiger *et al.*, 2001). Fibroblasts isolated from Paxillin null mice display defects in focal adhesion signaling, together with reduced cell migration and impaired cell spreading on fibronectin (Hagel *et al.*, 2002). Paxillin is one of the earliest proteins recruited into adhesions at the leading edge of ruffling cells (Laukaitis *et al.*, 2001) and becomes tyrosine phosphorylated after integrin ligation (Burridge *et al.*, 1992). Tyrosine phosphorylation of Paxillin is necessary for focal adhesion formation and the reorganization of the actin cytoskeleton in motile cells (Nakamura *et al.*, 2000). As a scaffold protein, Paxillin recruits several structural and signaling proteins into focal adhesions (reviewed in Turner, 2000).

We have addressed the mechanism through which Crk adapter proteins promote the spreading of colonies of epithelial cells. We report herein that the microinjection of CrkII or CrkL into colonies of epithelial cells promotes the formation of lamellipodia together with relocalization of Paxillin into focal complexes. The association of Crk with Paxillin is important for epithelial cell spreading and correlates with enhanced CrkII/Paxillin/GIT2/β-PIX complex formation in Madin-Darby canine kidney (MDCK) cells overexpressing CrkII. Paxillin mutants that fail to associate with Crk or GIT2, or fail to target to focal adhesions, inhibit Crk-dependent lamellipodia formation and cell spreading. We suggest that the coupling of Crk with Paxillin and their relocalization to focal contacts is important for the remodeling of the actin cytoskeleton and cell spreading, events critical for cell migration and invasion.

MATERIALS AND METHODS

Materials and Antibodies

A polyclonal p130Cas antibody was obtained from Dr. Michel Tremblay (McGill University, Montreal, QC, Canada). Antibodies to p1306 CrkII and Paxillin were purchased from Transduction Laboratories (Lexington, KY). CrkL and Cbl antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HA.11 and c-Myc (9E10) antibodies were obtained from Babco (Richmond, CA). FLAG-M2 antibodies were purchased from Sigma (Oakville, ON, Canada). An antibody raised against PKL, the chicken homolog of GIT2, was described previously (West *et al.*, 2001). Alexa Fluor 488-phalloidin, Texas Red-X-phalloidin, and secondary antibodies conjugated to Alexa Fluor 488 were purchased from Molecular Probes (Eugene, OR). Secondary antibodies conjugated to CY3 were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Human HGF was generously provided by Dr. George Vande Woude (Van Andel Research Institute, Grand Rapids, MI) and human epidermal growth factor (EGF) was purchased from Roche Diagnostics (Laval, QC, Canada). Y27632 was purchased from Calbiochem (La Jolla, CA).

Plasmids

Expression plasmids for CrkI/II and CrkL were obtained from Dr. Bruce Mayer (University of Connecticut Health Center, Farmington,

CT) and Dr. John Groffen (Childrens Hospital of Los Angeles Research Institute, Los Angeles, CA), respectively. pcDNA3-p130Cas, pRK5-mycN17Rac1, and pcdef3-β-PIX plasmids were obtained from Dr. Michel Tremblay (McGill University), Dr. Alan Hall (University College London, London, United Kingdom), and Dr. Arthur Weiss (University of California, San Francisco, CA), respectively. pcDNA3-Paxillin, pcDNA3-Paxillin Y31/118F, pcDNA3-Paxillin Δ263–282 (ΔLD4), pcDNA3-Paxillin Δ444–494 (ΔLIM3), and GFP-PKL expression plasmids were reported previously (Brown *et al.*, 1996; Turner *et al.*, 1999; Petit *et al.*, 2000).

Microinjection

MDCK cells were maintained in DMEM containing 10% fetal bovine serum (FBS) and 50 μg/ml gentamicin (Invitrogen Canada, Burlington, ON, Canada). 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 Chemical, 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 5 h 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. Cell permeabilization with CSK was performed as described previously (Lamorte *et al.*, 2002b). 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. 9E10 antibodies were diluted 1:800, CrkL antibodies were diluted 1:200, and Paxillin and FLAG-M2 antibodies were diluted 1:1000. All secondary antibodies were diluted 1:1000. Alexa Fluor 488-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. Donkey αrabbit antibodies conjugated to Alexa Fluor 488 were used to detect cells injected with rabbit immunoglobulin G. For experiments where monoclonal antibodies were used for costaining, CrkL was used instead of CrkII because the CrkL antibody is polyclonal. This was justified as both CrkII and CrkL promote a similar phenotype when microinjected into MDCK colonies (Lamorte *et al.*, 2002b; Figure 2). 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 an AxioVert 135 microscope (Carl Zeiss Canada, 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 and MDCK cells overexpressing CrkII were grown to ~90% confluence and serum starved for 6 h in DMEM containing 0.02% FBS. Cells were lysed with 1.0% Triton X-100 lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM EGTA, 1.5 mM $MgCl_2$, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 50 mM NaF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Immunoprecipitations and Western blotting were performed as described previously (Fixman *et al.*, 1996).

RESULTS

Paxillin Relocalizes to Focal Contacts in Response to HGF Stimulation or Crk Overexpression

HGF promotes cell spreading through lamellipodia formation, reorganization of the actin cytoskeleton, and the formation of nascent focal complexes within the lamellipodia (Ridley *et al.*, 1995; Royal *et al.*, 2000). To define the requirements for the spreading of colonies of epithelial cells, we examined the changes that occur in response to HGF, which promotes cell spreading when compared with EGF, which fails to do so. In unstimulated MDCK cells, Paxillin was predominantly cytoplasmic (Figure 1A). After stimulation with HGF, a pool of Paxillin accumulated within focal contacts, with the remainder of Paxillin remaining in the cytoplasm, possibly in the Golgi (Figure 1A). At higher magnification, Paxillin is observed within focal complexes in the lamellipodia at the leading edge of colonies (Figure 1B, arrow) and within focal adhesions at the ends of actin stress fibers (Figure 1B, arrowhead). In contrast, EGF failed to promote the spreading of MDCK cells and Paxillin displayed a diffuse distribution within the cytoplasm (Figure 1A), similar to unstimulated MDCK cells (Figure 1A).

