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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

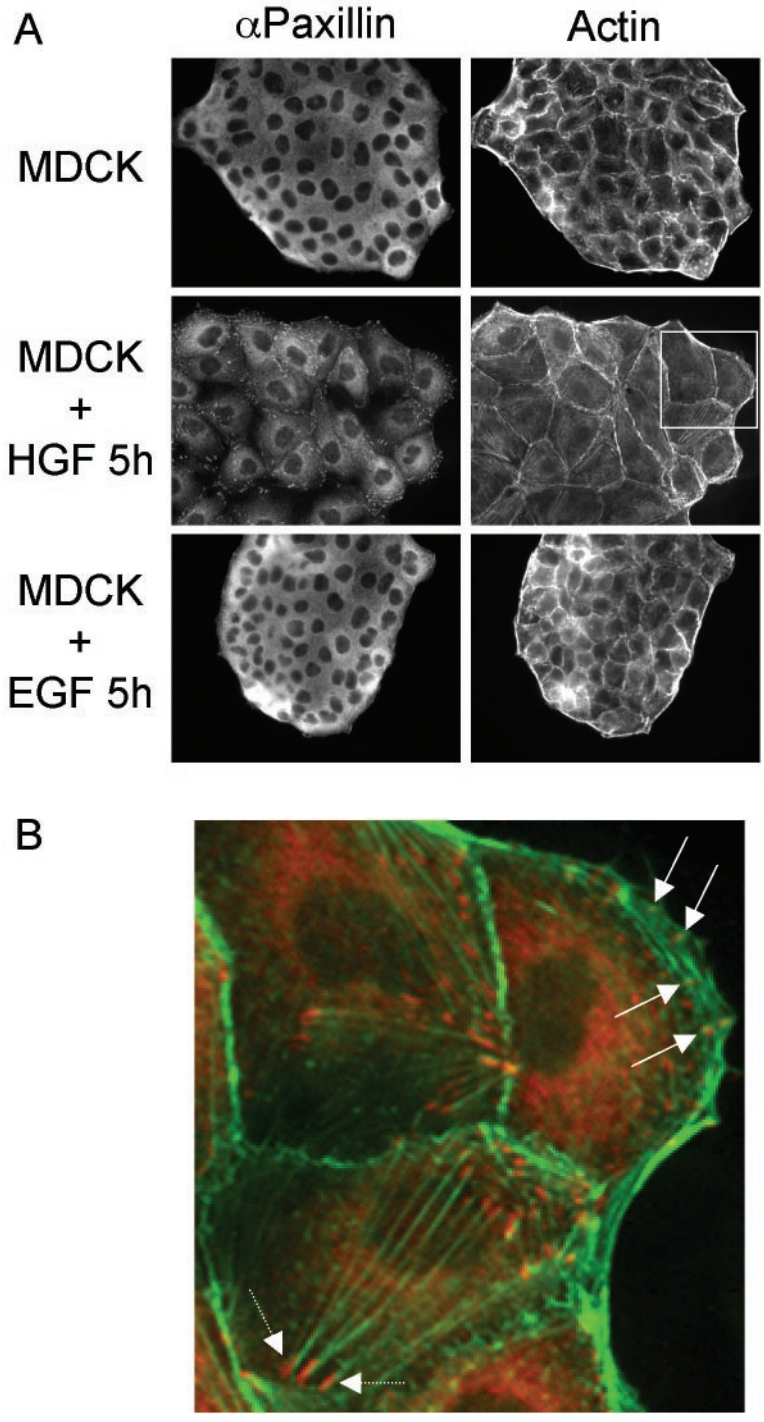

Figure 1. HGF but not EGF promotes the relocalization of Paxillin to lamellipodia and to the ends of actin stress fibers. (A) MDCK cells were left untreated or stimulated with 5 U/ml HGF or 100 ng/ml EGF for 5 h and fixed. Cells were processed for indirect immunofluorescence by using αPaxillin/αmouse-CY3 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

