RESEARCH COMMUNICATION

Activation of Rac1 by a Crk SH3-binding protein, DOCK180

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DOCK180 is involved in integrin signaling through CrkII-p130^{Cas} complexes. We have studied the involvement of DOCK180 in Rac1 signaling cascades. DOCK180 activated JNK in a manner dependent on Rac1, Cdc42Hs, and SEK, and overexpression of DOCK180 increased the amount of GTP-bound Rac1 in 293T cells. Coexpression of CrkII and p130^{Cas} enhanced this DOCK180-dependent activation of Rac1. Furthermore, we observed direct binding of DOCK180 to Rac1, but not to RhoA or Cdc42Hs. Dominant-negative Rac1 suppressed DOCK180-induced membrane spreading. These results strongly suggest that DOCK180 is a novel activator of Rac1 and involved in integrin signaling.

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The Rho family of low-molecular-weight G proteins, which consists of the Rho, Rac, and Cdc42 subfamilies, regulates the actin cytoskeleton (Hall 1998). Microinjection of constitutive active forms of these proteins into serum-starved fibroblasts demonstrated that Rho stimulates the organization of actin stress fibers, Rac stimulates the formation of lamellipodia or membrane ruffling, and Cdc42 induces the formation of filopodia (Nobes and Hall 1995).

Like other G proteins, the Rho-family proteins are activated by guanine nucleotide exchange proteins (GEPs) and inactivated by GTPase-activating proteins (GAPs) (Lamarche and Hall 1994; Nobes and Hall 1994). The catalytic domains of GEPs for Rho share amino-acid sequences with the Dbl-oncogene product; thus, this domain is designated as the Dbl-homology domain (Cerione and Zheng 1996). It has been also reported that PIP₂ and lipid kinases bind to and activate Rho family members (Tolias et al. 1995, 1998; Zheng et al. 1996). A third group of Rho regulator is RhoGDI, a GDP-dissociation

inhibitor of Rho (Sasaki and Takai 1998). RhoGDI binds to GDP-bound Rho proteins and retains them in the cytoplasm in an inactive form (Sasaki et al. 1993).

CrkII is a cellular homolog of the v-Crk oncogene product and consists almost entirely of SH2 and SH3 domains (Matsuda et al. 1992). Two major CrkII SH2-binding proteins are paxillin and p130^{Cas}, both of which are components of focal adhesions (for review, see Kiyokawa et al. 1997). More recently, it has been shown that CrkII-p130^{Cas} complexes regulate integrin-mediated cell movement and spreading (Vuori et al. 1996; Klemke et al. 1998). The amino-terminal SH3 domain of CrkII binds to several proteins; among the most prominent are C3G and DOCK180 (Matsuda and Kurata 1996).

Homologs of *DOCK180* have recently been isolated from *Drosophila melanogaster* and *Caenorhabditis elegans* and designated as *mbc* and *ced-5*, respectively (Erickson et al. 1997; Wu and Horvitz 1998). *ced-5* mutants are defective in the engulfment of cell corpses and the migration of the gonadal distal tip cells. The latter phenotype was rescued by the expression of human DOCK180. *mbc* was originally isolated as a gene that was essential for muscle development, suggesting that *mbc* is also involved in cellular motility.

Human DOCK180 also appears to be involved in cytoskeletal reorganization. Expression of membrane-targeted DOCK180 induced spreading of NIH-3T3 cells (Hasegawa et al. 1996). DOCK180 forms a complex with CrkII and p130^{Cas} only after integrin stimulation of NIH-3T3 cells. Expression of DOCK180 with CrkII and p130^{Cas} induces cell spreading and accumulation of DOCK180 at focal adhesions (Kiyokawa et al. 1998). However, the biochemical function of DOCK180 is unknown. Here, we demonstrate that DOCK180 activates and associates with the Rac1 GTPase and that DOCK180 requires Rac1 for the induction of cell spreading in NIH-3T3 cells.