We have previously demonstrated that the stable overexpression of CrkII or CrkL in colonies of epithelial cells promotes lamellipodia formation, cell spreading, and breakdown of adherens junctions (Lamorte *et al.*, 2002b). These are similar to the changes that occur after HGF stimulation (Ridley *et al.*, 1995; Royal and Park, 1995; Potempa and Ridley, 1998; Royal *et al.*, 2000; Figure 1). To understand the mechanism involved in Crk-mediated cell spreading, we compared the localization by indirect immunofluorescence of Paxillin and p130Cas, proteins associated with cell spreading and reorganization of the actin cytoskeleton and known to bind CrkII and CrkL (Feller, 2001). As shown above, in unstimulated cells, Paxillin displayed a diffuse cytoplasmic distribution in colonies of epithelial cells (Figure 1A), whereas in cells microinjected with CrkII expression plasmids, a pool of Paxillin relocalized to focal complexes present throughout the cell and within large lamellipodia at the edge of the colony (Figure 2A). Relocalization of Paxillin was also observed in MDCK cells microinjected with CrkL expression plasmids (Figure 2B). In contrast, there was no detectable relocalization of p130Cas to focal complexes in cells microinjected with CrkII (Figure 2C). Moreover, Paxillin failed to relocalize in cells microinjected with p130Cas expression plasmids (Figure 2D), consistent with the inability of p130Cas overexpression to promote cell spreading in MDCK cells (Lamorte *et al.*, 2002b). Hence, the overexpression of CrkII or CrkL, as well as stimulation of colonies of MDCK cells with HGF, promotes the redistribution of Paxillin to focal complexes at the leading edge of spreading cells.

Functional Crk SH2 and SH3 Domains Are Required for Paxillin Relocalization

To define the requirements for Paxillin redistribution in response to Crk, plasmids encoding Crk proteins with a mutation in the SH2 (R38K) or amino-terminal SH3 (W170K) domain were microinjected into MDCK cells. CrkI, an alternatively spliced form of CrkII lacking the carboxy-terminal SH3 domain (Matsuda *et al.*, 1992), promoted cell spreading and Paxillin redistribution to the leading edge (Figure 3). However, mutations within either the SH2 or SH3 domains of CrkI failed to promote cell spreading and Paxillin relocalization (Figure 3). Hence, although the carboxy-terminal SH3 domain of Crk is dispensable for cell spreading and the

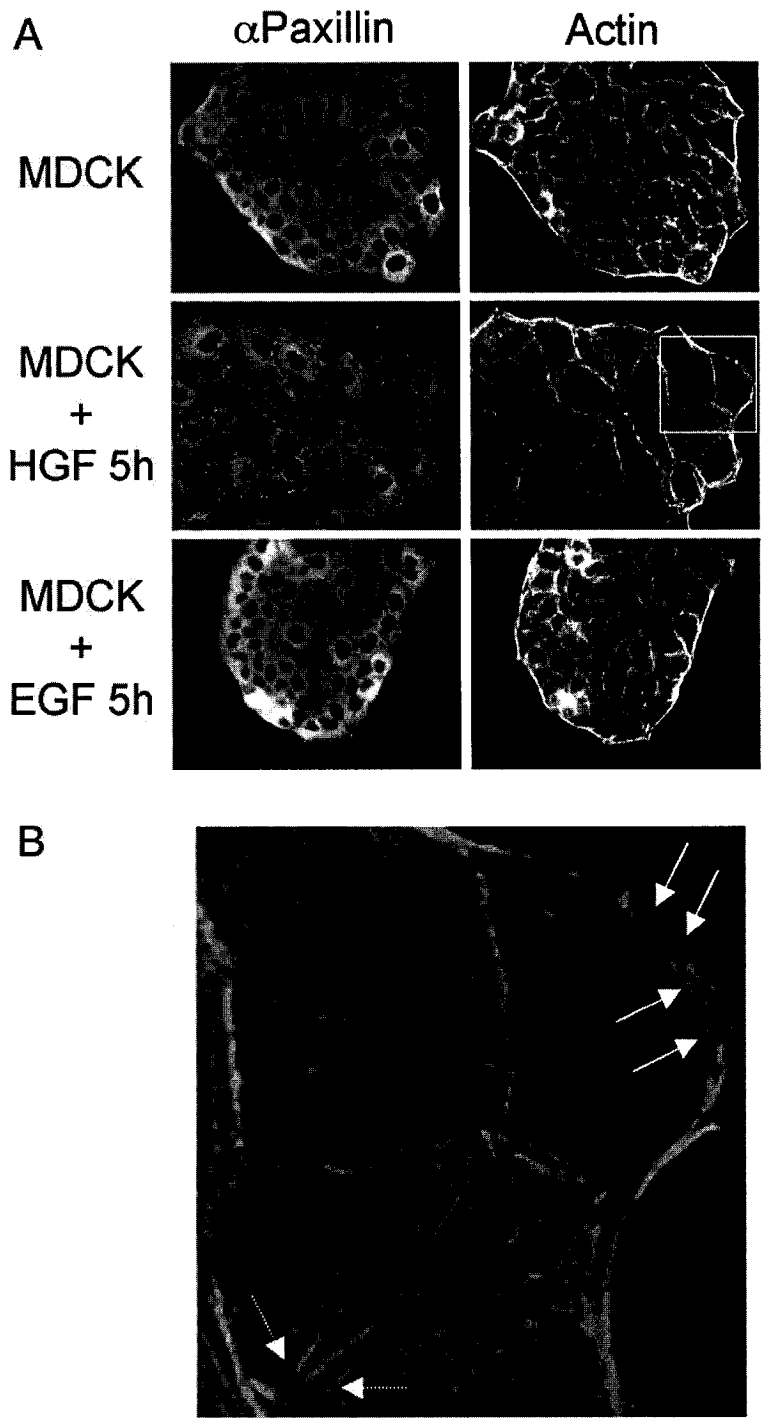

Figure 1. HGF but not EGF promotes the relocalization of Paxillin to lamellipodia and to the ends of actin stress fibers. (A) MDCK cells were left untreated or stimulated with 5 U/ml HGF or 100 ng/ml EGF for 5 h and fixed. Cells were processed for indirect immunofluorescence by using αPaxillin/αmouse-CY3 and phalloidin-Alexa 488. (B) The αPaxillin and Phalloidin staining from the region highlighted in A were merged and enlarged. The solid arrow highlights Paxillin staining present within lamellipodia and the dotted arrow highlights Paxillin staining at the ends of actin stress fibers.

redistribution of Paxillin, both the SH2 and amino-terminal SH3 domains are required.

Crk-stimulated Paxillin Redistribution Is Rac Dependent

The spreading of colonies of epithelial cells in response to HGF requires the coordinated regulation of Rho GTPases and is inhibited by the expression of a mutant Rac1 protein unable to bind guanine nucleotides (N17Rac1) (Ridley *et al.*, 1995; Royal *et al.*, 2000). The involvement of Rac1 in Crk-induced Paxillin relocalization was examined by coinjecting cells with plasmids that express CrkL and dominant negative Rac1 (N17Rac1). Consistent with the ability of N17Rac1 to inhibit Crk-dependent lamellipodia formation and cell

