Crk associates with ERM proteins and promotes cell motility toward hyaluronic acid

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Running title: Association of Crk and ERM
SUMMARY

Cell migration is a well-organized process regulated by the extracellular matrix (ECM) –mediated cytoskeletal reorganization. The signalling adaptor protein Crk has been shown to regulate cell motility, but its precise role is still under the investigation. Here we report that Crk associates with ERM family proteins including ezrin, radixin, and moesin, activates RhoA, and promotes cell motility towards hyaluronic acid. The binding of Crk with ERMs was demonstrated both by transient and stable protein expression systems in 293T cells and 3Y1 cells, and it was shown that v-Crk translocated the phosphorylated form of ERMs (pERMs) to microvilli in 3Y1 cells by immunofluorescence and immunoelectron microscopy. This v-Crk-dependent formation of microvilli was suppressed by inhibitors of Rho-associated kinase (ROCK), and the activity of RhoA was elevated by coexpression of c-Crk-II and ERMs in 3Y1 cells. In concert with the activation of RhoA by Crk, Crk was found to associate with Rho-GDI, which has been shown to bind to ERMs. Furthermore, upon hyaluronic acid treatment, coexpression of c-Crk-II and ERMs enhanced cell motility, while the sole expression of c-Crk-II or either of the ERMs decreased the motility of 3Y1 cells. These results suggest that Crk may be involved in regulation of cell motility by a hyaluronic acid-dependent mechanism through an association with ERMs.
INTRODUCTION

The extracellular matrix (ECM) plays an important role in various cellular responses (1-3). ECM drives the spatio-temporal reorganization of the cytoskeleton which is involved in physiological cell migration, tumor cell invasion, and metastasis. Multiple cell surface molecules have been shown to participate in this ECM-dependent signalling mechanism. One of the major molecules is CD44, a transmembrane receptor for hyaluronic acid (4,5), which associates with the actin cytoskeleton through the ERM family proteins (ERMs) including ezrin, radixin, and moesin. The cleavage of CD44 at the extracellular domain by membrane-associated metalloproteinases plays a crucial role in efficient cell detachment during cell migration (6,7). The binding of CD44 and ERMs is controlled by the threonine-phosphorylation of ERMs through Rho-associated kinase (ROCK), and also by N-terminal phospholipid modification of ERMs (8).

The signalling adaptor protein Crk, which is composed of an SH2 and two SH3 domains, is considered to be involved in the cytoskeletal regulation. Crk has been shown to interact with components of focal adhesion, such as p130Cas and paxillin (9,10), which were tyrosine-phosphorylated mainly by integrin stimulation. Crk transmits signals to downstream effecters through Crk-SH3 binding proteins, C3G and Dock180, which exert a guanine-nucleotide exchange factor (GEF) activity on Rap-1/R-Ras and Rac, respectively (11-13). Thus, Crk may regulate cytoskeletal movement thorough these GEFs and small GTPases. In fact, studies of C3G knockout mice have suggested the regulation of cell adhesion by C3G through Rap-1 (14). The phagocytosis, membrane ruffling, and lamellipodia formation has been shown to be regulated by a Dock180-ELMO-Rac-dependent mechanism (15,16).

Besides the identification of the activation of Rap-1 or Rac, we and others previously
found that Crk could activate another GTPase, RhoA in rat fibroblasts 3Y1 cells or PC12 neuronal cells (17-19). However, the mechanism of Crk-induced RhoA activation and its role were still under the investigation.

In this study, we analyzed the mechanism and significance of the Crk-induced RhoA activation, and found the novel association of Crk and ERMs. Unlike the known Crk-associated molecules, these bindings were not dependent on solely the SH2 or SH3 domain. In agreement with the activation of RhoA by Crk, Rho-GDI was colocalized and coprecipitated with Crk. Thus, the dissociation of Rho-GDI and RhoA by Crk was supposed to be involved in RhoA activation. Furthermore, Crk and the phosphorylated form of ERMs cooperatively induced microvilli formation in fibroblasts. By the hyarulonic acid treatment, the activation of RhoA was enhanced in cell lines stably expressing Crk and ERMs. Finally, Crk and ERMs cooperatively regulated cell motility towards hyarulonic acid. These results suggest that Crk associated with ERMs and activated RhoA possibly through Rho-GDI. Through this mechanism, Crk plays a role for hyaluronic acid-induced enhancement of cell motility.
EXPERIMENTAL PROCEDURES