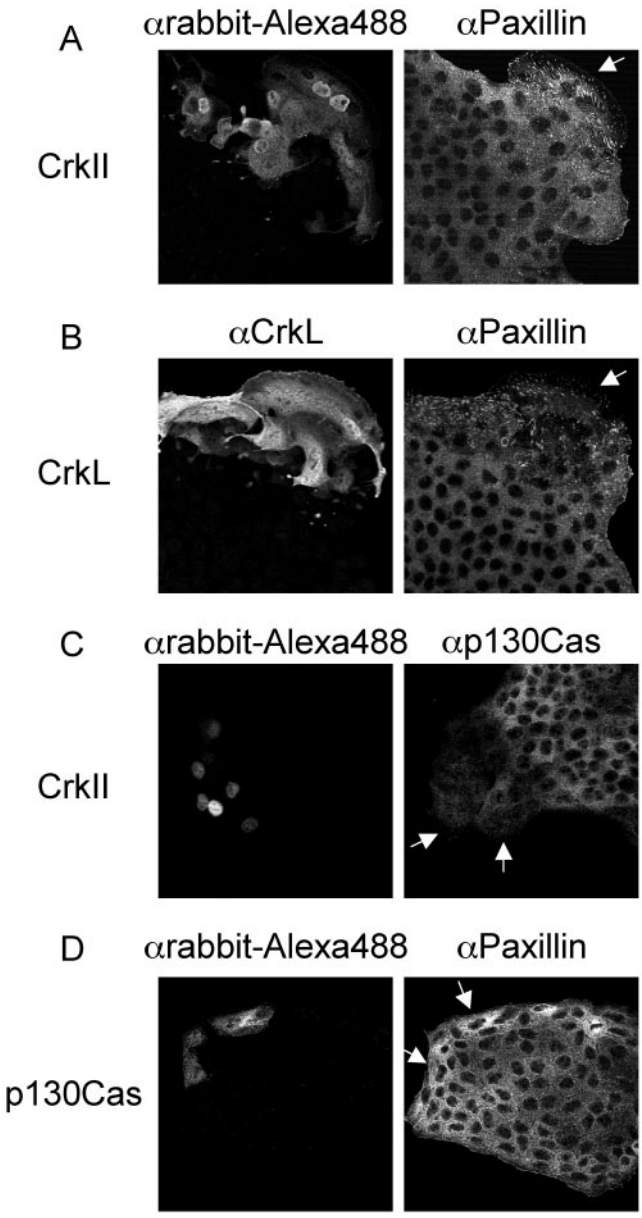

Figure 2. Paxillin but not p130Cas is redistributed in cells microinjected with CrkII or CrkL. (A and B) CrkII expression plasmids (50 ng/μl) and rabbit immunoglobulin G (0.6 μg/μl) (A) or CrkL expression plasmids (50 ng/μl) (B) were microinjected into the nuclei of MDCK cells. Cells were fixed after 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.