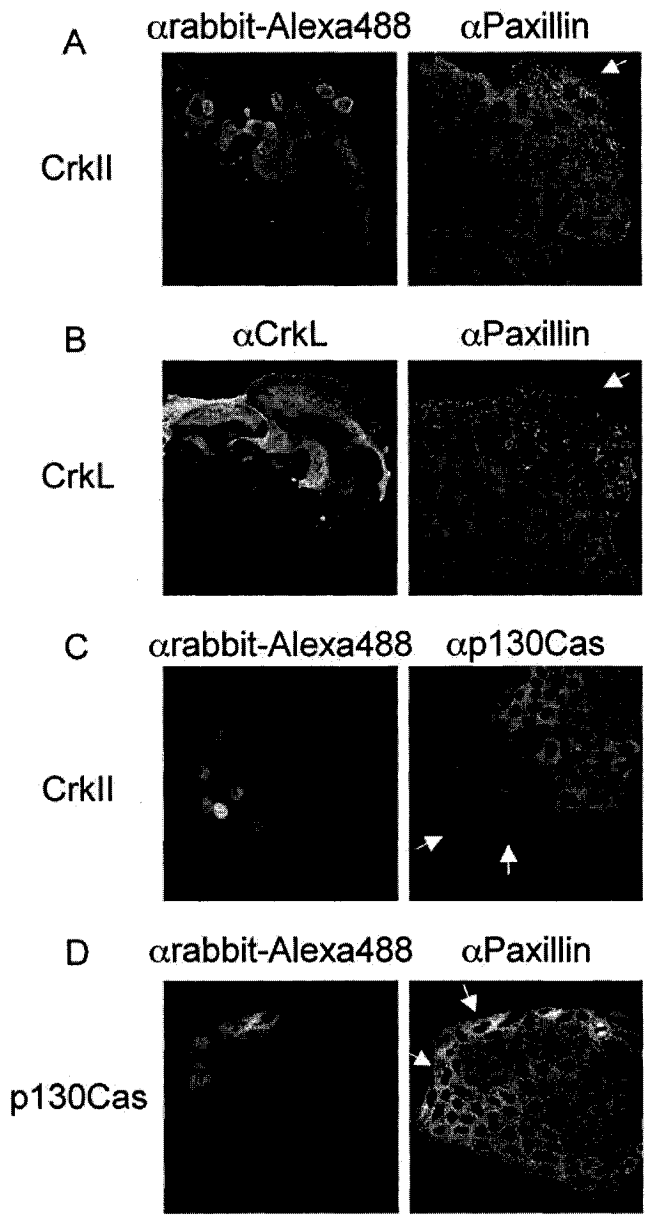

Figure 2. Paxillin but not p130Cas is redistributed in cells microinjected with CrkII or CrkL. (A and B) CrkII expression plasmids (50 ng/μl) and rabbit immunoglobulin G (0.6 μg/μl) (A) or CrkL expression plasmids (50 ng/μl) (B) were microinjected into the nuclei of MDCK cells. Cells were fixed after a 5-h incubation and double stained with αrabbit-Alexa Fluor 488 (A) or αCrkL/αrabbit-Alexa 488 (B) and αPaxillin/αmouse-CY3. (C) The nuclei of MDCK cells were microinjected with CrkII expression plasmids (50 ng/μl) and rabbit immunoglobulin G (0.6 μg/μl) and incubated for 5 h. After fixation, cells were stained with αrabbit-Alexa488 and αp130Cas/αmouse-CY3. (D) The nuclei of MDCK cells were microinjected with p130Cas expression plasmids (100 ng/μl) and rabbit immunoglobulin G (0.6 μg/μl). After a 5-h incubation, cells were fixed and stained with αrabbit-Alexa Fluor 488 and αPaxillin/αmouse-CY3. Arrows indicate microinjected cells.

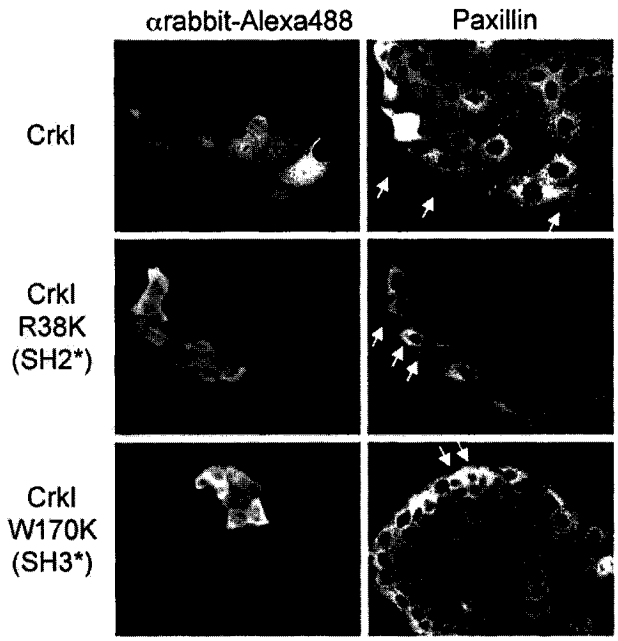

Figure 3. Crk mutants lacking functional SH2 or SH3 domains fail to promote cell spreading and the relocalization of Paxillin to focal complexes. Expression plasmids (100 ng/μl) encoding CrkI, CrkI R38K, and CrkI W170K were microinjected together with rabbit immunoglobulin G (0.6 μg/μl) into the nuclei of MDCK cells. Cells were fixed after a 5-h incubation and double stained with αrabbit-Alexa Fluor 488 and αPaxillin/αmouse-CY3. Arrows indicate microinjected cells.

spreading (Lamorte *et al.*, 2002b; Figure 4), Paxillin failed to relocalize to focal complexes in cells microinjected with CrkL and N17Rac1 (Figure 4).

Although Paxillin redistribution to focal adhesions is RhoA-dependent (Manser *et al.*, 1997), pharmacological in-

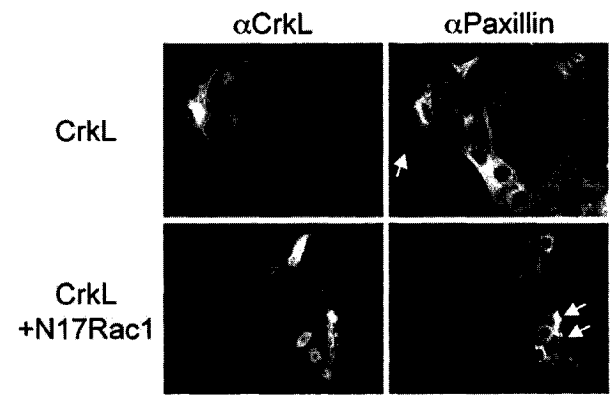

Figure 4. Rac is required for CrkII-induced Paxillin redistribution. CrkL plasmids (50 ng/μl) were coinjected into the nuclei of MDCK cells with vector (20 ng/μl) or N17 Rac1 (20 ng/μl). Cells were fixed after a 5-h incubation and double stained with αCrkL/αrabbit AlexaFluor488 and αPaxillin/αmouse-CY3. Arrows indicate microinjected cells.

