Tyrosine 221 in Crk regulates adhesion-dependent membrane localization of Crk and Rac and activation of Rac signaling

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The adaptor protein CrkII plays a central role in signal transduction cascades downstream of a number of different stimuli. We and others have previously shown that CrkII mediates attachment-induced JNK activation, membrane ruffling and cell motility in a Rac-dependent manner. We report here that cell attachment leads to tyrosine phosphorylation of CrkII on Y221, and that CrkII-Y221F mutant demonstrates enhanced association with the Crk-binding partners C3G and paxillin. Despite this enhanced signaling complex formation, CrkII-Y221F fails to induce JNK and PAK activation, membrane ruffling and cell migration, suggesting that it is defective in activating Rac signaling. Wild-type CrkII has no effect on adhesion-induced GTP loading of Rac, but its expression results in enhanced membrane localization of Rac, which is known to be required for Rac signaling. In contrast, CrkII-Y221F is deficient in enhancing membrane localization of Rac. Mutations in Rac and CrkII-Y221F that force membrane targeting of these molecules restore Rac signaling in adherent cells. Together, these results indicate that the Y221 site in CrkII regulates Rac membrane translocation upon cell adhesion, which is necessary for activation of downstream Rac signaling pathways.

Keywords: adhesion/Crk/integrin/Rac/Rho-family GTPase

Introduction

The Crk family of adaptor proteins, which include CrkI, CrkII and CrkL, function in signal transduction by mediating the timely formation of multi-protein signaling complexes upon a variety of extracellular stimuli. Crk proteins consist predominantly of Src homology 2 (SH2) and SH3 domains, with no obvious catalytic domains (Matsuda and Kurata, 1996). The N-terminal SH3 domain of the widely expressed CrkII interacts with several proteins that share a P-x-x-P-x-K binding motif, including the tyrosine kinase Abl, the guanine nucleotide exchange factors C3G and Sos, the Ste20-like kinase HPK1, c-Jun N-terminal kinase (JNK) and DOCK180 (Feller, 2001; Girardin and Yaniv, 2001). To date, no binding partner for the C-terminal SH3 domain of CrkII has been identified. The preferred consensus sequence for the Crk SH2 domain, pY-x-x-P, is found in a number of cytoplasmic signaling molecules, such as Cas, Cbl, paxillin and Gab1,

all of which have been shown to bind to Crk upon stimuli-induced tyrosine phosphorylation. The SH2 domain of Crk also binds directly to several tyrosine-phosphorylated growth factor receptors, such as epidermal growth factor receptor, platelet-derived growth factor receptor and insulin receptor (Feller, 2001).

The pY-x-x-P binding motif is also found in the 'spacer region' between the N- and C-terminal SH3 domains within CrkII (Y221) and CrkL (Y207). Several lines of evidence suggest that phosphorylation of Crk at this site leads to an intramolecular association between the SH2 domain of Crk and the phosphorylated tyrosine residue, and that this intramolecular folding negatively regulates Crk association with other proteins. Purified Abl kinase has been shown to phosphorylate CrkII on Y221, and to prevent the SH2 and SH3 domains of CrkII from interacting with their binding partners (Feller *et al.*, 1994). Epidermal growth factor, insulin and nerve growth factor stimulation of certain cells also leads to tyrosine phosphorylation of CrkII, which correlates with dissociation of CrkII from its SH2 and SH3 binding partners (Beitner-Johnson and LeRoith, 1995; Khwaja *et al.*, 1996; Ribon and Saltiel, 1996; Blakesley *et al.*, 1997; Hashimoto *et al.*, 1998; Okada *et al.*, 1998). Furthermore, NMR studies have demonstrated that the SH2 domain of purified CrkII binds to phosphorylated Y221 residue (Rosen *et al.*, 1995). Taken together, two different outcomes with respect to Crk signaling could occur after activation of intracellular tyrosine kinases. On one hand, binding of Crk through its SH2 domain to tyrosine-phosphorylated signaling proteins probably leads to active transmission of signals. On the other hand, tyrosine phosphorylation of CrkII on Y221 leads to dissociation of these complexes and downregulation of Crk signaling. It has been hypothesized that both of these events can take place in temporal succession, first an active signal, followed by downregulation of the signal by Crk folding, resulting in transient activation peak and subsequent desensitization of the Crk signaling pathway (Feller, 2001).

The emerging paradigm from both *in vitro* and *in vivo* studies indicates an important conserved function for Crk in biological processes ranging from cytoskeletal dynamics, cell migration and phagocytosis to activation of mitogen-activated protein kinases (MAPKs). In tissue culture cells, expression of wild-type CrkII enhances, and dominant-negative forms of CrkII inhibit, stimuli-induced lamellipodia formation, cell migration, phagocytosis and activation of the MAPK JNK (Dolfi *et al.*, 1998; Klemke *et al.*, 1998; Cheresh *et al.*, 1999; Tosello-Tramont *et al.*, 2001). The stimulatory effects of CrkII can be blocked by a dominant-negative form of Rac, a member of the Rho-family GTPases, suggesting that CrkII acts upstream of Rac pathways (Dolfi *et al.*, 1998; Klemke *et al.*, 1998; Tosello-Tramont *et al.*, 2001). Genetic studies in

Fig. 1. Adhesion-dependent tyrosine phosphorylation of CrkII on Y221. (A) Serum-starved COS-7 cells were either held in suspension for 1 h and lysed (S) or held in suspension for 1 h and plated on FN-coated dishes for the indicated periods of time (in minutes), and cell lysates were immunoprecipitated with anti-Crk antibody, followed by immunoblotting with anti-phosphotyrosine antibody ('anti-pY', upper panel). The membranes were stripped and reprobed with anti-Crk antibodies (lower panel). (B) Lysates prepared from cells that had been either held in suspension as above or plated on FN for 30 min were subjected to immunoprecipitation with anti-pY221 antibody, which recognizes CrkII that is phosphorylated on Y221, followed by immunoblotting with anti-Crk antibodies (upper panel). Total cell lysate samples were immunoblotted with anti-Crk antibodies to ensure that equal amounts of CrkII protein were used for immunoprecipitation (lower panel).

Caenorhabditis elegans have confirmed an evolutionary conserved pathway leading from Crk (*C.elegans* CED-2) and its binding partner DOCK180 (CED-5) via Rac (CED-10) to regulation of actin cytoskeleton changes and processes such as cell motility and phagocytosis (Reddien and Horvitz, 2000; Gumienny *et al.*, 2001; Lundquist *et al.*, 2001; Reddien *et al.*, 2001). At present, it remains unclear how Crk and DOCK180 regulate Rac signaling. DOCK180 has been shown to directly interact with Rac and to enhance Rac GTP loading when expressed in cells, but neither Crk nor DOCK180 is known to possess exchange factor activity for Rac (Kiyokawa *et al.*, 1998).