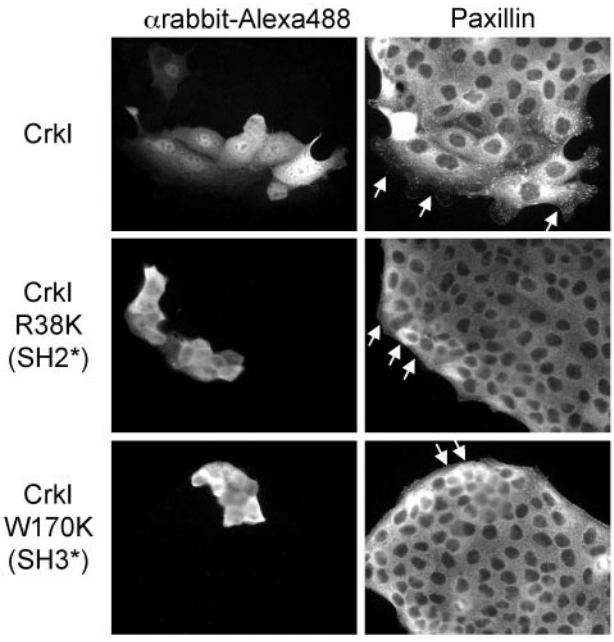

Figure 3. Crk mutants lacking functional SH2 or SH3 domains fail to promote cell spreading and the relocalization of Paxillin to focal complexes. Expression plasmids (100 ng/μl) encoding CrkI, CrkI R38K, and CrkI W170K were microinjected together with rabbit immunoglobulin G (0.6 μg/μl) into the nuclei 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-

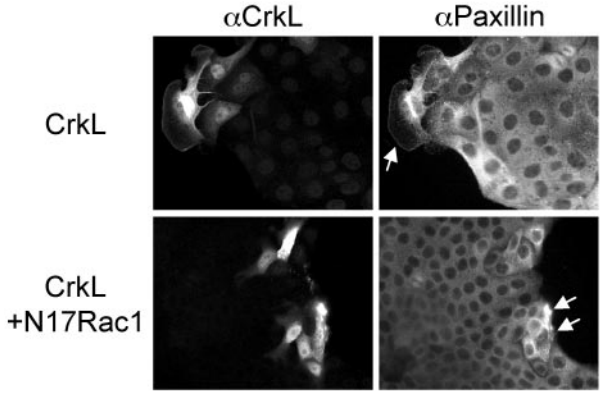

Figure 4. Rac is required for CrkII-induced Paxillin redistribution. CrkL plasmids (50 ng/μl) were coinjected into the nuclei of MDCK cells with vector (20 ng/μl) or N17 Rac1 (20 ng/μl). Cells were fixed after a 5-h incubation and double stained with αCrkL/αrabbit AlexaFluor488 and αPaxillin/αmouse-CY3.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