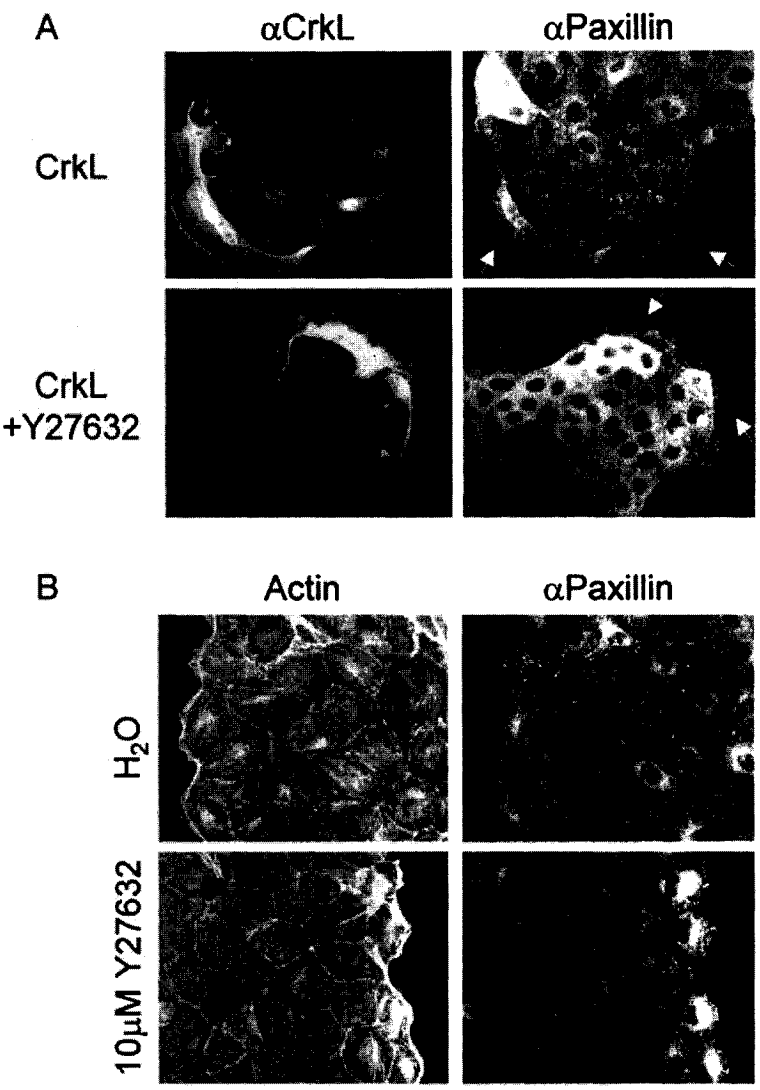

Figure 5. Rho-Kinase is required for HGF-dependent Paxillin relocalization to the ends of actin stress fibers but is dispensable for HGF- and Crk-dependent Paxillin redistribution to focal complexes. (A) CrkL expression plasmids (50 ng/μl) were microinjected into the nuclei of MDCK cells pretreated for 30 min with H_2O or 10 μM Y27632. After a 5-h incubation, cells were fixed and double stained with αCrkL/αrabbit-Alexa488 and αPaxillin/αmouse-CY3. Arrows indicate microinjected cells. (B) MDCK cells were treated with H_2O or 10 μM Y27632 for 30 min before stimulation with 5 U/ml HGF for 5 h. After fixation, cells were processed for indirect immunofluorescence by using αPaxillin/αmouse-CY3 and phalloidin-Alexa 488.

hibition of Rho-Kinase with 10 μM Y27632 (Uehata *et al.*, 1997) did not inhibit CrkL-induced Paxillin relocalization nor did it inhibit the formation of lamellipodia or cell spreading (Figure 5A). Dominant negative mutants of RhoA could not be used because they promote HGF-independent cell spreading and dispersal in MDCK cells (Ridley *et al.*, 1995). Y27632 inhibited HGF-induced actin stress fiber formation (Figure 5B), confirming that Y27632 inhibited Rho-kinase activity. Consistent with the localization of Paxillin to the ends of actin stress fibers in cells stimulated with HGF (Figure 1B), the presence of Paxillin-containing focal adhesions within the interior of HGF-stimulated colonies was significantly reduced in cells treated with Y27632 (Figure 5B). However, Y27632 did not inhibit HGF-stimulated relocalization of Paxillin within lamellipodia in cells at the edge of the colony (Figure 5B).

CrkII Associates with Paxillin/GIT2/β-PIX Complexes upon Overexpression

MDCK cell lines that overexpress CrkII display enhanced cell spreading in the absence of HGF stimulation (Lamorte *et al.*, 2002b; Figure 6A). Moreover, in these cell lines, Paxillin was localized to insoluble complexes within the lamellipodia that are retained after solubilization with CSK buffer (Figure 6A). The Crk SH2 domain and SH3 domains interact with multiple proteins (Feller, 2001). We have previously shown that in MDCK cells, Crk associates with several phosphotyrosine containing proteins, including Paxillin and p130Cas and that its association with these proteins as well as with Cbl and Gab1 are increased after HGF stimulation (Lamorte *et al.*, 2002b). To establish whether the overexpression of CrkII enhanced the coupling of Crk with specific tyrosine phosphorylated proteins, CrkII was immunoprecipitated from MDCK and MDCK cells overexpressing CrkII, and Western blotted with Paxillin, p130Cas, and Cbl antibodies (Figure 6B). Although the binding of Cbl to CrkII was decreased in MDCK cells overexpressing CrkII (Figure 6B), enhanced binding of Paxillin and p130Cas to CrkII was observed in MDCK cells overexpressing CrkII compared with control cells (Figure 6B).