Similar to other GTPases, Rac is active when bound to GTP, which allows Rac to interact with a host of effectors, depending on the signal and cell type (Hall, 1998). Recent studies have shown that in addition to GTP loading of Rac, appropriate subcellular localization of Rac is necessary for proper activation of downstream pathways. del Pozo *et al.* (2000) reported that in order for Rac to activate the Ste20-like kinase PAK, activated Rac needs to translocate to the membrane. While the precise mechanism for Rac translocation remains to be determined, del Pozo *et al.* concluded that integrin-mediated cell adhesion is required for this process. Our studies reported here provide evidence for a novel adhesion-dependent mechanism of regulation of Rac signaling, i.e. phosphorylation of the adaptor protein CrkII on tyrosine. Our results indicate that phosphorylation of CrkII on Y221 upon cell attachment has a crucial role in recruiting not only CrkII, but also Rac, to the cell membrane compartments, which is necessary for activation of downstream Rac signaling pathways.

Results

Cell adhesion on fibronectin leads to tyrosine phosphorylation of CrkII on Y221

Integrin-mediated cell adhesion leads to activation of intracellular signaling pathways and to an increase in tyrosine phosphorylation of a number of signaling proteins, including the Crk-binding proteins Cas and paxillin (Burrige *et al.*, 1992; Nojima *et al.*, 1995; Petch

et al., 1995; Vuori and Ruoslahti, 1995; Harte *et al.*, 1996). We sought to determine whether cell adhesion also regulates tyrosine phosphorylation of Crk. To this end, serum-starved COS-7 cells were either held in suspension or plated on fibronectin (FN) for the indicated time periods, and cell lysates were subjected to anti-Crk immunoprecipitation, followed by anti-phosphotyrosine immunoblot analysis (Figure 1A). When immunoprecipitated from suspended cells, CrkII exhibited a very low level of tyrosine phosphorylation. CrkII became tyrosine phosphorylated within 15 min of cell attachment to FN, and its tyrosine phosphorylation remained stable for at least 1 h in FN-adherent cells. Sequential immunoprecipitation, dissociation of the immunocomplexes and reprecipitation with anti-Crk antibodies, followed by anti-phosphotyrosine immunoblot analysis was carried out to confirm that the phosphorylated 42 kDa protein was CrkII, rather than a co-precipitating tyrosine-phosphorylated protein (data not shown). Cell adhesion to polylysine, to which cells adhere in an integrin-independent manner, did not induce tyrosine phosphorylation of CrkII (data not shown).

Several extracellular stimuli are known to induce CrkII tyrosine phosphorylation on Y221 (Hashimoto *et al.*, 1998; Escalante *et al.*, 2000; Kain and Klemke, 2001), and we investigated whether this is also the case for adhesion-induced tyrosine phosphorylation of CrkII. Serum-starved COS-7 cells were either kept in suspension or plated on FN for 30 min, and cell lysates were immunoprecipitated with an antibody that specifically recognizes CrkII phosphorylated on Y221 (Hashimoto *et al.*, 1998), followed by immunoblotting with anti-Crk antibody. As shown in Figure 1B, anti-pY221 antibody immunoprecipitated CrkII in FN-adherent, but not in suspended cells. Similar results were obtained in a reverse experiment, in which immunoprecipitation was carried out by anti-Crk antibodies, followed by immunoblotting with anti-pY221 antibodies (data not shown).

Complex formation between Crk and its SH2- and SH3-binding partners is regulated by CrkII Y221 phosphorylation in FN-adherent cells

Phosphorylation of CrkII on Y221 is thought to lead to an intramolecular association with its own SH2 domain, preventing the SH2 and SH3 domains from interacting with their cognate binding partners (see Introduction). In order to examine whether adhesion-induced tyrosine phosphorylation of CrkII on Y221 affects CrkII binding to its target proteins, plasmids encoding wild-type CrkII and CrkII with a conservative tyrosine to phenylalanine mutation in Y221 (CrkII-Y221F) were transiently expressed in COS-7 cells. The two proteins were expressed equally well, at about the same level as the endogenous CrkII protein in the experiments (data not shown). Serum-starved transfected cells were either held in suspension or plated on FN for the indicated time periods, and the tyrosine phosphorylation status of the expressed proteins and their interactions with the various binding partners were examined. As shown in Figure 2A, transfected wild-type CrkII exhibited a tyrosine phosphorylation pattern that was similar to that observed for the endogenous CrkII molecule in Figure 1. The CrkII-Y221F mutant in turn failed to undergo any detectable tyrosine phosphorylation upon cell attachment. Wild-type CrkII also demonstrated

Fig. 2. CrkII-Y221F mutant demonstrates enhanced interaction with paxillin. (A) COS-7 cells transfected with 3 μ g of either wild-type CrkII or CrkII-Y221F mutant were serum starved and either held in suspension (S) for 1 h or held in suspension for 1 h and plated on FN-coated dishes for the indicated periods of time. The cells were lysed and immunoprecipitated with anti-Crk antibody, followed by immunoblotting with anti-phosphotyrosine, anti-paxillin and anti-Crk antibodies. (B) Quantitative analysis of the interactions between CrkII and paxillin was carried out as described in Materials and methods. Interaction observed in wild-type CrkII-expressing cells in suspension was arbitrarily defined as '1'. * $P < 0.01$ between wild-type CrkII and CrkII-Y221F samples in suspension and at 15 and 30 min time points; ** $P < 0.05$ between wild-type CrkII and CrkII-Y221F samples at 60 min time point. Results are shown for experiments independently carried out three times.

binding to paxillin in an adhesion-dependent manner, as reported previously (Buensuceso and O'Toole, 2000; Schaller and Schaefer, 2001). Compared with wild-type CrkII, the CrkII-Y221F mutant demonstrated an ~2.5-fold increase in its interactions with paxillin in FN-adherent cells (Figure 2). CrkII-Y221F also displayed a slightly enhanced, ~1.5-fold, binding to C3G compared with wild-type CrkII (data not shown). Taken together, these studies suggest that Y221 is the major, and possibly the exclusive, site in CrkII that becomes tyrosine phosphorylated in response to cell attachment. These data also support the notion that tyrosine phosphorylation of CrkII on Y221 negatively regulates interactions of CrkII with its SH2- and SH3-binding partners, and demonstrate that a conservative tyrosine to phenylalanine mutation on Y221 enhances multi-protein complex formation by CrkII in FN-adherent cells.