Cells and antibodies—Rat fibroblasts 3Y1 cells (JCRB0734) and 293T human embryonal kidney cells with SV40 large T antigen were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). Establishment of the v-Crk-inducible 3Y1 cell line (3Y1 21-2-1) by the tetracycline inducible system (Tet-on system, CLONTECH) has been described (19). The v-Src transformed 3Y1 cell line (SR-3Y1) was established in the Hanafusa Lab at The Rockefeller University, New York. The mouse monoclonal antibody (mAb) against viral gag protein (clone 3C2) has been described (19) and used to detect v-Crk. The rat monoclonal antibody for phospho-ERM (297S) was a gift from Dr. Sachiko Tsukita (Kyoto Univ., Japan). Anti- RhoA (119) and Rho-GDI (A-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-Rac1 and anti-Crk antibodies were from BD Transduction Laboratories (Lexington, KY), anti-Flag M2 antibody was from Sigma (Saint Louis, MO), anti-myc antibody (9E10) was a gift from Dr. Hiroshi Ariga (Hokkaido Univ., Japan), and anti-CD44cyto antibody was a gift from Dr. Hideyuki Saya (Kumamoto Univ., Japan).

Plasmids—pGEX-rhotekin-RBD was generated by using PCR. The cDNAs of ezrin, radixin, and moesin, a gift from Dr. Sachiko Tsukita (Kyoto University, Japan), were subcloned into the pCXN2-Flag vector. The cDNA for Rho-GDI was a gift from Dr. Yoshimi Takai (Osaka University, Japan). The following are Crk mutants: pCAGGS-Myc-CrkII-R38V as an SH2 mutant of c-Crk-II, and W169L as an SH3 mutant of of c-Crk-II; pCAGGS-Myc-vCrk-N273 as an SH2 mutant of v-Crk, and W405K as an SH3 mutant of v-Crk.

Establishment of cell lines—To establish stable cell lines expressing Crk and/or ERM, mammalian expression plasmids of pCAGGS-Myc-Crk II and/or pCXN2-Flag-ezrin, -radixin, or -moesin were transfected to 3Y1 cells using Fugene 6 transfection reagent (Roche,
Indianapolis, IN, USA). Following the selection with 400 µg/ml of G418 (Calbiochem, Demstat, Germany), the expression levels of Crk and ERMs were examined by immunoblotting using anti-Myc and anti-Flag antibody, respectively.

**Immunoprecipitaion and immunoblotting**—Immunoprecipitation and immunoblotting were performed by the method described elsewhere. For detection of CD44 by immunoblotting, cells were pretreated by proteasome inhibitor as described previously (19).

**Confocal laser scanning**—For analysis of the subcellular localization of v-Crk, cells were fixed with 3% paraformaldehyde for 15 min at room temperature (RT), permeabilized with 0.1% Triton X-100 for 4 min at RT, and then refixed with 70% methanol for 5 min at -20°C.

Anti-gag mAb, 3C2 (1:50 dilution) and Alexa 594-conjugated goat anti-mouse immunoglobulin (Ig) antibody (1:200 dilution; Molecular Probes) were used as primary and secondary antibodies, respectively. For analysis of the localization of pERMs, cells were fixed with 10% trichloroacetic acid for 15 min on ice. Anti-pERM Ab (297S, culture supernatant without dilution) and Alexa 488-conjugated goat anti-rat immunoglobulin (Ig) antibody (1:200 dilution; Molecular Probes) were used as primary and secondary antibodies, respectively. The samples were observed using a confocal laser-scanning microscope (FV-300, OLYMPUS, Japan) equipped with a computer.

**Pull-down assay for RhoA and Rac**—These methods were described previously (19,20). Briefly, for Rho assay, serum-starved v-Crk inducible 3Y1 cells with or without Dox were lysed by lysis buffer composed of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1mM EDTA, 1% Nonidet P-40, 5 mM MgCl₂, 10% glycerol, 50 mM NaF, 1 mM Na₃VO₄, 1 mM dithiothreitol, 50 µg/ml PMSF, 10 µg/ml leupeptin, and 10 µg/ml pepstatin. Cell lysates were clarified by 5,000 x g centrifugation at 4°C for 5 min, and the supernatants were incubated with 10 µg of GST-rhotekin-RBD fusion protein pre-conjugated with glutathione-beads at
4°C for 1h. For Rac assay, cells were lysed with lysis buffer composed of 1% NP40, 25 mM HEPES pH7.4, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 10 mM MgCl₂, 1 µg/ml aprotinin, and 1 mM PMSF. Lysates were clarified by 12,000 rpm at 4°C for 1 min, and the supernatant was incubated with 10 µg of purified GST-PAK2-RBD and glutathione-beads at 4°C for 1h. In both Rho and Rac assays, the beads were washed three times with each lysis buffer and subjected to SDS-PAGE with a 12% gel. Precipitated RhoA or Rac1 was detected by immunoblotting using anti-RhoA or Rac1Ab.