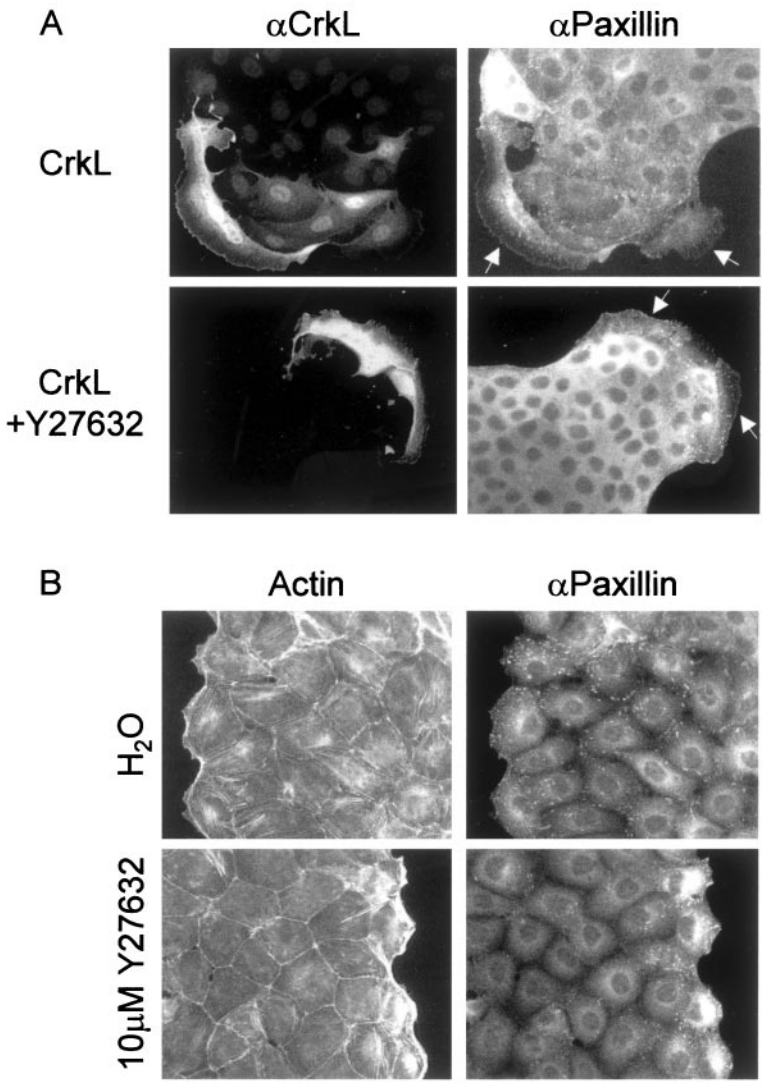

Figure 5. Rho-Kinase is required for HGF-dependent Paxillin relocalization to the ends of actin stress fibers but is dispensable for HGF- and Crk-dependent Paxillin redistribution to focal complexes. (A) CrkL expression plasmids (50 ng/μl) were microinjected into the nuclei of MDCK cells pretreated for 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).

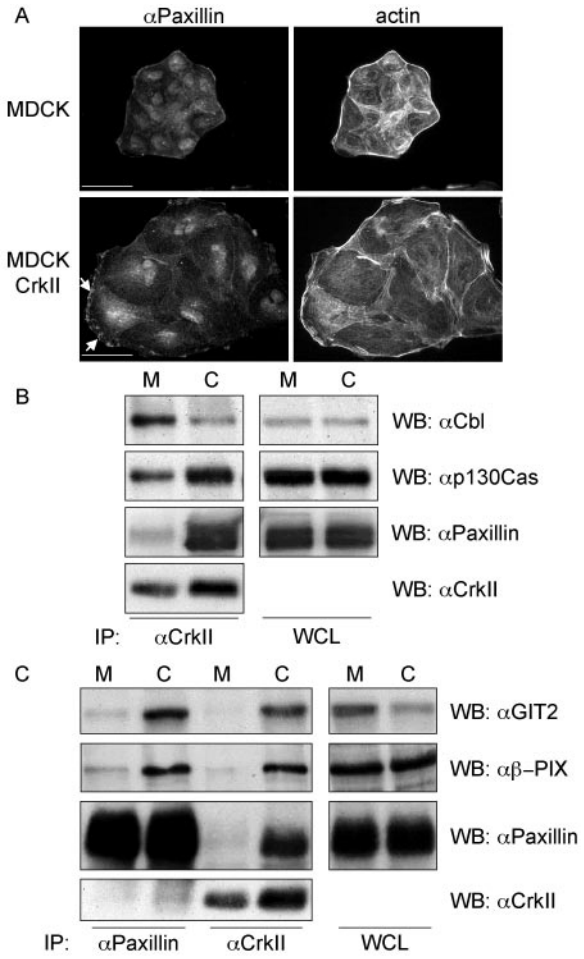

Figure 6. Altered cell morphology in MDCK cells overexpressing CrkII correlates with increased CrkII/Paxillin/GIT2/β-PIX coupling. (A) MDCK and MDCK cells overexpressing CrkII were grown on glass coverslips in DMEM containing 10% FBS for 48 h. Cells were solubilized with 0.25× CSK buffer for 10 min and 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