Results

Morphologic resemblance between NIH-3T3 cells expressing membrane-targeted DOCK180 and constitutively active Rac1

Cells expressing constitutively active Rac1 or Tiam-1, a GEP for Rac1, show a pancake-shaped phenotype (Michiels et al. 1995), reminiscent of NIH-3T3 cells expressing membrane-targeted DOCK180 (Hasegawa et al. 1996). Therefore, we expressed membrane-targeted DOCK180, constitutively active Rac1 (RacV12), or constitutively active Cdc42Hs (Cdc42QL) in NIH-3T3 cells to compare directly the effect of these proteins on cell morphology. Expression of farnesylated DOCK180, RacV12, or Cdc42QL changed the spindle NIH-3T3 cells to flat polygonal cells with ruffling at the periphery (Fig. 1). The cells expressing farnesylated DOCK180, RacV12, and Cdc42QL were indistinguishable from each other.

[Key Words: DOCK180; Crk; Rac; mbc; ced-5; guanine nucleotide exchange factor]

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Figure 1. Morphology of cells expressing membrane-targeted DOCK180, constitutively active Rac1, and constitutively active Cdc42Hs. NIH-3T3 cells were microinjected with pEF-HA-RacV12 (RacV12), pEBG-Cdc42Hs-QL (Cdc42QL), pCA-DOCK-F (DOCK180), or pEBG (GST), incubated with anti-HA antibody for RacV12, anti-GST antibody for Cdc42Hs and GST, or anti-DOCK180 antibody for DOCK180. White bars, 23 μ m.

Activation of JNK by DOCK180

Both Rac1 and Cdc42Hs are known to activate JNK [Jun (amino) N-kinase] (Coso et al. 1995; Minden et al. 1995). Therefore, we wanted to determine whether DOCK180 activates JNK as well. GST-tagged JNK was expressed in 293T cells with or without DOCK180, and the *in vitro* kinase activity of JNK was examined by use of c-Jun as a substrate. JNK was activated by DOCK180 to a similar extent as the constitutively active Cdc42HsQL mutant (Fig. 2A). Another MAP kinase, ERK, was not activated by DOCK180. Coexpression of dominant-negative mutants of Rac1 (RacN17), Cdc42Hs (Cdc42N17), or SEK (SEKDN) inhibited DOCK180-dependent activation of JNK (Fig. 2B). Thus, DOCK180 appears to activate the JNK pathway in a manner dependent on Rac1, Cdc42Hs, and SEK. Furthermore, activation of JNK by DOCK180 was enhanced by the expression of CrkII, suggesting that signaling from tyrosine kinases may activate JNK through the CrkII-DOCK180 complex (Fig. 2C).

Activation of Rac1 by DOCK180

Next we directly examined whether DOCK180 activates Rac1. For measurement of the GTP/GDP ratio of Rac1, we employed the same system that we developed for the measurement of the GTP/GDP ratio of Rap1 in cells (Gotoh et al. 1995; Ichiba et al. 1997). In our assay system, the basal level of GTP-bound Rac1 was $\sim$ 2.0% in 293T cells (Fig. 3A). Coexpression of DOCK180 increased the GTP-bound Rac1 to 6.2%. The effect of DOCK180 on RhoA was also examined; GTP-bound RhoA was $\sim$ 15% with or without expression of DOCK180. We also tried to measure the GTP/GDP ratio on Cdc42Hs by the same method; however, 32 P-labeled guanine nucleotides bound poorly to Cdc42Hs, preventing us from quantifying the amount of GTP-loaded Cdc42Hs by this method.

The binding of GTP to Rac1 was also examined by quantifying the amount of [32 P]GDP bound to Rac1, by use of an assay developed previously to demonstrate the guanine nucleotide exchange activity of Vav, a Dbl-

homology domain-containing GEP for Rac1 (Crespo et al. 1997). The transfected cells were serum and phosphate starved for 16 hr, labeled with 32 P_i for 2 hr, and Rac1 was examined for the bound [32 P]GDP (Fig. 3B). Under these conditions, the increase in GTP-bound Rac1 by DOCK180 was not remarkable as seen in Figure 3A. However, the expression of DOCK180 increased the amount of [32 P]GDP bound to Rac1 as did the expression of Vav (Fig. 3B). We observed the most prominent increase in the Rac1-bound [32 P]GDP by the coexpression of DOCK180 and Vav. Similarly, we examined the effect of DOCK180 on the guanine nucleotide exchange of Cdc42Hs (Fig. 3C). [32 P]GDP on Cdc42Hs was not increased by the expression of DOCK180. Thus, promotion