Functional consequences of CrkII Y221 phosphorylation upon cell attachment

Based on the biochemical data obtained above, we hypothesized that tyrosine-phosphorylated CrkII probably represents a functionally inactive, and the CrkII-Y221F mutant an 'activated' form of CrkII. We and others have previously shown that adhesion-induced JNK activation, membrane ruffling and haptotactic cell migration are mediated via Crk in a Rac-dependent manner (Dolfi *et al.*, 1998; Klemke *et al.*, 1998). In order to examine the functional significance of adhesion-induced tyrosine phosphorylation of CrkII, we examined the effect of transient expression of CrkII-Y221F on JNK and PAK activation and haptotactic migration upon cell attachment on FN.

As shown in Figure 3A and as published previously, cell attachment on FN leads to an ~2-fold increase in JNK kinase activity towards glutathione *S*-transferase (GST)-c-jun, which was used as a specific substrate for JNK. Transient expression of wild-type CrkII led to a dose-dependent increase in JNK activity with maximum enhancement being observed when 500 ng of CrkII were used for transfection. Surprisingly, the CrkII-Y221F mutant failed to induce JNK activity above background; in fact, it appeared to inhibit adhesion-induced JNK activation in a dose-dependent manner with maximum inhibition observed when 1 μ g of CrkII-Y221F was transfected. MTT assay demonstrated that transient transfection of CrkII-Y221F did not have an adverse effect on cell survival, excluding the possibility that toxicity would contribute to the lack of JNK activity in the cells (data not shown).

Similar findings were obtained when the activity of another kinase, PAK, was monitored. PAK is an effector of activated Rac, and mediates Rac-induced JNK activation (Bagrodia *et al.*, 1995; Brown *et al.*, 1996). Thus, transient co-expression of wild-type CrkII and PAK reproducibly led to a dose-dependent increase in myelin basic protein (MBP) phosphorylation by PAK with maximum activation seen with 500 ng of transfected CrkII (Figure 3B). The CrkII-Y221F mutant in turn failed to induce PAK activity towards MBP above background; rather, it again inhibited adhesion-induced PAK activation when expressed at high concentrations.

We next examined the capability of CrkII to modulate haptotactic cell migration. As reported previously, transient expression of wild-type CrkII was found to lead to an increase in haptotactic cell migration towards FN in a modified Boyden Chamber assay (Figure 3C). Expression of CrkII-Y221F mutant in turn failed to enhance haptotactic cell migration at all transfection concentrations and assay times tested. Similar findings were obtained when the capability of CrkII to enhance adhesion-induced membrane ruffling and lamellipodia formation was examined. In these experiments, COS-7 cells expressing wild-type CrkII or CrkII-Y221F mutant were allowed to adhere on FN, and phalloidin staining was used to visualize the actin cytoskeleton. As reported previously (Dolfi *et al.*, 1998; Klemke *et al.*, 1998), ~50–60% of the cells expressing CrkII displayed characteristics of a migratory phenotype, such as enhanced membrane ruffling and enhanced lamellipodia formation (data not shown). Cells expressing CrkII-Y221F in turn did not contain any detectable membrane ruffles or lamellipodia in the cell

Fig. 3. CrkII-Y221F mutant negatively regulates Crk signaling upon cell attachment. COS-7 cells were transiently transfected with the indicated amounts (in μg) of wild-type CrkII or CrkII-Y221F mutant, together with 0.25 μg of HA-tagged JNK (A) or myc-tagged PAK (B). The cells were then held in suspension (white bar) for 5 min [in (A)] or for 3 h [in (B)] and then plated on FN (black bars) for 15 min. Kinase assays, densitometric and statistical analysis were carried out as described in Materials and methods. * $P < 0.01$ between the indicated samples and HA-JNK/Myc-PAK alone-transfected cells plated on FN; ** $P < 0.005$ between the indicated samples and HA-JNK/Myc-PAK alone-transfected cells plated on FN. (C) COS-7 cells were transiently transfected with 0.5 μg of GFP ('Control'), wild-type GFP-CrkII or GFP-CrkII-Y221F constructs. The cells were subjected to a haptotactic migration assay on FN as described in Materials and methods. The cells that had migrated to the underside of the Transwell membrane were fixed, and GFP-positive cells were visualized and photographed. At least six different fields were counted per membrane and the results were normalized to the transfection efficiency. In (A) and (B), results are shown for experiments independently carried out three times. In (C), bars indicate SD in a representative experiment carried out in triplicate.

periphery, and when transfected at high concentrations, CrkII-Y221F significantly decreased cell spreading compared with control-transfected cells (data not shown). In summary, these results indicate that the CrkII-Y221F mutant, despite enhanced complex formation with Crk SH2- and SH3-binding proteins, fails to enhance Crk-dependent biochemical and biological signaling events, such as activation of JNK and PAK kinases and cell migration. Instead, CrkII-Y221F appears to function as a dominant-negative molecule when expressed at high concentrations and to inhibit Crk-dependent intracellular signaling.

Effect of wild-type CrkII and CrkII-Y221F on Rac activity and Rac localization

An explanation for the results obtained above could be that the CrkII-Y221F mutant, despite enhanced signaling complex formation, might somehow be deficient in activating Rac. As a corollary of this, phosphorylation of CrkII on Y221 might have a previously unidentified crucial role in regulating Rac activity. In order for Rac and its downstream effectors to be activated, two separate and distinct events need to occur. First, Rac itself needs to be activated by binding to GTP and, once bound to GTP, Rac needs to translocate to the appropriate membrane compartments (del Pozo *et al.*, 2000). In order to determine the effect of CrkII and the significance of Y221 site of CrkII on Rac signaling, we examined both GTP loading and subcellular localization of Rac in cells expressing either wild-type CrkII or CrkII-Y221F.

A GST fusion of the p21-binding domain (PBD) of PAK-1, which specifically interacts with the GTP-bound, but not the GDP-bound, form of Rac (Benard *et al.*, 1999), was used to pull down active myc-tagged Rac in cells expressing either wild-type CrkII or the CrkII-Y221F mutant (Figure 4). The extent of active Rac was assessed by probing PBD pull-downs with anti-myc antibody. As shown previously (del Pozo *et al.*, 2000), cell adhesion on FN was found to result in an ~2-fold increase in Rac GTP loading compared with cells kept in suspension. Co-expression of wild-type CrkII with Rac failed to further activate Rac on FN over a wide concentration range tested. Expression of CrkII-Y221F similarly failed to have an effect on GTP loading of Rac. As a positive control, expression of Vav2, an exchange factor for Rac, reproducibly increased GTP loading of Rac by ~2-fold, as shown previously (Abe *et al.*, 2000).