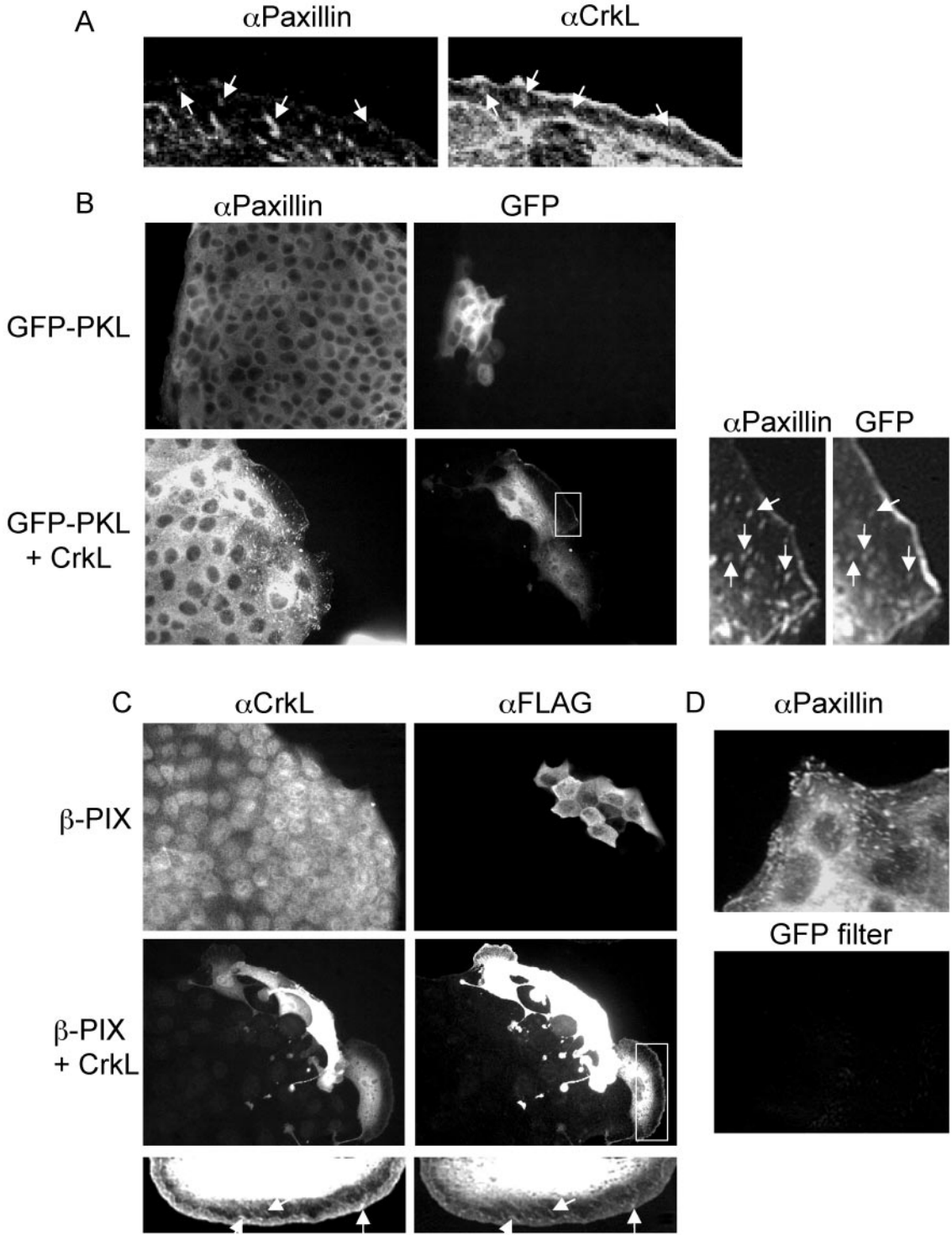

Figure 7. CrkL, Paxillin, PKL, and β-PIX colocalize to focal complexes in cells microinjected with CrkL. (A) The nuclei of MDCK cells were microinjected with CrkL expression plasmids (50 ng/μl), fixed 5 h later, and double stained with αCrkL/αrabbit Alexa Fluor 488 and αPaxillin/αmouse-CY3. (B) The nuclei 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.