Figure 2. Activation of JNK by DOCK180. (A) 293T cells were transfected with pZipNeo-RasV12, pcDNA3-Cdc42QL, pCA-DOCK180, or empty vector with either pEBG-ERK2 or pEBG-JNK. Thirty-two hours after transfection, medium was changed to DMEM containing 0.5% BSA. After 16 hr starvation, cells were lysed and incubated with glutathione-Sepharose. Beads were washed and incubated in reaction buffer containing [γ - 32 P]ATP and either MBP for ERK kinase assay or GST-c-Jun for JNK kinase assay. The reaction mixture was separated by SDS-PAGE. Phosphorylated MBP (phospho-MBP) or GST-c-Jun (phospho-c-Jun) was visualized and quantitated with a Molecular Imager (Bio-Rad). Comparable expression of GST-ERK or GST-JNK was examined by Western blotting by use of anti-GST antibody. The graphs show the average and standard deviation of at least three experiments. (B) pCA-DOCK180 and pEBG-JNK were transfected into 293T cells with empty vector, pCMV-RacN17, pCMV-Cdc42HsN17, or pEBG-SEK-DN, and processed as in A. (C) pCA-DOCK180 and pEBG-JNK were transfected into 293T cells with or without pCA-myc-CrkII, and processed as in A.

Figure 3. Activation of Rac1 by DOCK180. (A) 293T cells were transfected with pEBG-Rac1 (Rac1) or pEBG-RhoA (RhoA) in combination with pCA-Flag-DOCK180 or empty vector (see Materials and Methods). The labeled guanine nucleotides bound to GST-tagged G proteins were separated by TLC and visualized and quantitated with a Molecular Imager. (Ori) Origin of the TLC plate. Data shown were obtained from three independent experiments. Bars, standard deviation. (B) 293T cells were transfected with pEBG-Rac1 in combination with pCA-Flag-DOCK180 or pSR-Vav and processed as in A, except that cells were serum and phosphate starved for 16 hr before $^{32}\text{P}_i$ labeling. Expression of GST-Rac1 was confirmed by Western blotting (middle). (C) 293T cells were transfected with pEBG-Cdc42Hs with or without pCA-Flag-DOCK180 and processed as in B. (D) 293T cells were transfected with pEBG-Rac1 in combination with pCA-Flag-DOCK180 or pCA-myc-CrkII and pSR α p130^{Cas} and processed as in B.

of the guanine nucleotide exchange reaction by DOCK180 was specific to Rac1.

We further examined the effect of CrkII and p130^{Cas} on the activation of Rac1, because expression of the CrkII-p130^{Cas} complex recruits DOCK180 to focal adhesions (Kiyokawa et al. 1998). As shown in Figure 3D, expression of CrkII and p130^{Cas} was sufficient to promote guanine nucleotide exchange of Rac1; however it was further promoted by the coexpression of DOCK180. This

result strongly suggests that DOCK180 transduces signals from integrin to Rac1.

Binding of DOCK180 to Rac1

The previous results imply that DOCK180 might be a direct activator of Rac1. Thus, we examined the association of DOCK180 with Rac1, Cdc42Hs, and RhoA. 293T cells were transfected with expression vectors, lysed, and GST-tagged proteins were collected on glutathione-Sepharose and examined for binding to DOCK180 by immunoblotting. DOCK180 coprecipitated with Rac1 only when cells were lysed in EDTA-containing buffer (Fig. 4A). Binding of DOCK180 to RhoA or Cdc42Hs was not observed. Binding of Rac1 to DOCK180 in the presence of EDTA strongly suggested that DOCK180 bound to the nucleotide-free Rac1 protein. To avoid possible artifacts of using GST-tagged proteins and to understand the role of guanine nucleotides on Rac1/DOCK180 association, we next used HA-tagged Rac1 proteins, RacN17 and RacV12. Both RacN17 and RacV12 bound to DOCK180 in the presence of EDTA when coexpressed in 293T cells (Fig. 4B). However, only RacN17 bound to DOCK180 when Mg^{2+} was included in the lysis buffer. RacN17 binds to guanine nucleotides less efficiently than does the wild-type Rac1; therefore, this also implied that DOCK180 binds to guanine nucleotide-free Rac1. We examined further whether DOCK180 directly bound to Rac1 (Fig. 4C, left). Only Rac1 bound to DOCK180, and the nucleotide-free Rac1, but not GTP- γ S- or GDP-loaded Rac, bound to DOCK180 (Fig. 4C, right).