Immunoelectron microscopy—Analysis was performed by the pre-embedding method with double immunostaining. v-Crk-induced 3Y1 cells were fixed with 0.1% glutaraldehyde in 0.1 M cacodylate buffer (CB) for 5 min on ice, and first incubated with a mixture of primary rat mAbs for pERM and a mouse mAb for v-Crk for 3 days at 4°C. After washing with PBS, the specimens were incubated with 10 nm gold-labeled anti-mouse Ig Ab for 1hr, followed by incubation for 1 hr with biotin-labeled anti-rat Ab, which was further reacted with peroxydase-labeled streptavisin. After re-fixation for 5 min, the enzyme reaction was visualized by using diaminobenzidine (DAB) as substrate. Cells were re-fixed with 2% OsO4 in 0.1M phosphate buffer for 50 min and then embedded in epon. Cells in epon block were sectioned into 1-µm thicknesses and stained with 1% toluidinblue for confirmation of the status of the cells. Then ultra-thin sections made by an ultramicrotome (Ultracut, Reihert-Jung, Co, Ansberg, Germany) were stained with 0.2% lead citrate for 5 min. Samples were observed by transmission electron microscopy (Hitachi 7100, Tokyo, Japan).

Wound healing assay—The method for the wound healing assay was described previously (19). Briefly, after the initial plating of cells for 48 h on uncoated, 10 µg/ml fibronectin- or hyaluronic acid-coated culture dishes, cells were scraped off/wounded by a yellow tip. Subsequently at 12 and 18 hours, the recovery percentage of the wounded portion was...
measured.
RESULTS

Association of Crk and ERMs- To examine the mechanism of Crk-mediated cytoskeletal movement, the association of Crk and ERMs was examined, because we reported the Crk-dependent activation of RhoA and the cleavage of CD44 (19), and ERMs are known to bind to CD44 regulating actin cytoskeleton. First, we found that anti-Crk antibody coprecipitated transiently expressed ERMs with endogenous Crk in human embryonal kidney 293T cells (Fig. 1A, lanes 1-3). Furthermore, with the overexpression of either v-Crk or human c-Crk-II and ERMs, the association of Crk and ERMs were efficiently demonstrated in 293T cells (Fig. 1A, lanes 4-9). To confirm the association of Crk and ERMs, we established 3Y1 cell lines stably expressing either c-Crk-II or ERMs alone, and c-Crk-II and either of the ERMs. In these cell lines, coprecipitations of EMRs with Crk were clearly demonstrated (Fig. 1B, lanes 6-8).

To analyze the binding mechanism of Crk and ERMs, we transiently transfected mutants of c-Crk-II with ERMs in 293T cells. Although the association of c-Crk-II and radixin/moesin seemed to be weakened when we expressed the SH2 or SH3 mutants of c-Crk-II (Fig. 1C, lanes 9, 10, 12, 13), we could not detect a remarkable suppression of the binding of SH2- or SH3-mutants of c-Crk-II and ERMs (Fig. 1C). Mutational analyses using SH2 or SH3 mutants of v-Crk and CrkL were also performed, and similar results were obtained (data not shown).

Activation of Rho by Crk and ERMs- To examine the effect of the association of Crk and ERMs on Crk-induced activation of RhoA, we performed a pull-down assay using the GST fusion form of Rhotekin-RBD in 293T cells. The activities of RhoA were augmented by coexpression of ERMs by 2.8-fold compared with Crk alone (Fig. 2A, upper panels and graph). In addition, coexpression of Crk and EMRs enhanced Rac activity (Fig. 2B, lower
panels and graph).