We next assessed the effect of CrkII on the subcellular localization of Rac by employing both confocal immunofluorescence microscopy and biochemical fractionation. COS-7 cells that had been transiently transfected with plasmids coding for GFP-tagged Rac alone or together with wild-type CrkII or CrkII-Y221F were plated on FN-coated coverslips and analyzed by confocal immunofluorescence microscopy. As shown in Figure 5A, GFP-Rac, when expressed alone, was mainly found in the cytoplasm, with some membrane staining observed. Consistent with the fact that wild-type CrkII activates Rac-dependent signaling events, expression of CrkII led to an enhanced lamellipodia formation and translocation of Rac to the plasma membrane. In contrast, Rac staining was exclusively cytosolic in cells that expressed CrkII-Y221F. Expression of CrkII-Y221F, especially at higher concen-

Fig. 4. Expression of CrkII does not affect GTP loading of Rac in FN-adherent COS-7 cells. COS-7 cells were transiently transfected with the indicated amounts (in μg) of wild-type CrkII, Crk-Y221F mutant or wild-type Vav2, together with 0.25 μg of myc-tagged wild-type Rac. The cells were held in suspension for 3 h, plated on FN for 15 min (black bars) or held in suspension for a further 15 min (white bar) and lysed in PBD lysis buffer. Cell lysates were incubated with GST-PBD beads, and bound GTP-Rac as well as the amount of myc-Rac in the lysate was detected by anti-myc immunoblotting. The amount of GTP-Rac signal in each sample was normalized to the amount of total myc-Rac expressed in the sample. Results are shown for experiments independently carried out three times.

trations, also led to inhibition of cell spreading on FN and to cell rounding. Subcellular localization of Rac and cell morphology correlated well with the localization of CrkII itself. Thus, a fraction of wild-type CrkII translocated to the cell membrane, while CrkII-Y221F was exclusively localized in the cytosol.

Similar results were obtained when the subcellular distribution of Rac was assessed by biochemical means. In these experiments, serum-starved COS-7 cells expressing myc-Rac alone or together with wild-type CrkII or CrkII-Y221F were plated on FN-coated dishes and subjected to subcellular fractionation as described in Materials and methods. As shown in Figure 5B, the bulk of myc-Rac partitions to the cytosolic fraction under all conditions studied. Co-expression of wild-type CrkII, however, led to a reproducible enhancement of Rac localization in the membrane fraction. Quantification of the relative ratio of myc-Rac in the membrane versus in the cytosol demonstrated an ~ 2 -fold enrichment of myc-Rac in the membrane fraction in cells co-expressing wild-type CrkII (Figure 5C). Furthermore, as observed by immunofluorescence microscopy (Figure 5A), wild-type CrkII, but not CrkII-Y221F, was also found in the membrane fraction (Figure 5B). Taken together, these data suggest that cell adhesion activates two signaling pathways, both of which are required for activation of signaling events downstream of Rac. Thus, cell adhesion induces GTP loading of Rac, presumably via activation of a guanine nucleotide exchange factor, and this signaling event appears to be unaffected by a transient expression of wild-type CrkII or CrkII-Y221F. In addition, cell adhesion induces

membrane translocation of activated Rac, which allows GTP-Rac to couple to its downstream effectors, and our results suggest that this event is regulated by CrkII and the Y221 site in it.

CrkII-mediated membrane localization is required for Rac to activate downstream signaling pathways

The results obtained above suggest that adhesion-mediated tyrosine phosphorylation of CrkII on Y221 regulates activation of Rac signaling by regulating the subcellular localization of Rac, and several lines of investigation were carried out to study this in more detail. First, we examined the possibility that co-expression of CrkII-Y221F with an activated form of Rac, RacV12, would inhibit translocation of RacV12 to the plasma membrane and, despite the fact that RacV12 is constitutively GTP bound, CrkII-Y221F would inhibit activation of signaling downstream of RacV12. Forced membrane targeting of RacV12 by utilizing an engineered myristylation tag (myr-RacV12 construct) should in turn overcome the inhibitory effect of CrkII-Y221F. As shown in Figure 6, this was indeed found to be the case. As published previously (Dolfini *et al.*, 1998), we found that expression of RacV12, and also of myr-RacV12, significantly enhanced activation of JNK in FN-adherent cells. While co-expression of wild-type CrkII slightly enhanced the capability of RacV12 to induce JNK activation, co-expression of CrkII-Y221F in turn resulted in a slight inhibition of RacV12-mediated JNK activation. Thus, there was a significant difference in the capability of RacV12 to activate JNK, depending on whether it was co-expressed with wild-type CrkII or with CrkII-Y221F. In contrast, there was no significant difference in the capability of myr-RacV12 to activate JNK when it was co-expressed with the two different Crk molecules. Thus, myr-RacV12 was largely refractory to the inhibitory effect by CrkII-Y221F, and its expression with Crk-Y221F led to an ~ 1.5 -fold increase in adhesion-dependent JNK activation compared with RacV12 expressed with Crk-Y221F. RacV12 and myr-RacV12 were approximately equally effective in activating JNK when co-expressed with wild-type CrkII.

Expression of either wild-type CrkII or CrkII-Y221F had no effect on the GTP loading of RacV12 or myr-RacV12, as measured by the PBD pull-down assay (data not shown). Instead, the effects of the two CrkII molecules on Rac signaling correlated well with their capabilities to regulate the subcellular localization of RacV12 (del Pozo *et al.*, 2000). Co-expression of wild-type CrkII resulted in co-localization of RacV12 and CrkII in the membrane regions. Co-transfection of CrkII-Y221F significantly inhibited translocation of RacV12 to the membrane in FN-adherent cells, and the two molecules were mainly localized in the cytosol. In accordance with its biochemical signaling capabilities (Figure 6), myr-RacV12 in turn demonstrated constitutive membrane localization, regardless of co-expression of CrkII-Y221F or wild-type CrkII (Figure 7B). Wild-type CrkII and myr-RacV12 demonstrated co-localization at the membrane, while CrkII-Y221F remained mainly cytosolic in myr-RacV12-expressing cells.