Effect of dominant-negative Rac1 on the localization of DOCK180

DOCK180 forms a complex with CrkII and p130^{Cas} and induces cell spreading of NIH-3T3 cells (Kiyokawa et al. 1998). In cells expressing DOCK180, DOCK180 was particularly concentrated at the site of membrane spreading and at focal adhesions (Fig. 5, second row). Therefore, we examined the role of Rac1 on the membrane spreading induced by the DOCK180-CrkII-p130^{Cas} complex by use of RacN17. Expression of RacN17 significantly suppressed both cellular spreading and recruitment of CrkII, p130^{Cas}, and DOCK180 to focal adhesions (Fig. 5, third and fourth rows). Close examination of RacN17-expressing cells revealed that expression of RacN17 and concentration of DOCK180 at the membrane were exclusive of each other (Fig. 5, fourth row). These results demonstrated that Rac1 is required for the DOCK180-induced morphological change and also for the recruitment of DOCK180 to sites of membrane spreading.

Discussion

In this study we have shown that DOCK180 is a novel upstream regulator of Rac1. DOCK180 binds to nucleotide-free Rac1, as do many of the guanine nucleotide exchangers for the low-molecular-weight G proteins (Miki et al. 1993; Miki 1995). DOCK180 does not contain the Dbl-homology domain, which is the catalytic domain of

Figure 4. Binding of DOCK180 to Rac1. (A) 293T cells were transfected with pEBG, pEBG-RhoA, pEBG-Rac1, or pEBG-Cdc42Hs, lysed, and GST-tagged proteins were collected by incubation with glutathione-Sepharose, separated by SDS-PAGE, and blotted with anti-DOCK180 antibody. Arrows and numbers at right indicate the position of DOCK180 (1), GST-RhoA (2), GST-Rac1 and Cdc42Hs (3), and GST (4). (B) 293T cells were transfected with pCA-Flag-DOCK180, together with pEB-HA-RacN17 or pEB-HA-RacV12, lysed, and incubated with anti-DOCK180 antibody and protein A-Sepharose for 2 hr. Immune complexes were washed, separated by SDS-PAGE, and blotted with anti-HA-antibody. (IP, NRS) Immunoprecipitation, normal rabbit serum, respectively. (C) DOCK180 purified from 293T cells was incubated with glutathione-Sepharose, which was preloaded with nucleotide-free (-), GDP-bound (GDP), or GTP γ S-bound GST-Rac1 (GTP). Proteins bound to beads were analyzed by immunoblotting with anti-His antibody.

most, if not all, of the previously reported GEP for Rho family proteins (Hart et al. 1994; Cerione and Zheng 1996). Because the expression of DOCK180 enhanced Vav-induced guanine nucleotide exchange of Rac1, DOCK180 may be a coactivator of the Dbl-domain containing GEP, although we could not detect interaction between Vav and DOCK180 in cells (E. Kiyokawa and M. Matsuda, unpubl.). DOCK180 may function to stabilize the guanine nucleotide-free Rac1, which is an intermediate during the guanine nucleotide exchange reaction catalyzed by GEPs.

There are numerous putative GEPs for Rac; however,

only a few have been shown to increase the GTP/GDP ratio on Rac in cells (Hawkins et al. 1995). This is partly due to the lack of an antibody that efficiently precipitates GTP-bound Rac, and partly due to the strong GAP activity for Rac in the cells. Thus, the guanine-nucleotide exchange activity of Vav for Rac1 was demonstrated by pulse labeling of cells with 32 P_i (Crespo et al. 1997). Expression of Vav increased the amount of [32 P]GDP-Rac1, which was thought to reflect the rapid turnover from [32 P]GTP-Rac1 to [32 P]GDP-Rac1. We have been using GST-tagged G proteins for measurement of the ratio of GTP/GDP bound to various G proteins (Gotoh et al. 1995; Ichiba et al. 1997). By using this method, we succeeded in the measuring of the GTP/GDP ratio on Rac1 and showed an increase in the GTP/GDP ratio of Rac1 when DOCK180 is expressed in cells.