To examine the mechanism of Crk-induced Rho activation, we focused on the association of Crk and Rho-GDI because no known Rho GEF has been reported to bind to Crk. In fact, Rho-GDI contains possible Crk-interacting sequences such as the YXXP motif for the SH2 domain and proline-rich region for the SH3 domain. The colocalization of these proteins was observed in 3Y1 cells by confocal microscopy (Fig. 2B) and force-expressed Crk was coprecipitated with Rho-GDI by using anti-Rho-GDI antibody in 293T cells (Fig. 2C, arrowhead). The association of ERMs and Rho-GDI was also observed, as reported previously (Fig. 2C, asterisk) (21). It should be noted that we failed to detect the association of endogenous Crk and Rho-GDI (data not shown).

**Association of Crk and pERMs and induction of microvilli formation** - As ERM proteins were known to be regulated by phosphorylation, the binding of Crk and the phosphorylated form of ERMs (pERMs) were examined by using a v-Crk-inducible 3Y1 cell line (clone 21-2-1)(19). In the presence of v-Crk, the association of Crk with pERMs was detectable in the cytoplasmic fraction of 3Y1 cells (Fig. 3A).

We then analyzed the subcellular localization of pERMs in a v-Crk-inducible 3Y1 cell line. pERMs were observed diffusely in the cytoplasm and partially at the edge of the cytoplasm of 3Y1 cells without v-Crk (Fig. 3B-a). However, with v-Crk induction, pERMs were demonstrated to translocate to cellular microvilli, and co-localization of v-Crk and pERMs was shown by a merged image (Fig. 3B, b-d). In addition, co-localization of v-Crk and pERMs was also detected as dotted patterns in the cytoplasm (Fig. 3B, b-d, arrowheads). To confirm the involvement of ROCK, which was known to phosphorylate ERMs in a v-Crk-dependent microvilli formation of pERMs, we utilized the ROCK inhibitor Y27632, and found that this reagent inhibited the localization of pERMs to microvilli, while the
remaining co-localization of v-Crk and pERMs was still detectable in the cytoplasm (Fig. 3B, e-g, arrowheads). With Y27632 treatment, phospho-threonine levels of ERM core especially on ezrin and radixin were decreased, but the detectable levels of the phosphorylated form of moesin should be noted (Fig. 3C, lane 3 and 4).

As the accumulation of phosphatidylinositol 4,5-bisphosphate (PIP2) in the plasma membrane through the activation of Rho/ROCK/PI4P-5 kinase (PI 4-phospho 5-kinase) cascade has been shown to be involved in the continuous activation of pERMs (22), we analyzed the effect of neomycin which inhibits the membrane accumulation of PIP2, and found that this reagent produced similar responses to Y27632 (Fig. 3B, h-j and 3C, lanes 5 and 6).

To confirm the immunofluorescence study, we performed immunoelectron microscopy using the double staining method. pERMs were visualized by using diaminobenzidine, in which they are recognized as electron-dense black substances by transmission electron microscopy (TEM), and the presence of anti-v-Crk Ab was demonstrated by 10 nm-gold particle-labeled secondary antibody. pERM labeled with DAB was recognized in the cytoplasm of vCrk expressing 3Y1 cells by light microscopy (Fig. 4a). Immunoelectron microscopy demonstrated that vCrk was colocalized with pERM at the microvilli, cytoplasmic edge, and filamentous structure in the cytoplasm of the cells (Fig. 4b-g).

*The cleavage of CD44 in 3Y1 cells expressing both Crk and ERM*-To analyze the involvement of Crk in the hyaluronic acid-CD44-dependent signalling mechanism, we examined whether Crk increased the cleavage of CD44. Without hyaluronic acid, there was no detectable level of cleavage product of CD44 in Crk or Crk/ERMs-expressing cells (Fig. 5A, upper panel, lanes 3, 7, 8). Upon hyaluronic acid coating, the cleavage products of
CD44 could be observed in Crk-expressing cells and coexpressing cells of c-Crk-II and ezrin or radixin (Fig. 5A, lower panel, lanes 3, 7, 8). Cleaved bands were not observed in Crk/moesin-coexpressing cells (Fig. 5A, lower panel, lane 9).