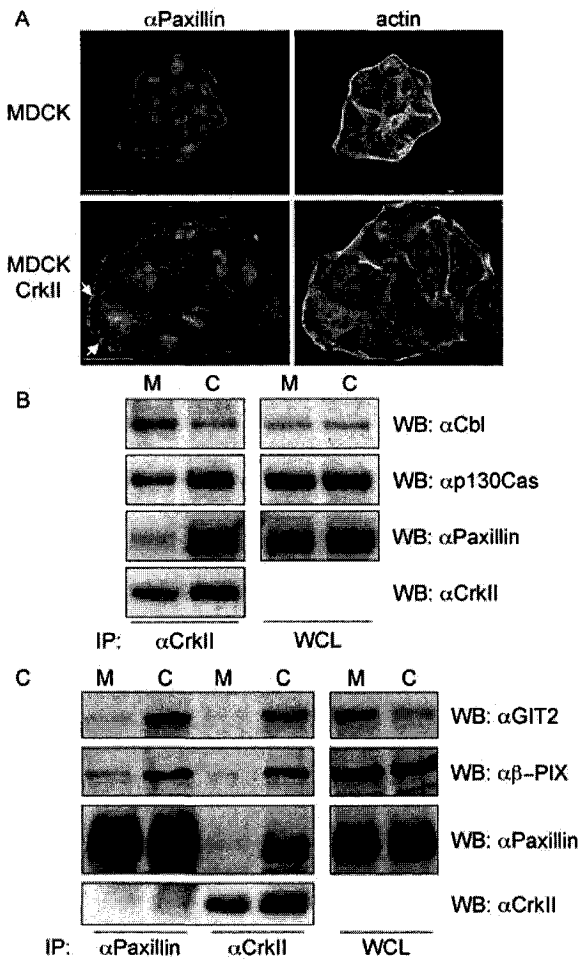

Figure 6. Altered cell morphology in MDCK cells overexpressing CrkII correlates with increased CrkII/Paxillin/GIT2/β-PIX coupling. (A) MDCK and MDCK cells overexpressing CrkII were grown on glass coverslips in DMEM containing 10% FBS for 48 h. Cells were solubilized with 0.25× CSK buffer for 10 min and fixed in formaldehyde. Cells were stained with αPaxillin/αmouse-CY3 and phalloidin-Alexa 488. The bar represents 25 μm. (B and C) MDCK and MDCK cells overexpressing CrkII were serum starved for 6 h and lysed. Cell lysate (2 mg) was used for immunoprecipitation with CrkII or Paxillin antibodies. The immunoprecipitates were washed and associated proteins together with 25 μg of whole cell lysate were resolved by SDS-PAGE. Proteins on the gel were transferred to a nitrocellulose membrane, immunoblotted with αp130Cas, αCbl, αPaxillin, and αCrk. M and C refer to MDCK and MDCK cells overexpressing CrkII, respectively.

The formation of a complex of Paxillin with GIT2 and β-PIX is promoted in a Rac-dependent manner in fibroblasts (Brown *et al.*, 2002). Because both cell spreading and the redistribution of Paxillin in cells microinjected with CrkL is dependent on Rac, we established whether the coupling of GIT2 and β-PIX with Paxillin was enhanced in MDCK cells overexpressing CrkII. The association of Paxillin with GIT2 and β-PIX in MDCK cells overexpressing CrkII was greatly enhanced over the levels of these proteins that coimmunoprecipitated with Paxillin in control MDCK cells (Figure 6C). Consistent with the ability of Crk to bind Paxillin (Birge *et al.*, 1993), the association of Crk with Paxillin, GIT2 and β-PIX was also increased in cells overexpressing CrkII (Figure 6C). This suggests that increased expression of CrkII promotes an increased association of Paxillin with GIT2 and β-PIX.

To establish whether the enhanced assembly of a Crk/Paxillin complex in cells overexpressing CrkII promotes the localization of Crk to focal complexes, MDCK cell colonies were microinjected with CrkL and the colocalization of CrkL with endogenous Paxillin was examined by indirect immunofluorescence. Although the majority of CrkL displayed a diffuse cytoplasmic distribution after microinjection (Figures 2B and 7A), CrkL localized to focal complexes at the edge of the lamellipodia and showed some colocalization with endogenous Paxillin (Figure 7A). Similarly, although no punctate GFP-PKL or β-PIX was observed in cells microinjected with vector (Figure 7, B and C), some colocalization of GFP-PKL with Paxillin (Figure 7B) and β-PIX with CrkL (Figure 7C) was observed in cells microinjected with CrkL expression plasmids. The colocalization of GFP-PKL with Paxillin was specific, because noninjected cells displaying punctate Paxillin localization did not display any staining when visualized with fluorescent excitation filters specific for GFP (Figure 7D).

Paxillin Mutants Impair Crk-dependent Lamellipodia Formation and Cell Spreading

To examine the potential contribution of Paxillin to Crk-mediated lamellipodia formation and cell spreading, Paxillin mutants were coinjected with CrkL into MDCK cells. The ΔLIM3 mutant lacks the LIM3 domain (amino acids 444–494) and displays significantly reduced targeting to focal adhesions (Brown *et al.*, 1996). The Y31/118F mutant contains tyrosine to phenylalanine mutations at residues 31 and 118, which represent Crk SH2 binding sites (Petit *et al.*, 2000). The ΔLD4 mutant lacks the LD4 domain (amino acids 263–282) and fails to bind PKL/GIT2 (Turner *et al.*, 1999). The microinjection of wild-type Paxillin did not impair CrkL-stimulated lamellipodia formation (Figure 8). In contrast, the microinjection of the ΔLIM3, Y31/118F, or ΔLD4 mutants diminished the effects of CrkL on lamellipodia formation and cell spreading (Figure 8) while promoting enhanced membrane ruffling for the Y31/118F and ΔLIM3 mutants (Figure 8). These effects were observed in >50% of injected colonies (Table 1).

DISCUSSION

We have previously demonstrated that the CrkII and CrkL adapter proteins are required for the spreading of epithelial colonies and the breakdown of adherens junctions in response to HGF (Lamorte *et al.*, 2002b). Despite a growing interest in the Crk adapter proteins as modulators of cell spreading and migration, the role of Crk in these processes is not completely defined. The goal of our study was to determine the mechanisms by which Crk adapter proteins regulate these cellular processes. Our results show that in colonies of epithelial cells, Crk promotes the redistribution of a pool of Paxillin from the cytoplasm to focal complexes within developing lamellipodia. Paxillin redistribution and