Genetic studies in *D. melanogaster* have revealed that *mbc* is required for muscle development. *mbc* mutant embryos exhibit defects in dorsal closure and cytoskeletal organization. It has been reported that this phenotype resembles that of the *Drosophila rac* (*Drac*) mutant, sug-

Figure 5. Effect of dominant-negative Rac1 on the DOCK180-induced morphologic change. NIH-3T3 cells were microinjected with expression vectors encoding proteins indicated at left. Cells were fixed and incubated with antibodies indicated at bottom. White arrowheads (rows 2 and 4) indicate cellular spreading. White bars, 27 μ m.

gesting that *mbc* lies in the same signaling pathways as *Drac* (Erickson et al. 1997). More recently, mutations in *mbc* have been shown to suppress Rough-eye phenotype evoked by the overexpression of wild-type *Drac* (J. Settleman, pers. comm.). These observations genetically support our finding that DOCK180 activates Rac1.

It remains undetermined how the dominant-negative mutants of both Rac1 and Cdc42Hs inhibit the DOCK180-dependent activation of JNK, when DOCK180 binds to and activates only Rac1. Dominant-negative mutants of the low-molecular-weight G proteins bind to and sequester their GEPs away from wild-type G proteins. Because many Dbl domain-containing GEPs have redundant substrate specificity (Sasaki and Takai 1998), it is likely that the dominant-negative Cdc42Hs inhibits GEP(s) that activate both Cdc42Hs and Rac. The GEP inhibited by Cdc42Hs might play a major role in the DOCK180-dependent Rac1 activation, leading to increased JNK activity.

It has been shown that CrkII-p130^{Cas} complexes regulate cell spreading after integrin stimulation and serve as a molecular switch for induction of cell migration (Vuori et al. 1996; Klemke et al. 1998). However, the downstream signaling molecules of CrkII have not been identified. There is substantial evidence that DOCK180 is the downstream effector of CrkII in integrin signaling (Kiyokawa et al. 1998). First, DOCK180 binds to CrkII after integrin stimulation; second, DOCK180 colocalizes with the CrkII-p130^{Cas} complexes at focal adhesions and at the sites of membrane spreading; and third, the expression of DOCK180 in 293T cells accelerates the formation of the CrkII-p130^{Cas} complexes. Because Rac1 is also known to be involved in cell migration and spreading (Hordijk et al. 1997; Keely et al. 1997), it is likely that the DOCK180 recruited to the CrkII-p130^{Cas} complexes on integrin stimulation transduces signals to Rac1 at focal adhesions, which eventually induces cell spreading.

Material and methods

Expression plasmids

pCA-DOCK180 and pCA-DOCK-F are expression plasmids encoding wild-type and farnesylated DOCK180, respectively (Hasegawa et al. 1996). pEBG encodes farnesylated GST. pCAGGS-His and pCAGGS-Flag are derivatives of the pCAGGS eukaryotic expression vector with His- and Flag-tag-coding sequences before the cloning sites, respectively (Niwa et al. 1991). The cDNA of DOCK180 was subcloned into pCAGGS-His or pCAGGS-Flag. The resulting vectors are indicated by the prefixes, pCA-His and pCA-Flag, respectively. pCAGGS-myc-CrkII has been described previously (Ota et al. 1998). Plasmids encoding RhoA, Rac1, and Cdc42Hs were generous gifts from J.S. Gutkind (Coso et al. 1995). The coding regions of RhoA, Rac1, and Cdc42Hs were subcloned into pEBG for the generation of GST-tagged proteins. pEB-HA-RacV12 and pEB-HA-RacN17 were gifts from K. Kaibuchi (NAIST, Nara, Japan); pEBG-ERK2, pEBG-JNK1, and pEBG-SEK-DN from B.J. Mayer (Children's Hospital, Boston, MA); pSS α -Cas, a p130^{Cas} expression vector, from H. Hirai (University of Tokyo, Japan). pSR-Vav, encoding full-length c-Vav, was a gift from Dr. H. Mano (Jichi Medical School, Tochigi, Japan).

Preparation of GST fusion proteins

pGEX-RhoA, Rac1, and Cdc42Hs were obtained from J.S. Gutkind and pGEX-c-jun from B.J. Mayer. Recombinant proteins fused to GST were expressed in *Escherichia coli* XL-2-Blue and purified by the use of glutathione-Sepharose as described previously (Smith and Johnson 1988).

Antibodies

Antibodies against DOCK180, CrkII, and GST were developed in our laboratory (Hasegawa et al. 1996). Anti-p130^{Cas} was a gift from H. Hirai. The anti-His, anti-vinculin, and anti-HA antibodies were purchased from Qiagen, Cymbus Bioscience, and Boehringer Mannheim, respectively.