As an additional approach, we rationalized that membrane targeting of CrkII-Y221F should result in membrane

Fig. 5. Subcellular localization of Rac and CrkII in wild-type CrkII- and CrkII-Y221F mutant-expressing cells. (A) Wild-type CrkII or CrkII-Y221F mutant was co-expressed with GFP-tagged wild-type Rac in COS-7 cells. The cells were detached and reattached on FN-coated coverslips for 15 min, followed by staining with anti-Crk antibodies. The cells were visualized and photographed by confocal microscopy. (B) COS-7 cells expressing myc-Rac or myc-Rac with wild-type CrkII or CrkII-Y221F were serum starved, detached and reattached to FN-coated dishes. The cells were fractionated as described in Materials and methods in order to obtain cytosolic and membrane fractions. Equal amounts of protein from these fractions were loaded on a gel and immunoblotted with anti-Rac or anti-Crk antibodies. (C) The bands corresponding to myc-Rac were densitometrically scanned and the ratio of the relative intensity is shown. The data are representative of three independent experiments. * $P < 0.005$ between wild-type CrkII and control samples.

Fig. 6. CrkII-Y221F inhibits RacV12-, but not myr-RacV12-induced JNK activation upon cell attachment. COS-7 were transiently transfected with 0.25 μ g of plasmids encoding either wild-type CrkII or CrkII-Y221F mutant, or 0.1 μ g of plasmids coding for RacV12 or myr-RacV12 together with 0.25 μ g of HA-JNK, as indicated. Transfected cells were detached and reattached on FN-coated dishes for 15 min. Cell lysates were subjected to immunoprecipitation with anti-HA antibodies, followed by immunocomplex kinase assay, as described in Materials and methods. * $P < 0.05$ between CrkII-Y221F/RacV12 and wild-type CrkII/RacV12 samples, and between CrkII-Y221F/myr-RacV12 and CrkII-Y221F/RacV12 samples. Results are shown for experiments independently carried out three times.

localization of both CrkII and Rac, and restore the capability of CrkII-Y221F to activate Rac signaling pathways. A myr-CrkII-Y221F construct engineered to harbor the myristylation tag in the N-terminus was transiently expressed in COS-7 cells together with GFP-Rac or GFP-RacV12. The cells were detached, allowed to adhere on FN-coverslips and stained with anti-Crk antibodies. As shown in Figure 8, myr-CrkII-Y221F was readily targeted to the membrane, and its expression restored the localization of both GFP-Rac and GFP-RacV12 (compare with CrkII-Y221F-expressing cells in Figures 5 and 7, respectively) to the membrane. In addition, expression of myr-CrkII-Y221F also induced enhanced membrane ruffling, as visualized by enhanced phalloidin staining at the membrane periphery (data not shown). As shown in Figure 9, myr-CrkII-Y221F readily induced activation of both JNK and PAK to the same extent as wild-type CrkII in FN-adherent cells. Transient expression of myr-CrkII-Y221F also enhanced haptotactic cell migration towards FN in COS-7 cells (data not shown). This enhanced signaling ability was Rac dependent, as dominant-negative Rac (RacN17) inhibited myr-CrkII-Y221F-induced JNK and PAK activation (data not shown). Similar to wild-type CrkII, myr-CrkII-Y221F failed to induce GTP loading of Rac on FN (Figure 9C), further supporting the notion that CrkII regulates Rac

Fig. 7. Subcellular localization of RacV12 and myr-RacV12 in cells expressing wild-type CrkII and CrkII-Y221F mutant. Wild-type CrkII or CrkII-Y221F was co-expressed with GFP-tagged RacV12 in (A), and GST-tagged CrkII or GST-tagged CrkII-Y221F was expressed with myc-tagged myr-RacV12 in (B) in COS-7 cells. The cells were detached, replated on FN-coated coverslips for 15 min, and stained with the indicated antibodies. The cells were visualized and photographed by confocal microscopy.

signaling via subcellular localization rather than by enhancing its GTP loading. Taken together, these results suggest that the Y221 site in CrkII is indispensable for the translocation of both CrkII and Rac to the plasma membrane, and for the activation of Rac signaling pathways upon cell attachment on FN.

Discussion

Previous studies have demonstrated an important and conserved role for the Crk signaling complex in regulating cytoskeletal dynamics, cell motility and phagocytosis in a Rac-dependent manner. Our findings reported here indicate that the Y221 site in CrkII, presumably via tyrosine phosphorylation, regulates adhesion-dependent membrane localization of both CrkII and Rac, and efficient coupling of Rac to its downstream effectors. These findings provide

Fig. 8. Membrane-targeted myr-CrkII-Y221F induces membrane localization of wild-type Rac and RacV12. COS-7 cells transiently expressing myr-CrkII-Y221F along with GFP-Rac and GFP-RacV12 were plated on FN-coated chamber slides for 15 min, followed by staining with anti-Crk antibodies. The cells were visualized and photographed using confocal microscopy.

evidence for a novel mechanism of regulation of Rac signaling and reveal a previously unidentified function of CrkII in cellular signaling pathways.

By utilizing antibodies that specifically recognize tyrosine phosphorylation of CrkII on Y221, we found that cell attachment on FN induces phosphorylation of CrkII at this site. While our results utilizing the CrkII-Y221F mutant suggest that this is the exclusive phosphorylation site in CrkII, we can not rule out the possibility that additional, minor phosphorylation sites would also be present. Furthermore, phosphorylation of these other sites could be affected by a mutation of Y221; additional studies are needed to address this. Several tyrosine kinases that are known to become activated upon integrin-mediated cell adhesion have been shown to phosphorylate CrkII. These include, for example, Abl and several receptor tyrosine kinases (Feller *et al.*, 1994; Beitner-Johnson and LeRoith, 1995; Khwaja *et al.*, 1996; Ribon and Saltiel, 1996; Blakesley *et al.*, 1997; Hashimoto *et al.*, 1998), and studies are under way to explore the pathways leading from integrins to regulation of CrkII phosphorylation.