**Analysis of cell motility of 3Y1 cells expressing Crk and ERMs** - To examine whether the association of Crk with ERMs affects cell motility, we performed wound healing assay by using 3Y1 cells stably expressing Crk and ERMs. Without extracellular matrix, the expression of Crk decreased cell motility as reported previously (19), and expression of either of the ERMs also suppressed motilities (Fig. 5B, upper panel). Coexpression of Crk and either of the ERMs slightly promoted motility, but the levels were still under those observed with wild type 3Y1 cells. However, upon hyaluronic acid stimulation, double expression of Crk and ERMs enhanced motility more than wild type level (Fig. 5B, lower panel). With integrin stimulation, Crk-expressing cells and the double expression of Crk and ERMs recovered the motility to the levels of wild type 3Y1 cells, but did not provide significant enhancement of motility higher than that of wild type 3Y1 cells (data not shown).

To confirm the involvement of the CD44 cleavage in v-Crk-regulated cell motility, we examined the effect of PI-3 kinase inhibitors because PI-3 kinase was known to up-regulate CD44 cleavage (23, 24). Wound healing assay demonstrated that PI-3 kinase inhibitors such as LY294002 and Wortmannin tend to suppress the motility of 3Y1 cell lines; however, this suppressive function was most prominently found in 3Y1 cells stably expressing Crk and ezrin (Fig. 5C). PI-3 kinase inhibitors did not affect the motility of cells expressing Crk and moesin (Fig. 5C).
DISCUSSION

Signalling adaptor protein Crk was originally found as an avian sarcoma encoding oncoprotein v-Crk(25). Since human c-Crk-II, the homologue of v-Crk, was isolated, the identification of Crk targets has suggested that Crk links between tyrosine phosphorylated proteins and guanine-nucleotide exchange factors for small GTPases, and regulates cytoskeletal reorganization. In particular, under fibronectin stimulation, the integrin-provoked signal has been shown to be mediated by Crk and transmitted to the downstream effector Dock180, leading to Rac activation. However, the mechanism of Crk-mediated cell migration or tumor cell invasion has still been under investigation.

In this study, we have found a novel interaction of Crk and the ERM family of proteins that is involved in activation of Rho and hyaluronic acid-CD44 dependent regulation of cell motility (Fig. 6). In contrast to the known Crk binding molecules such as p130Cas, paxillin, C3G, and Dock180, the interaction of Crk and EMRs were not solely SH2- or SH3-dependent. Mutation analysis showed that both of SH2 and SH3 mutants of Crk significantly attenuates the association of Crk and EMRs, while exceptionally, Crk SH2 mutant binds to ezrin as much as wild type Crk. Thus, Crk may bind to EMRs by both the SH2 and SH3 domains. Alternatively, the entire conformation of Crk might be required for this association. Determination of the Crk binding site(s) of EMRs may reveal the mechanism.

According to our previous results, v-Crk activated RhoA in fibroblasts and coexpression of Crk, and EMRs enhanced the activity of RhoA in 293T cells. As no known Rho-GEF was found to bind to Crk, the mechanism of Crk-dependent activation of Rho was the missing link. Rho-GDI has been shown to bind to the N-terminal FERM domain of EMRs (21), and these data led us to hypothesize that upon ECM stimulation, Crk binds to the
negative regulator of RhoA such as Rho-GDI, inactivates of Rho-GDI, and leads to the activation of RhoA shown as Fig. 6. Thus, we examined the association of Rho-GDI and Crk. In this study, the association of force-expressed Crk and Rho-GDI was observed in 293T cells, but we failed to show the association of endogenous Crk and Rho-GDI (data not shown). In 293T cells, we did not examine the inhibition of the function of negative regulator Rho-GDI, because the simple expression of Rho-GDI did not significantly suppress the activity of RhoA measured by pull-down assay. Furthermore, we also tried to test the effect of Crk on another negative regulator of RhoA, Rho-GAP. However, we did not observe a significant activation of RhoA by the double expression of Crk and Rho-GAP (data not shown). Establishment of a deficient cell line for the negative regulator of RhoA may reveal the Crk-dependent activation mechanism of Rho in future studies.

As it is known that ERMs were phosphorylated in the cytoplasm and translocated to membrane, and that the phosphorylated form of ERMs (pERMs) link between CD44 and actin cytoskeleton, we analyzed the localization of pERMs in v-Crk inducible fibroblasts. In 3Y1 cells, v-Crk translocated pERMs and induced microvilli formation by a ROCK-dependent signalling mechanism. Although we expected the induction of v-Crk-induced phosphorylation of ERMs, we failed to demonstrate such increased phosphorylation of ERMs by Crk in our system (Fig. 3C). We speculated the relatively high levels of pERMs in the cytoplasm of wild type 3Y1 cells may mask further phosphorylation of ERMs.