Microinjection and cell staining

NIH-3T3 cells were plated subconfluently on fibronectin-coated glass dishes (Mat Tek) 16 hr before microinjection. Expression plasmids were injected into the nucleus by use of an Automated Microinjection System (Carl Zeiss). After 16 hr, cells were fixed with PBS containing 4% paraformaldehyde and 0.33 M sucrose at 25°C for 15 min, permeabilized with PBS containing 0.2% Triton X-100 for 3 min, preincubated in 1% BSA for 10 min, and incubated with indicated antibodies for 1 hr. The cells were further incubated with anti-rabbit antibody conjugated with FITC or Cy5 (Molecular Probes), mounted in 90% glycerol containing 1,4-diazabicyclo (2.2.2) octane (DABCO) (Sigma), and observed by confocal microscopy (Carl Zeiss).

In vitro MAP kinase assay

pEBG-ERK2 or pEBG-JNK1 was transfected into 293T cells with pCA-DOCK180, pcDNA3-Cdc42QL, pZipNeo-RasV12, or empty vector. After 36 hr, cells were serum-starved for 12 hr, washed three times with ice-cold TBS-V buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM Na₃VO₄], and lysed in KLB buffer [25 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 50 mM NaF, 1 mM Na₃VO₄, 10 mM sodium pyrophosphate, 10 mM β -glycerophosphate, 10% glycerol, 5 μ g/ml aprotinin, 1 mM PMSF]. Cleared cell lysates were incubated with glutathione-Sepharose for 30 min at 4°C. The beads were washed twice with KLB buffer and once with kinase buffer [10 mM HEPES (pH 7.4), 5 mM MgCl₂], and incubated in 10 μ l of kinase buffer containing 2 μ g of substrate (myelin basic protein for ERK2 or GST-c-Jun for JNK), 100 μ M ATP, and 10 μ Ci of [γ -³²P]ATP (6000 mCi/mmol, NEN) for 15 min at 30°C. The kinase reaction was stopped by addition of 10 μ l of 2 $\times$ SDS sample buffer. Samples were heat denatured for 10 min at 95°C and then separated by SDS-PAGE.

Measurement of guanine nucleotides bound to Rho-family GTPases

Quantitation of guanine nucleotides bound to low-molecular-weight G proteins was carried out as described previously (Gotoh et al. 1995). Briefly, pEBG-RhoA or pEBG-Rac1 was transfected into 2×10^5 293T cells in a 35-mm diam. dish with or without pCA-FLAG-DOCK180. Forty-eight hours after transfection, cells were labeled with 0.05 mCi of ³²P_i (NEN) in 0.5 ml of phosphate-free MEM (GIBCO-BRL) for 2 hr and lysed in lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 20 mM MgCl₂, 1 mM Na₃VO₄, 0.5% Triton X-100, 5 μ g/ml aprotinin, 1 mM PMSF].

Binding of GTPases to DOCK180 in vivo

293T cells were transfected with pEBG-RhoA, pEBG-Rac1, or pEBG-Cdc42Hs. After 48 hr, cells were lysed in EDTA buffer [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 0.5 mM Na₃VO₄, 5 μ g/ml aprotinin, 1 mM PMSF] or Mg buffer [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 10 mM NaF, 0.5 mM Na₃VO₄, 5 μ g/ml aprotinin, 1 mM PMSF]. The cleared cell lysate was incubated with glutathione-Sepharose for 30 min at 4°C. Proteins collected on glutathione-Sepharose were washed three times with lysis buffer, separated by SDS-PAGE, and analyzed by Western blot with anti-DOCK180 antibody or anti-GST antibody and detected by ECL. Similarly, pEB-HA-RacV12 and pEB-HA-RacN17 were cotransfected with pCA-Flag-DOCK180 and lysed in the buffer described above. Cleared cell lysates were incubated with normal rabbit serum or anti-DOCK180 antibody and protein A-Sepharose at 4°C for 2 hr. Immune complexes were washed with lysis buffer several times, separated by SDS-PAGE, and analyzed by immunoblotting with anti-DOCK180 antibody and anti-HA antibody.