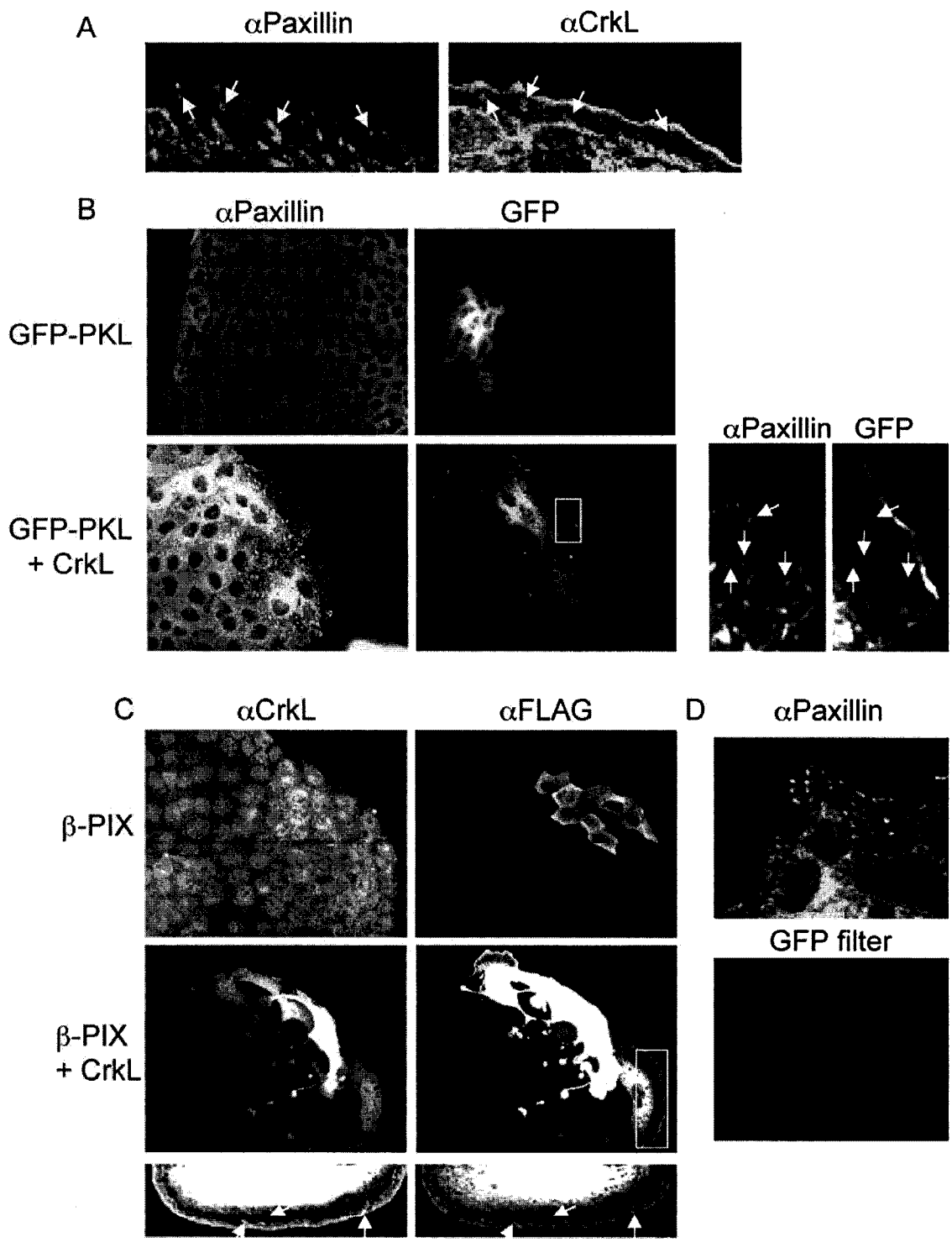

Figure 7. CrkL, Paxillin, PKL, and β-PIX colocalize to focal complexes in cells microinjected with CrkL. (A) The nuclei of MDCK cells were microinjected with CrkL expression plasmids (50 ng/μl), fixed 5 h later, and double stained with αCrkL/αrabbit Alexa Fluor 488 and αPaxillin/αmouse-CY3. (B) The nuclei of MDCK cells were microinjected with GFP-PKL expression plasmids (20 ng/μl) and vector (50 ng/μl) or CrkL expression plasmids (50 ng/μl). After a 5-h incubation, cells were fixed and stained with αPaxillin/αmouse-CY3. (C) The nuclei of MDCK cells were microinjected with FLAG-β-PIX expression plasmids (20 ng/μl) and vector (50 ng/μl) or CrkL expression plasmids (50 ng/μl). After a 5-h incubation, cells were fixed and stained with αFLAG/αmouse-CY3 and αCrkL/αrabbit Alexa Fluor 488. (D) Noninjected cells from B that contained punctate Paxillin staining were excited with a GFP-specific filter and photographed. Arrows indicate colocalization.

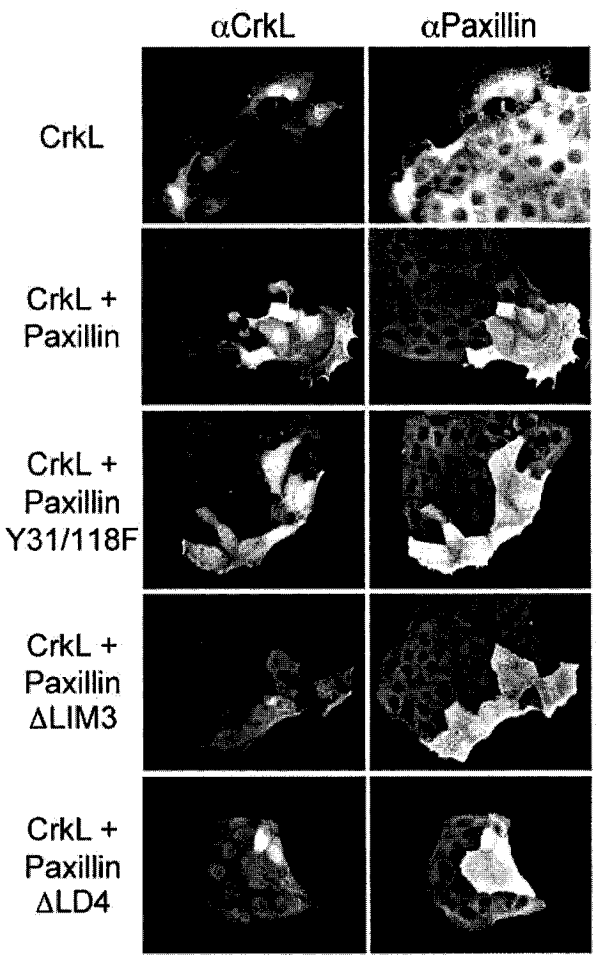

Figure 8. Paxillin mutants lacking Crk SH2-binding sites, the LIM3 domain or the LD4 domain impair Crk-stimulated lamellipodia formation and cell spreading. The nuclei of MDCK cells were microinjected with CrkL expression plasmids (50 ng/μl) and vector (A), wild-type Paxillin (B), PaxillinY31/118F (C), PaxillinΔLIM3 (D), or PaxillinΔLD4 (E), each at 100 ng/μl. Cells were fixed 5 h later and double stained with αCrkL/αrabbit Alexa Fluor 488 and αPaxillin/αmouse-CY3.

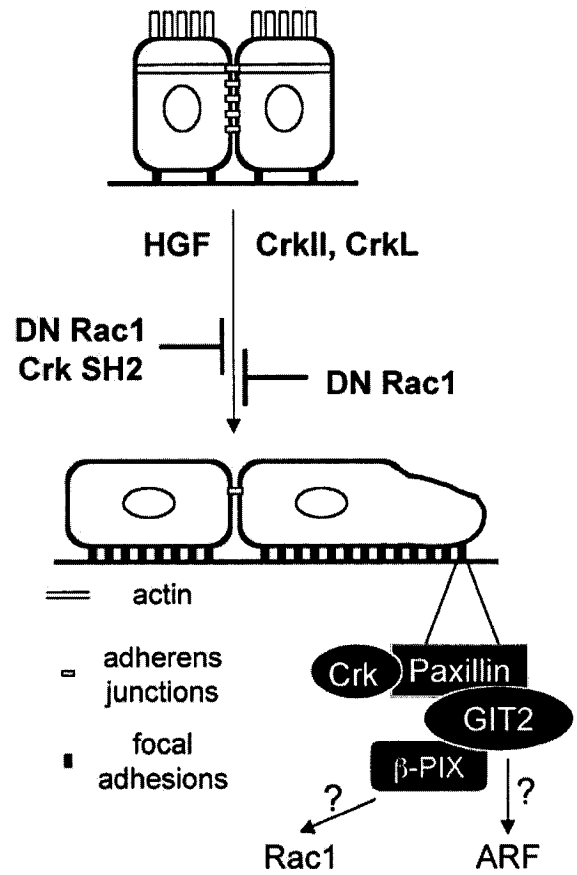

Figure 9. Overexpression of Crk adapter proteins in MDCK cells promotes lamellipodia formation and cell spreading, mirroring the response of cells at the edge of the colony to HGF stimulation. Similarly, overexpression of Crk or stimulation of MDCK cells with HGF promotes the redistribution of Paxillin to focal contacts throughout the cell and within lamellipodia. In cells overexpressing CrkII, the assembly of a Crk/Paxillin/GIT2/β-PIX complex that relocalizes to focal complexes at the leading edge contributes to lamellipodia formation and cell spreading, possibly by influencing the activities of the Rac and ARF GTPases.