In this study, we showed that the association of Crk and ERMs was involved in the hyaluronic acid-CD44 signalling mechanism to promote cell motility. Considering the mechanism of Crk-dependent enhancement of CD44 cleavage, Crk may also regulate the transcriptional levels of matrix-metalloproteinases (MMPs). As Crk is also known to activate PI-3 kinase (26) which was reported to control the transcriptional levels of MMPs by
a Rac-Cdc25-dependent mechanism (23,24), we examined the effect of PI-3 kinase inhibitors on Crk/ERM-dependent motility of 3Y1 cells on an HA-coated plate. The data shown in Figure 5C suggest that PI-3 kinase may be involved in the motility of Crk/ezrin- and Crk/radixin-expressing 3Y1 cells. Additionally, the Ras/ERK pathway may be involved in Crk-dependnet expression of MMPs, as v-Crk has been reported to activate the secretion of matrix metalloproteinase through Ras and ERK (27).

Recently we and others have reported that overexpression of Crk, especially Crk-I, was observed in human malignant tumors such as glioblastoma and lung cancer (28-30). Crk has been shown to be related to the malignant feature of tumor cells. Thus, we should reveal the significance of our present findings, in which Crk regulates cell motility through hyarulonic acid/CD44/ERM, in relation to the invasiveness of human cancers in future studies.
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LEDGENDS TO FIGURES

Fig. 1  Association of Crk and ERM.  A.  293T cells were transiently transfected by the expression vector of ERMs with Flag tag (lanes 1-3), by both ERMs and v-Crk (lanes 4-6), or by c-Crk-II with myc tag (lanes 7-9).  Lane 10, vector control.  Upper panel, lysates were immunoprecipitated by anti-Crk Ab, and ERM proteins bound to Crk were detected by immunoblotting using anti-Flag tag Ab.  E, R, M stand for ezrin, radixin, and moesin, respectively.  The expression levels of exogeneous ERMs were examined by anti-Flag Ab (middle panel), and those of v-Crk and c-Crk-II were by anti-Crk Ab (lower panel).  B. Association of c-Crk-II with ERMs in 3Y1 cell lines.  The following were stably expressed in 3Y1 cells:  Lane 1, C as control vector; lane 2, c-Crk-II; lane 3, ezrin; lane 4, radixin; lane 5, moesin; lane 6, c-Crk-II and ezrin; lane 7, c-Crk-II and radixin; lane 8, c-Crk-II and moesin.  c-Crk-II was tagged with myc and ERMs were with Flag.  Using cell lysates, immunoprecipitation of anti-Crk Ab was performed and precipitants were probed with anti-Flag Ab (upper panel).  The expression levels of exogenous c-Crk-II and ERMs are shown (middle and lower panels).  C.  Association of ERMs and Crk mutants.  293T cells were transiently transfected with the expression vectors as follows: Lane 1, vector control; lanes 2-4, either of the ERMs alone; lanes 5-7, either of the ERMs and wild-type c-Crk-II; lanes 8-10, either of the ERMs and SH2-mutant of c-Crk-II, R38V; lanes 11-13, either of the ERMs and SH3-mutant of c-Crk-II, W169L.  Cell lysates were immunoprecipitated by anti-Crk Ab and probed with anti-Flag Ab (upper panel).  The expression levels of exogenous ERMs and c-Crk-II were examined (middle and lower panels).

Fig. 2  A.  Analysis of RhoA and Rac activity in Crk and ERMs-expressing cells.  293T cells were transiently transfected with expression vectors of myc-tagged c-Crk-II and
Flag-tagged ERMs as follows: Lane 1, c-Crk-II; lane 2, ezrin; lane 3, radixin; lane 4, moesin; lane 5, c-Crk-II and ezrin; lane 6, c-Crk-II and radixin; lane 7, c-Crk-II and moesin.

GTP-bound forms of endogenous RhoA and Rac were precipitated by GST-rhotekin-RBD and GST-PAK-RBD, respectively, and detected by immunoblotting by using anti-RhoA and Rac antibodies. Protein expression levels of total RhoA or Rac were also examined and ratios of the GTP-form of RhoA/Rac and total RhoA/Rac were calculated and described as a bar graph.