Our results utilizing the CrkII-Y221F mutant indicate that adhesion-dependent tyrosine phosphorylation of CrkII on Y221 negatively regulates interactions of CrkII with its SH2- and SH3-binding partners. In addition to regulating protein-protein interactions mediated by the SH2 and SH3 domains, several lines of evidence suggested that the Y221 site of CrkII is crucial for adhesion-dependent membrane localization of both CrkII and Rac, and for subsequent activation of Rac signaling. First, we found that exogenously expressed wild-type CrkII was localized to the plasma membrane in FN-adherent cells, and it induced membrane localization of Rac and activation of Rac-dependent signaling events, such as JNK and PAK activation, membrane ruffling and cell migration. The CrkII-Y221F mutant in turn failed to localize to the plasma membrane. Instead, both CrkII-Y221F and Rac were found in the cytosol in CrkII-Y221F-expressing cells, and CrkII-Y221F inhibited activation of Rac signaling

pathways induced by FN attachment and by an activated form of Rac, RacV12. It should be noted here that when RacV12 was expressed at high concentrations, the capability of CrkII-Y221F to inhibit Rac signaling was greatly diminished (data not shown). This is in accordance with the data from del Pozo *et al.* (2000), indicating that high levels of RacV12 expression lead to adhesion-independent (and, presumably, Crk-independent) activation of PAK. Secondly, we found that the capability of CrkII-Y221F to inhibit RacV12 signaling could be overcome by membrane targeting of RacV12, demonstrating that the CrkII-Y221F mutant specifically interferes with Rac signaling at the level of membrane targeting. Thirdly, the capability of CrkII-Y221F to positively regulate Rac signaling was restored when the construct was targeted to the membrane by an added myristylation signal, demonstrating that the lack of membrane localization was the sole reason why the CrkII-Y221F mutant was incapable of activating Rac signaling. The conservative tyrosine to phenylalanine mutation is not expected to change the overall confirmation of the CrkII molecule; thus, we anticipate that the functional deficiency of the CrkII-Y221F mutant is due to its incapability to undergo tyrosine phosphorylation on Y221 upon cell attachment. As mentioned above, adhesion-dependent activation of Rac signaling pathways requires both GTP loading of Rac and membrane translocation of GTP-Rac to occur. While our studies indicated that CrkII and the Y221 site in it contribute to membrane translocation of Rac, expression of either wild-type CrkII or CrkII-Y221F failed to have an effect on GTP loading of Rac in adherent cells. At present, the signaling events leading from integrins to a putative guanine exchange factor for Rac and Rac GTP loading remain unknown.

That tyrosine phosphorylation of CrkII on Y221 would positively regulate CrkII membrane translocation and Rac signaling seems contradictory with the finding that tyrosine phosphorylation of CrkII negatively regulates its interactions with its SH2- and SH3-binding partners. Previously, we and others have reported that interactions

of Crk with its SH2- and SH3-binding partners are required for activation of Rac-dependent signaling events (Dolfi *et al.*, 1998; Klemke *et al.*, 1998). At present, we do not know whether a pool of CrkII exists that is both tyrosine phosphorylated and complexed with its binding partners, nor do we know the subcellular location of this putative subpopulation of phosphorylated CrkII. We estimate that <10% of CrkII is tyrosine phosphorylated in FN-adherent cells in steady-state conditions (data not shown), and attempts to characterize protein-protein interactions mediated by the tyrosine-phosphorylated CrkII in a quantitative manner have proven to be difficult. Interestingly, however, Hashimoto *et al.* (1998) utilized the antibody that specifically recognizes CrkII when phosphorylated at position Y221 in an immunofluorescence analysis, and found that tyrosine-phosphorylated CrkII is mainly localized at the periphery of the cells; our unpublished data confirm these findings. Thus, our hypothesis is that the phosphorylation cycle of CrkII and of its SH2-binding partners regulates the subcellular localization and the dynamic turnover of the Crk multi-protein complex, and both of these events are critical for Rac activation and cytoskeletal dynamics during processes such as cell motility. In a simplistic model, tyrosine phosphorylation of CrkII may be instrumental for the initial recruitment of CrkII to the membrane, while subsequent (or further) binding of CrkII to its tyrosine-phosphorylated SH2-binding partners (which may require dephosphorylation of CrkII by an as yet undefined phosphatase) may be necessary for keeping CrkII and its associated signaling proteins at the membrane sites, allowing efficient membrane recruitment of Rac and activation of Rac signaling. Conceivably, a Crk-binding protein, such as DOCK180, which directly binds to Rac (Kiyokawa *et al.*, 1998), could mediate a ternary complex formation and recruitment of Rac to the membrane. It is likely that activation of Rac signaling further enhances CrkII complex formation and membrane localization; thus, expression of a dominant-negative form of Rac has been shown to reduce interaction between Cas and CrkII (Girardin and Yaniv, 2001) and membrane localization of CrkII (our unpublished data). Upon cessation of the stimuli that are needed for tyrosine phosphorylation of Crk-SH2-binding proteins, unphosphorylated CrkII would be released from the membrane, and signaling downstream of CrkII would be terminated. As per this model, a purely 'inductive' signal, such as the one exemplified by the CrkII-Y221F mutant, would be incapable of activating Rac signaling due to its incapability to translocate to the

Fig. 9. Membrane-targeted myr-CrkII-Y221F induces activation of JNK and PAK. COS-7 cells were transiently transfected with 0.25 μ g of the indicated CrkII constructs together with 0.25 μ g of HA-tagged JNK (A) or myc-tagged PAK (B). The cells were held in suspension for 5 min (A) or 3 h (B) and then plated on FN for 15 min. Kinase assays and densitometric analysis were carried out as described in Materials and methods. * P < 0.01 between CrkII-Y221F and myr-CrkII-Y221F samples; ** P < 0.025 between Crk-Y221F and myr-CrkII-Y221F samples. (C) Serum-starved COS-7 cells expressing myc-Rac with or without wild-type CrkII or myr-CrkII-Y221F were detached, held in suspension for 3 h and plated on FN-coated dishes for 15 min. The cells were lysed and analyzed for Rac-GTP loading as described in Materials and methods. Results are shown for independent experiments carried out three times.

membrane, and this construct would function as a dominant-negative by being a 'sink' for SH2- and SH3-binding partners in the cytosol.

The molecular mechanism(s) by which Y221 induces CrkII membrane translocation under this model remains to be explored. Phosphorylation of CrkII on Y221 could lead to a protein-protein interaction *in trans* that would function to recruit CrkII to the plasma membrane. Alternatively, tyrosine phosphorylation of Y221 may lead to a release of an inhibitory interaction that keeps CrkII in the cytosol. In either case, the interaction could be directly mediated by Y221 in CrkII, or phosphorylation of Y221 could regulate other protein-protein interaction sites in CrkII. Interestingly, Anafi *et al.* (1996) have demonstrated that a proline-rich insert within the Crk SH2 domain constitutes an SH3 domain-binding site that can be regulated by binding of CrkII-Y221 phosphopeptide to the Crk SH2 domain. Furthermore, the 'spacer region' and the C-terminal SH3 domain of CrkII have been shown to contain regulatory elements that can be uncovered by structural alterations; tyrosine phosphorylation of Y221 within the spacer region could similarly induce changes in these elements (Zvara *et al.*, 2001).