the formation of focal complexes is dependent on Rac activity and correlates with an increase in the formation of a multiprotein complex containing Crk and Paxillin, as well as an ARF-GAP, GIT2, and a Rac1 exchange factor, β-PIX (Figure 9). Paxillin mutants that fail to bind Crk or fail to associate with GIT2 inhibit Crk-dependent lamellipodia formation, supporting a role for this multiprotein complex in lamellipodia formation and cell spreading, processes critical for cell migration (Figure 9).

Table 1. Effect of Paxillin on CrkL-dependent lamellipodia

Plasmid	% Lamellipodia and cell spreading	SD
Wt Paxillin	80.6	9.8
Paxillin ΔCrk	37.6	13.3
Paxillin ΔLIM3	44.8	4.5
Paxillin ΔLD4	47.9	5.0

Paxillin plays an important role in focal adhesion signaling (Turner, 2000) and is critical for efficient cell spreading and motility (Hagel *et al.*, 2002). In colonies of epithelial cells, Paxillin is predominantly localized to a cytosolic compartment (Figure 1A). However, unlike Vinculin (Lamorte *et al.*, 2002b), Paxillin is not detected within established focal adhesions present at the edge of the colony (Figure 1A). In response to HGF, Paxillin redistributes to newly forming focal adhesions at the ends of actin stress fibers and to focal complexes within lamellipodia at the leading edge of the colony (Figure 1B). Similarly, the relocalization of Paxillin to membrane ruffles was observed in mIMCD-3 cells in response to HGF (Liu *et al.*, 2002b). In contrast, EGF, which

fails to stimulate the formation of large lamellipodia or the spreading of epithelial cell colonies, fails to promote the redistribution of Paxillin (Figure 1A), demonstrating that the relocalization of Paxillin correlates with cell spreading. Consistent with the ability of the Crk adapter protein to promote lamellipodia formation and cell spreading in colonies of epithelial cells (Lamorte *et al.*, 2002b; Figure 2), the microinjection of Crk expression plasmids promotes the redistribution of Paxillin and Vinculin into focal complexes throughout the cell and within developing lamellipodia (Figure 2; our unpublished data). Noninjected cells surrounding the injected cells also display Paxillin relocalization (Figures 2, 3, and 5), indicating that the Crk-dependent loss of adherens junctions (Lamorte *et al.*, 2002b) would favor the spreading of neighboring cells and subsequently, the redistribution of Paxillin to focal contacts.

Rac but Not Rho-kinase Is Required for Crk-dependent Paxillin Relocalization

There are several distinct classes of cell-matrix adhesions. Focal adhesions localize to the ends of actin stress fibers on the basal surface of the cell and their formation is dependent on RhoA activity (Ridley and Hall, 1992), whereas focal complexes are generally smaller in size, localize within lamellipodia or filopodia, and are Rac1 or Cdc42 dependent, respectively (Nobes and Hall, 1995). Pretreatment of cells with a pharmacological inhibitor of Rho-kinase, Y27632 (Uehata *et al.*, 1997), blocked HGF-stimulated actin stress fiber formation and Paxillin relocalization in cells within the interior of the colony, consistent with a requirement for RhoA activity in Paxillin relocalization and tyrosine phosphorylation (Barry and Critchley, 1994; Manser *et al.*, 1997; Clark *et al.*, 1998). In contrast, Y27632 failed to inhibit the extensive relocalization of Paxillin observed in response to HGF in cells at the periphery of the colony, indicating that HGF-dependent Paxillin relocalization is differentially regulated. Notably, in response to HGF, cells at the edge of the colony develop large lamellipodia that contain Rac-dependent focal complexes (Figure 5B). The pretreatment of cells with Y27632 failed to inhibit Crk-induced lamellipodia formation and Paxillin relocalization to focal complexes (Figure 5A), indicating that pathways downstream of Rho-Kinase are dispensable for these events, implicating a possible role for Rac in Crk-dependent Paxillin relocalization. In support of this, we have previously shown that CrkII overexpression enhances the basal activity of Rac in MDCK cells (Lamorte *et al.*, 2002b). Moreover, dominant negative mutants of Rac1 inhibit Crk-dependent Paxillin relocalization as well as lamellipodia formation and spreading of cells at the edge of the colony (Figure 4; Lamorte *et al.*, 2002b). Hence, the overexpression of Crk mirrors the response of cells at the edge of the colony to HGF, further supporting a role for Crk adapter proteins in HGF-mediated epithelial-mesenchymal transitions.

Enhanced Assembly and Association with CrkII of a Multiprotein Paxillin/GIT2/β-PIX Complex

Using Crk mutant proteins, we have shown that Crk-dependent cell spreading and Paxillin relocalization requires both an intact Crk SH2 domain and an intact amino terminal Crk SH3 domain (Figure 3). This indicates that the association of the Crk SH2 domain with tyrosine phosphorylated proteins and the Crk SH3 domain with proline-rich domain containing proteins is required to initiate signals that promote lamellipodia formation, cell spreading, and Paxillin relocalization. Paxillin that is present within focal adhesions and at the cell periphery is tyrosine phosphorylated at Y31 and Y118 (Nakamura *et al.*, 2000; West *et al.*, 2001). These phosphorylated tyrosine residues form consensus binding sites for the Crk SH2 domain (Petit *et al.*, 2000; Schaller and Schaefer, 2001). Consistent with this, HGF stimulation enhances Crk/Paxillin coupling (Lamorte *et al.*, 2002b). Moreover, in cells overexpressing CrkII, the association of CrkII with Paxillin is enhanced (Figure 6, B and C) and after microinjection, CrkL relocalizes to Paxillin containing focal complexes present within lamellipodia (Figure 7A).