B. Association of Crk with Rho-GDI. In 3Y1 cells stably expressing CrkII and ezrin, the localizations of Rho-GDI and CrkII were analyzed by immunofluorescence microscopy using anti-Rho-GDI Ab (a, green) and anti-Crk Ab (b, red). Co-localization of Rho-GDI and c-Crk-II was demonstrated by merged imaging (c, yellow). C. 293T cells were transiently transfected with the expression vector of c-Crk-II (lane 1), Rho-GDI (lane 2), both c-Crk-II and Rho-GDI (lane3), c-Crk-II, Rho-GDI, and either of the ERMs (lane 4-6). c-Crk-II and ERMs were tagged with Flag. The lysates were immunoprecipitated by anti-Rho-GDI Ab and probed with anti-Flag antibody, demonstrating coprecipitated c-Crk-II (upper panel, arrowheads) and ERMs (upper panel, asterisk). The expression levels of exogenous c-Crk-II and ERMs are shown in the lower panel.

**Fig. 3** Association of Crk and the phosphorylated form of ERMs. A. Analysis of the binding between v-Crk and pERMs in the v-Crk-inducible 3Y1 cell line (clone 21-2-1). After the treatment of doxycycline (Dox) to induce v-Crk, total cell lysates (lane 1) and each fraction of cell lysates indicated above the panel (lane 2-4) were examined by immunoprecipitation using anti-pERM Ab (297S), and probed with anti-gag Ab (3C2) for detection of v-Crk (upper panel). The expression level of v-Crk in each lysate was analyzed by immunoblotting by using 3C2 Ab (lower panel). B. Immunofluorescent analysis of
pERMs by induction of v-Crk. The v-Crk-induced 3Y1 cells (clone 21-2-1) were treated with Dox for 48 hours (photographs, b-j). Cells with no Dox treatment are shown as photo a. Within 48 hours, cells were also treated with ROCK inhibitor, Y27632 (e, f, and g) or PIP2 inhibitor, neomycin (h, i, and j). Localizations of pERMs and v-Crk were analyzed by anti-pERM Ab (297S) (green) and anti-gag Ab (red), respectively. Arrowheads indicate the colocalization of v-Crk and pERMs. C. The phosphothreonine levels of ERMs in 3Y1 21-2-1 cells in the absence (lane 1, 3, and 5) or the presence (lane 2, 4, and 6) of Dox were examined by immunoblotting by anti-pERM Ab. The cells were also treated with Y27632 (lane 3 and 4) or neomycin (lane 5 and 6).

**Fig. 4** Immunoelectron microscopical analysis of the association of pERMs and v-Crk.

a Immunopositive reaction of pERM in v-Crk-expressing 3Y1 cells observed by light microscopy. pERMs were visualized by enzymatic reaction using DAB as a brown color after counterstaining with toluidine blue. b-e Transmission electron microscopy for the colocalization of v-Crk as 10-nm gold particles and pEMR as electron-dense substance at microvilli. The areas in the boxes of panels b and d were enlarged and displayed as panels c and e, respectively. f and g Colocalization of v-Crk and pERM at the filamentous structure in the cytoplasm (panel f) and at the cytoplasmic edge (panel g). Insets of panels f and g are enlarged images of 10-nm gold particles of each panel. Scale bars: b, 300 nm; d, 1µm; and f and g, 100 nm.

**Fig. 5** A. Cleavage of CD44 in coexpressing cells of Crk and ERMs. A. The levels of full-length and cleavage products of CD44 were investigated by anti-CD44cyto Ab. The cells were cultured on un-coating (upper panel) or hyaluronic acid-coating (lower panel)
dishes. Lane 1, parental 3Y1; lane 2, vector control; lane 3, c-Crk-II; lane 4, ezrin; lane 5, radixin; lane 6, moesin; lane 7, c-Crk-II and ezrin; lane 8, c-Crk-II and radixin; lane 9, c-Crk-II and moesin; lane 10, v-Src expressing 3Y1 (SR-3Y1). Arrows indicate the full length of CD44, and arrowheads indicate the cleavage products of CD44. B. Analysis of cell motility of Crk and EMRs-expressing 3Y1 cells by wound healing assay. The cells were plated on uncoated (upper graph) or hyaluronic acid-coated (lower graph) dishes. Light microscopical analyses of the cells were performed at 0 h, 12 h, and 18 h after wound formation. The percentage of recovery of wound formation was calculated, and the averages of three independent experiments were plotted as the graph. C. Effect of PI-3 kinase inhibitors in 3Y1 cell motility by wound healing assay. 3Y1 cells stably expressing either of the EMRs or Crk or both of the EMRs or Crk, indicated below the graph, were plated on HA-coated culture dishes, and wound healing assay was performed with or without PI-3 kinase inhibitors, 10 µM LY294002 or 200 nM Wortmannin. Twelve hours after scratching off the plates, the distances of the moved cells were measured. The distance of each cell line without the treatment of inhibitor was designated as 1.0 (bar graph not shown), and the relative distance of the moved cells of each cell line with the inhibitor was described as a bar graph.