Our finding that CrkII Y221 regulates adhesion-dependent Rac membrane translocation and activation of Rac signaling is not cell type specific; in addition to the COS-7 cell system used in this study, similar results were obtained when NIH 3T3 cells were utilized (data not shown). It remains to be determined whether Y221 in CrkII similarly regulates activation of Rac signaling downstream of other cellular stimuli, such as activation of growth factor receptors. Escalante *et al.* (2000) have reported that the CrkII-Y221F mutant inhibits nerve growth factor-induced neuritegenesis, and this may well result from lack of activation of Rac signaling. Of note, the *C.elegans* Crk homolog CED-2 lacks a tyrosine residue at the site that corresponds to Y221 in CrkII, but it nevertheless appears to regulate Rac signaling via the *C.elegans* DOCK180 homolog CED-5 (Reddien and Horvitz, 2000; Gumienny *et al.*, 2001; Reddien *et al.*, 2001). As such, the overall molecular basis for Crk-mediated regulation of Rac activation is likely to be complex, and it will be important to identify the molecular mechanisms that mediate the recruitment of the Crk signaling complex and Rac activation in various organisms and cellular models.

Materials and methods

Constructs and antibodies

Myc-tagged wild-type CrkII and CrkII-Y221F mutant constructs in pCAGGS vector have been described previously (Hashimoto *et al.*, 1998) and were obtained from Dr Michiyuki Matsuda. GFP-CrkII and GFP-CrkII-Y221F constructs were generated by performing a partial digest of the pCAGGS-CrkII and CrkII-Y221F vectors with *EcoRI* and *PvuII*, followed by subcloning of the full-length CrkII sequences into *EcoRI*-*SmaI*-digested pEGFP-C2 vector (Clontech Laboratories). The myr-CrkII-Y221F construct was generated by a partial digestion of the pCAGGS-CrkII-Y221F plasmid with *EcoRI* and *PvuII*, followed by subcloning of the CrkII sequences into a pcDNA3.myr (Invitrogen) vector digested with *EcoRI* and *EcoRV*. GST-tagged wild-type CrkII and GST-tagged CrkII-Y221F constructs were generated by subcloning full-length CrkII and CrkII-Y221F constructs into pEBG vector. Myc-tagged wild-type Rac, constitutively active RacV12 and dominant-negative RacN17 constructs in pEXV vector were obtained from Dr Alan Hall. myr-RacV12 construct was generated by digesting the pEXV-RacV12 plasmid with *EcoRI* and then ligating the Rac sequences in-frame with the

myristylation sequence into an *EcoRI*-digested pcDNA3.myr vector. The GFP-Rac and GFP-RacV12 constructs have been described previously (del Pozo *et al.*, 1999) and were obtained from Drs Martin Schwartz and Miguel del Pozo. Myc-tagged PAK-1 construct was obtained from Dr Jonathan Chernoff. PCR was used to amplify the region of the PDB of PAK-1 (amino acids 67–150), which was subsequently cloned into the bacterial expression vector pGEX4T-1 in-frame with GST. The pEGFP-Vav2 construct was obtained from Dr Keith Burridge. The HA-JNK expression plasmid has been described previously (Dolfi *et al.*, 1998). Anti-Crk monoclonal antibody, anti-paxillin monoclonal antibody and horseradish peroxidase-conjugated anti-phosphotyrosine antibody (pY20-HRP) were purchased from Transduction Laboratories. Anti-C3G polyclonal antibody and anti-Myc and anti-HA monoclonal antibodies were purchased from Santa Cruz Biotechnology. The anti-CrkII pY221 antibody was obtained from Dr Michiyuki Matsuda.

Cell culture and transfections

COS-7 cells were maintained at 37°C and 5% CO₂ in DME high glucose medium (Irvine Scientific) supplemented with 10% calf serum, penicillin, streptomycin and gentamycin (Gibco-BRL). For transient transfections, cells were seeded on tissue culture dishes at 75–80% confluence the day before and transfections were carried out using LipofectAMINE (Gibco-BRL). Twenty-four hours after transfections, the cells were washed and serum starved for an additional 24 h. The cells were then processed for the various assays and experiments, as described below. For all the experiments, the cells were checked for the expression of the transfected constructs by immunoblotting with the appropriate antibodies.

Cell attachment on FN, immunoprecipitations and immunoblotting

Serum-starved cells were detached with 0.05% trypsin-EDTA buffer and diluted in serum-free medium containing 0.5% bovine serum albumin (BSA). The cells were centrifuged at 500 g and the cell pellet was gently resuspended in serum-free medium containing 0.2% BSA. The cells were kept in suspension for 1 h and then replated for the time periods indicated on dishes that had been coated with 10 µg/ml FN that had been purified from serum as described previously (Ruoslahti *et al.*, 1982). Attached cells were washed with phosphate-buffered saline (PBS) and then lysed in radioimmunoprecipitation buffer (RIPA; 50 mM Tris pH 7.5, 150 mM sodium chloride, 5 mM EDTA, 1 mM sodium vanadate, 50 mM sodium fluoride, 0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100) containing protease inhibitors [10 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Cell lysates were scraped off, kept on ice for 10 min and centrifuged at 21 000 g for 10 min at 4°C. The protein content of the supernatants was measured by the Bio-Rad D_C protein assay kit and the supernatants were adjusted for equal amount of protein and incubated with the indicated antibodies for 2 h at 4°C, followed by precipitation with protein A-Sepharose for an additional 1 h. The immunoprecipitates were washed three times with RIPA buffer, resuspended in Laemmli sample buffer, heated at 95°C for 5 min prior to being loaded on 4–20% pre-cast SDS-polyacrylamide gel (Novex), and immunoblotted with the primary antibody followed by secondary antibodies covalently linked to horseradish peroxidase. Antibody binding was detected by using ECL reagents (Pierce). Where indicated, the films were scanned and the bands were densitometrically quantitated using the NIH Image software.