In addition to its ability to associate with Crk, Paxillin acts as a scaffold for other proteins, including GIT2/PKL, a member of the ARF-GAP family (Turner *et al.*, 1999), which also includes GIT1, PAP/PAG3, ASAP1, and ACAP1/2 (Turner *et al.*, 2001). GIT2/PKL binds β-PIX (Turner *et al.*, 1999), a Rac1 exchange factor (Bagrodia *et al.*, 1998; Manser *et al.*, 1998), and β-PIX binds PAK (Bagrodia *et al.*, 1998; Manser *et al.*, 1998). Together, this complex is thought to act in a synergistic manner to recruit PAK to focal complexes (Manser *et al.*, 1998) where it could promote focal complex disassembly (Manser *et al.*, 1997) and participate in Rac-dependent actin reorganization (Obermeier *et al.*, 1998), thereby promoting cell spreading. In support of this, *Drosophila* PAK is involved in dorsal closure, together with Rac1 and Cdc42 (Harden *et al.*, 1996).

We provide evidence that CrkII overexpression enhances the levels of a Paxillin/GIT2/β-PIX complex in cells (Figure 6C) and in turn these proteins localize to focal complexes in cells microinjected with CrkL expression plasmids (Figure 7). Paxillin/GIT2/β-PIX complexes are present within CrkII immunoprecipitates in stable cell lines overexpressing CrkII (Figure 6C), indicating that CrkII associates with this multiprotein complex. Due to poor specificity of available PAK sera, we were unable to detect endogenous PAK within the Paxillin/GIT2/β-PIX complex in MDCK cells overexpressing CrkII. However, from the tight association observed between PAK and β-PIX, we would predict that PAK is recruited to this complex. Because the activation of Rac and Cdc42 enhances the association of PKL with Paxillin (Brown *et al.*, 2002), the enhanced association of the Paxillin/GIT2/β-PIX multiprotein complex in cells overexpressing CrkII is consistent with the elevated levels of Rac activity observed in these cells (Lamorte *et al.*, 2002b). Similarly, V12Rac stimulates the redistribution of a related ARF-GAP, GIT1/APP1, to focal complexes (Zhao *et al.*, 2000; Matafora *et al.*, 2001).

Members of the ARF family of small GTP binding proteins have been implicated in the reorganization of the actin cytoskeleton. ARFs regulate membrane traffic between endosomes and the Golgi (Chavrier and Goud, 1999). Moreover, ARF1 has been reported to mediate the recruitment of Paxillin to focal adhesions in fibroblasts (Norman *et al.*, 1998), and ARF6 promotes the relocalization of Rac1 to the plasma membrane (Radhakrishna *et al.*, 1999; Zhang *et al.*, 1999; Boshans *et al.*, 2000). Several ARF-GAP proteins associate with focal adhesion protein complexes, suggesting that these proteins and their associated ARF GTPases are important regulators of signaling pathways during cell spreading and

migration (de Curtis, 2001). Although dominant negative mutants of ARF1 or ARF6 impaired HGF-stimulated cell spreading, their comicroinjection with Crk failed to inhibit Crk-stimulated cell spreading and Paxillin relocalization (Lamorte and Park, submitted), suggesting that these proteins may act upstream or in a pathway parallel to Crk. Hence, the increased assembly of a Paxillin/GIT2/β-PIX complex after CrkII overexpression, together with the Crk-dependent recruitment of these proteins to focal complexes (Figure 7), supports a role for this complex in Crk-dependent lamellipodia formation and cell spreading. Consistent with this, mutants of Paxillin that fail to associate with Crk (Y31/118F), or GIT2 (ΔLD4), or do not target to focal adhesions (ΔLIM3), impaired CrkL-dependent lamellipodia formation and cell spreading (Figure 8). With the exception of cells microinjected with PaxillinΔLD4, cells microinjected with the other Paxillin mutants displayed elevated membrane ruffling (Figure 8) consistent with Rac activation. Hence, both the association of Crk with Paxillin/GIT2 complexes and the targeting of Crk/Paxillin complexes to focal complexes are required for the ability of Crk to stimulate lamellipodia formation and cell spreading. In a similar manner, expression of a PaxillinY31/118F mutant inhibited the migration of NBT-II bladder carcinoma cells on collagen type I (Petit *et al.*, 2000) and PaxillinΔLD4 inhibited IGF-1-dependent cell spreading and lamellipodia formation (Turner *et al.*, 1999). Moreover, CHO.K1 cells overexpressing PaxillinΔLD4 are defective in directed motility (West *et al.*, 2001), and overexpression of the LD4 motif perturbs directed motility (Turner *et al.*, 1999; Zhao *et al.*, 2000). Thus, the coupling of Crk proteins with Paxillin and the assembly of Paxillin/GIT2/β-PIX complexes may represent an important mechanism for cell spreading and migration, enabling the localization and activation of downstream pathways such as Rac1, sustaining lamellipodia formation and cell spreading. However, additional mechanisms for activating Rac1 and promoting lamellipodia formation, involving p130Cas/Crk and/or Gab1/Crk complexes must exist as not all cells microinjected with the Paxillin mutants failed to promote Crk-dependent lamellipodia formation (Table 1). Moreover, HGF-dependent lamellipodia formation and cell spreading are not inhibited by the microinjection of the different Paxillin mutants (our unpublished data). Thus, the coupling of Crk with Paxillin is dispensable for HGF-dependent cell spreading, suggesting that additional pathways can compensate for the loss of these signals.

The binding of the Crk SH2 domain to Paxillin would enable the recruitment of Crk to Paxillin-containing focal contacts, possibly targeting Crk SH3 binding proteins to focal complexes and promoting localized Rac activation. In support of this, DOCK180, a Crk amino-terminal SH3 binding protein, functions as a two-component Rac1 exchange factor through its interaction with ELMO (Brugnera *et al.*, 2002). Furthermore, the coexpression of p130Cas, CrkII, and DOCK180 promotes the spreading of single cells and the accumulation of these complexes to focal adhesions (Kiyokawa *et al.*, 1998b). We have described the formation of a distinct complex involving Crk/Paxillin/GIT2/β-PIX that may behave similarly (Figure 9). Although CrkII/p130Cas complex formation is enhanced in cells overexpressing CrkII, p130Cas does not detectably relocalize to focal contacts in cells overexpressing Crk (Figure 2C). However, we cannot exclude a role for Crk/p130Cas interactions in lamellipodia formation and cell spreading. Moreover, the ability of CrkII/p130Cas coupling to regulate cell migration and invasion (Klemke *et al.*, 1998; Cho and Klemke, 2000; Spencer *et al.*, 2000) indicates that these complexes may have a similar role in enhancing the invasiveness of MDCK cells (Lamorte *et al.*, 2002a).

In conclusion, our results identify a novel role for Crk in promoting the relocalization of Paxillin to focal complexes. Both Rac activation and the targeting of Crk/Paxillin complexes to focal complexes are essential for lamellipodia formation and cell spreading in cells overexpressing Crk adapter proteins (Figure 9). Recruitment of Paxillin binding proteins, such as GIT2- and GIT2-associated proteins (β-PIX and PAK) to these focal complexes enables lamellipodia formation and cell spreading, possibly through the regulation of Rac and ARF activity (Figure 9). These results provide further insights into the mechanisms involved in the regulation of epithelial-mesenchymal transitions, events critical for tumor cell migration and metastasis.

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