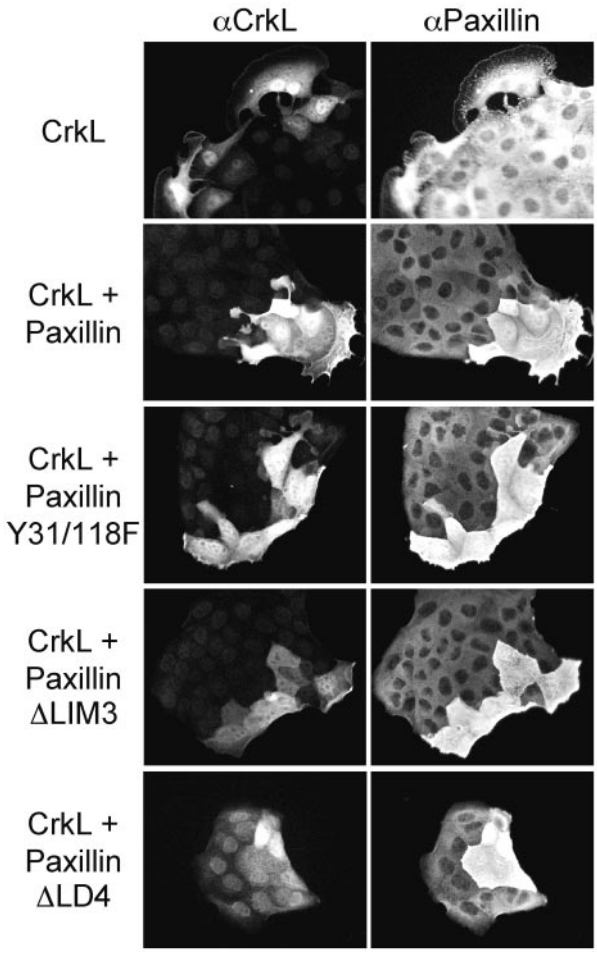

Figure 8. Paxillin mutants lacking Crk SH2-binding sites, the LIM3 domain or the LD4 domain impair Crk-stimulated lamellipodia formation and cell spreading. The nuclei of MDCK cells were microinjected with CrkL expression plasmids (50 ng/μl) and vector (A), wild-type Paxillin (B), PaxillinY31/118F (C), 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.

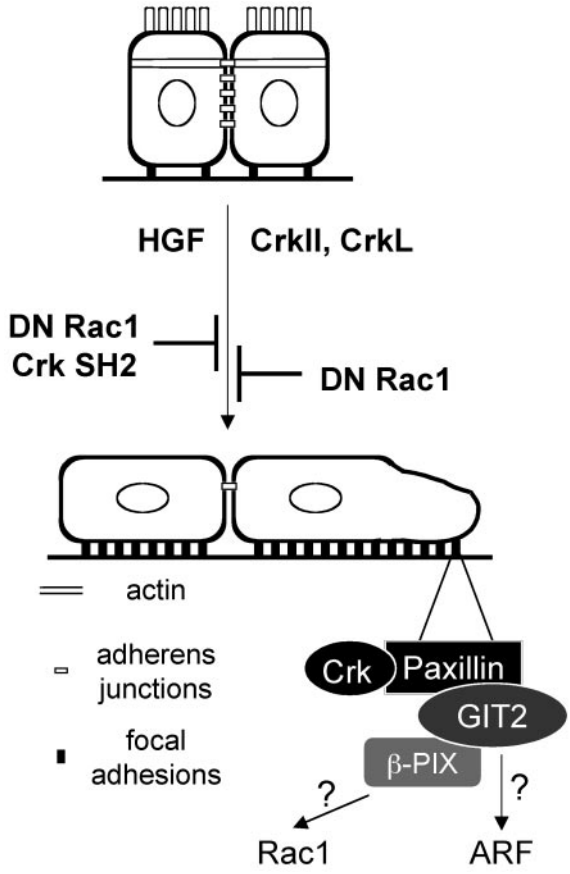

Figure 9. Overexpression of Crk adapter proteins in MDCK cells promotes lamellipodia formation and cell spreading, mirroring the response of cells at the edge of the colony to HGF stimulation. Similarly, overexpression of Crk or stimulation of MDCK cells with HGF promotes the redistribution of Paxillin 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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