PBD pull-down assay

Cells were transfected with the indicated amounts of plasmids coding for CrkII and its various mutants (Figure 4) along with 0.25 µg of plasmid encoding myc-tagged Rac. The total amount of DNA transfected per dish was 3 µg, the remainder of which was compensated with empty vector. Twenty-four hours after transfection, the cells were serum starved for 16–18 h and the cells were detached with trypsin-EDTA, held in suspension for 3 h in plastic dishes coated with 0.5% BSA and then seeded on FN-coated plastic dishes for 15 min at 37°C. The cells were washed in PBS, and lysed in PBD lysis buffer (20 mM HEPES pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 10% glycerol, 0.5% NP-40 and protease inhibitors). The lysates were incubated with 20 µg of glutathione beads linked to PAK PBD for 30 min at 4°C. The beads were washed with PBD buffer and the proteins were eluted with sample buffer and loaded on 4–20% pre-cast polyacrylamide gels. The gels were transferred to nitrocellulose membrane and probed with anti-myc monoclonal antibody as described above.

In order to make GST-PBD fusion protein, DH5α bacterial cells harboring pGEX4T-PBD plasmid were grown in 100 ml of LB-Amp and induced with IPTG for 2 h. The cells were collected by centrifugation,

resuspended in 3–4 ml of lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, 10 µg/ml aprotinin and 10 µg/ml leupeptin), sonicated and centrifuged at 11 000 g for 15 min. The soluble proteins were incubated with glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech) for 30 min at 4°C and washed three times with lysis buffer. The purified proteins were checked and quantitated on a gel using known amounts of BSA as a standard and stored at 4°C.

JNK and PAK kinase assays

Approximately 300 000 cells were seeded per well of a 6-well tissue culture plates. The cells were transfected with 2 µg of total DNA, which included the indicated amount of CrkII constructs along with 0.25 µg of plasmids encoding HA-JNK or Myc-PAK. Empty vector was used to compensate for the remainder of the DNA. Where indicated, 0.1 µg of RacV12 and myr-RacV12 were co-transfected. Twenty-four hours after transfection, the cells were serum starved for 16–18 h, detached, held in suspension in 0.5% BSA-coated dishes (5 min for JNK and 3 h for PAK), and then seeded on FN-coated plastic dishes for 15 min. The cells were washed in PBS and lysed in MAP kinase lysis buffer as described previously (Garcia-Guzman *et al.*, 1999). The lysates were immunoprecipitated with 0.5 µg of anti-HA or anti-Myc monoclonal antibodies, and the immunocomplexes were washed twice with the lysis buffer and twice with kinase wash buffer (50 mM HEPES pH 7.5, 10 mM MgCl₂). The immunocomplex was subjected to an immunocomplex kinase assay either containing 2 µg of GST–c-jun (Stratagene) for JNK or 5 µg of MBP (Sigma) for PAK as a substrate in kinase assay buffer (50 mM HEPES pH 7.5, 10 mM MgCl₂, 100 µM cold ATP, 10 µCi of [³²P]ATP) at 30°C for 20 min. The reactions were terminated by the addition of sample buffer, samples were loaded on a 4–20% gradient gel, and Coomassie Blue-stained gels were exposed to PhosphorImager screens for ~24–48 h. The phosphoscreens were scanned using the Molecular Dynamics scanner and the bands were quantitated using the ImageQuant software (Molecular Dynamics). For all the experiments, equal amounts of cell lysates were fractionated on a gel and immunoblotted with anti-HA, anti-Myc and anti-Crk antibodies to ensure equal expression of the various constructs.

Immunofluorescence

COS-7 cells were transfected with 0.5 µg of wild-type CrkII or its various mutant expression plasmids along with 0.2 µg of GFP-tagged wild-type Rac, GFP-tagged RacV12 or myc-tagged myr-RacV12. The cells were detached as described above and then seeded on 4- or 8-well chamber slides (Lab-Tec) coated with 10 µg/ml FN, allowed to spread for 15–30 min and fixed in 4% paraformaldehyde. The cells were permeabilized in PBS/0.2% Triton X-100, blocked with goat serum or 0.5% BSA, and stained with anti-CrkII and anti-Myc, or rhodamine–phalloidin (Molecular Probes), followed by FITC-conjugated anti-mouse antibody or TRITC-conjugated anti-mouse antibody (Sigma). The cells were visualized with a laser confocal microscope (model 1024; Bio-Rad) and photographed.

Biochemical fractionation

COS-7 cells grown on 15-cm dishes were transfected with 2 µg of wild-type myc-tagged Rac alone or together with 5 µg of wild-type CrkII or CrkII-Y221F. The cells were detached with 0.05% trypsin–EDTA as described above and seeded on 15-cm plastic dishes coated with 10 µg/ml FN for 15 min. The cells were scraped in fractionation buffer (10 mM MES pH 6.5, 5 mM EDTA, 1 mM sodium vanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 1 mM PMSF), transferred to a Teflon glass homogenizer, and homogenized on ice using 15 strokes. The homogenate was transferred to Eppendorf tubes, centrifuged for 3 min at 425 g and the supernatants were transferred to new Eppendorf tubes and subjected to further centrifugation for 30 min at 21 000 g. The supernatant, designated as the ‘cytosolic fraction’, was kept on ice and the pellet was solubilized in lysis buffer (25 mM MES pH 6.5, 150 mM NaCl, 1% Triton X-100, 1 mM sodium vanadate, 5 mM EDTA and protease inhibitors) for 20 min on ice and subjected to sucrose step gradient centrifugation as described previously (Xavier *et al.*, 1998). The Triton-insoluble fraction was designated as the ‘membrane’ fraction.

Migration assay

Cell migration assays were performed using Costar-modified Boyden Chambers (Costar; Cat 3422), essentially as described previously (Klemke *et al.*, 1998). The underside of the transwell membranes was coated with 10 µg/ml FN for 2 h at 37°C. COS-7 cells (8 × 10⁵) in 10-cm dishes were transfected with 0.5 µg of GFP, GFP–CrkII or various GFP-tagged CrkII mutant plasmids, and the cells were detached with

trypsin–EDTA and resuspended in serum-free medium/0.5% BSA. The cells were washed in serum-free medium/0.5% BSA, counted and adjusted to 10⁶ cells/ml. One hundred microliters of the cell suspension were placed on the top chambers and the cells were allowed to migrate to the underside for 3 h at 37°C. The cells on the top chamber were removed with a cotton swab and the cells migrating to the underside of the filter were fixed in 4% paraformaldehyde. The filters were cut, mounted on a coverslip and the GFP-positive cells were visualized and photographed using a Nikon immunofluorescence microscope. As a control for transfection efficiency and cell spreading, 5 × 10⁵ cells were allowed to spread on FN-coated chamber slides for 30 min and then fixed and visualized under the microscope.

Statistical analysis

Comparison of the data was performed using Student's *t*-test. Unless indicated otherwise, data are from at least three independent experiments.

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