**Fig. 6** Scheme of the regulation of hyaluronic acid-CD44-dependent cell motility by Crk. Crk bound to EMRs possibly with Rho-GDI and promoted hyaluronic acid-CD44-dependent cell motility. The association of Crk and the negative regulator for Rho such as Rho-GDI may suppress the function of Rho-GDI, resulting in the activation of Rho. The following activation of ROCK leads to the phosphorylation of EMRs which link CD44 to actin fibers.
Fig. 2

A

|         | Crk | E  | R  | M  | E  | R  | M  |
|---------|-----|----|----|----|----|----|----|
| total RhoA |     |    |    |    |    |    |    |
| RhoA GTP  |     |    |    |    |    |    |    |
| ratio     | 1.3 | 1.2| 1.5| 1.0| 1.8| 3.7| 1.9|

|         | 1   | 2   | 3   | 4   | 5   | 6   | 7   |
|---------|-----|-----|-----|-----|-----|-----|-----|
| total Rac1 |    |    |    |    |    |    |    |
| Rac1 GTP  |    |    |    |    |    |    |    |
| ratio     | 1.7 | 1.1| 1.4| 1.0| 1.9| 2.6| 1.2|

|         | Crk | E  | R  | M  | E  | R  | M  |
|---------|-----|----|----|----|----|----|----|
| Fold intensity of GTP-RhoA | 0   | 0.5| 1 | 1.5| 2 | 2.5| 3.0|

|         | Crk | E  | R  | M  | E  | R  | M  |
|---------|-----|----|----|----|----|----|----|
| Fold intensity of GTP-Rac1 | 0   | 0.5| 1 | 1.5| 2 | 2.5| 3.0|

B

α Rho-GDI  α Crk  merge

C

ERM - - - E  R  M
Rho-GDI + + + + + +
Crk + - + + + + +

| 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| IP: α Rho-GDI | Flag-ERM | Flag-Crkl |

IB: α Flag
Fig. 3

A

IB: α-v-Crk

IP-αpERM

total cell lysate
cytosol
nucleus,
cytoskeleton
membrane-skeletal

(K)

1 2 3 4

total cell
lysate
|          | Dox | Y27632 | neomycin |
|----------|-----|--------|----------|
| Untreated| -   | -      | -        |
| + Dox   | +   | -      | -        |
| Y27632  | +   | +      | -        |
| Neomycin| -   | -      | +        |

(kDa) 1 2 3 4 5 6

IB: α p-ERM

**Fig. 3**

- **B** α p-ERM
- **C** α gag merge

**NT**

**Y27632**

**neomycin**

(kDa) 75 1 2 3 4 5 6

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Fig. 4

|   | a | b | c | d | e | f | g |
|---|---|---|---|---|---|---|---|

300 nm

1 μm

100 nm
Fig. 5

A

Figures showing results of no treatment and hyaluronic acid (kDa) treatments.

B

Graphs illustrating un-coating and HA-coating recovery with time (h).

Lines represent different treatments:
- Cont.
- Ezrin
- Radixin
- Moesin
- Crk
- Ezrin & Crk
- Radixin & Crk
- Moesin & Crk
Fig. 5

C

![Bar graph showing the distance (ratio) for different proteins under 10 µM LY294002 and 200 nM Wortmannin conditions.](image)

- Ezrin
- Radixin
- Moesin
- Crk
- Ezrin & Crk
- Radixin & Crk
- Moesin & Crk

- 10 µM LY294002
- 200 nM Wortmannin
Crk associates with ERM proteins and promotes cell motility toward hyaluronic acid
Masumi Tsuda, Yoshinori Makino, Toshinori Iwahara, Hiroshi Nishihara, Hirofumi Sawa,
Kazuo Nagashima, Hidesaburo Hanafusa and Shinya Tanaka

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