Purification of DOCK180 from 293T cells

293T cells were transfected with pCA-His-DOCK180 as described above. After 48 hr, cells were disrupted by freeze-thawing, suspended in PBS containing 10 mM imidazole and protease inhibitors, and centrifuged at 12,000g for 30 min. The supernatant was loaded on HiTrap Chelating Column preloaded with NiSO₄ (Pharmacia), washed with 50 mM imid-

azole (pH 8.0) containing 100 mM NaCl, and eluted with 200 mM imidazole (pH 8.0) containing 100 mM NaCl.

In vitro binding assay

Binding of DOCK180 to Rac1 was examined essentially as described previously (Hart and Powers 1995). GST or GST-Rac was bound to glutathione-Sepharose, washed three times with C buffer [20 mM Tris-HCl (pH 7.5), 5% glycerol, 50 mM NaCl, 0.1% Triton X-100, 1 mM DTT], incubated in E buffer [20 mM Tris-HCl (pH 7.5), 5% glycerol, 50 mM NaCl, 0.1% Triton X-100, 1 mM DTT, 10 mM EDTA], and incubated for 20 min at 25°C. The glutathione-Sepharose was kept at 4°C or collected by brief centrifugation and resuspended in 500 μ l of M buffer [20 mM Tris-HCl (pH 7.5), 5% glycerol, 50 mM NaCl, 0.1% Triton X-100, 1 mM DTT, 10 mM MgCl₂] containing either 200 μ M GDP or 20 μ M GTP γ S for 20 min at 25°C. Beads were again collected by centrifugation and resuspended in 500 μ l of E or M buffer containing recombinant His-DOCK180 for 2 hr at 4°C. After washing in either E or M buffer, proteins bound to the beads were separated by SDS-PAGE and analyzed by Western blotting with anti-His antibody.

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References

- Cerione, R.A. and Y. Zheng. 1996. Dbl family of oncogenes. *Curr. Opin. Cell Biol.* **8**: 216–222.
- Coso, O.A., M. Chiariello, J.C. Yu, H. Teramoto, P. Crespo, N. Xu, T. Miki, and J.S. Gutkind. 1995. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* **81**: 1137–1146.
- Crespo, P., K.E. Schuebel, A.A. Ostrom, J.S. Gutkind, and X.R. Bustelo. 1997. Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. *Nature* **385**: 169–172.
- Erickson, M.S., B.J. Galletta, and S.M. Abmayr. 1997. Drosophila myoblast city encodes a conserved protein that is essential for myoblast fusion, dorsal closure, and cytoskeletal organization. *J. Cell Biol.* **138**: 589–603.
- Gotoh, T., S. Hattori, S. Nakamura, H. Kitayama, M. Noda, Y. Takai, K. Kaibuchi, H. Matsui, O. Hatase, H. Takahashi, T. Kurata, and M. Matsuda. 1995. Identification of Rap1 as a target for Crk SH3-binding guanine nucleotide-releasing factor, C3G. *Mol. Cell. Biol.* **15**: 6746–6753.
- Hall, A. 1998. Rho GTPases and the actin cytoskeleton. *Science* **279**: 509–514.
- Hart, M. and S. Powers. 1995. Ras-Cdc25 and Rho-Dbl binding assays: Complex formation in vitro. *Methods Enzymol.* **255**: 129–135.
- Hart, M.J., A. Eva, D. Zangrilli, S.A. Aaronson, T. Evans, R.A. Cerione, and Y. Zheng. 1994. Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogene product. *J. Biol. Chem.* **269**: 62–65.
- Hasegawa, H., E. Kiyokawa, S. Tanaka, K. Nagashima, N. Gotoh, M. Shibuya, T. Kurata, and M. Matsuda. 1996. DOCK180, a major CRK-binding protein, alters cell morphology upon translocation to the cell membrane. *Mol. Cell. Biol.* **16**: 1770–1776.
- Hawkins, P.T., A. Eguinoa, R.G. Qiu, D. Stokoe, F.T. Cooke, R. Walters, S. Wennstrom, L. Claesson-Welsh, T. Evans, M. Symons, and L. Stephens. 1995. PDGF stimulates an increase in GTP-Rac via activation of phosphoinositide 3-kinase. *Curr. Biol.* **5**: 393–403.
- Hordijk, P.L., J.P. ten Klooster, R.A. van der Kammen, F. Michiels, L.C.J.M. Oomen, and J.G. Collard. 1997. Inhibition of invasion of epithelial cells by Tiam 1-Rac signaling. *Science* **278**: 1464–1466.
- Ichiba, T., Y. Kuraishi, O. Sakai, S. Nagata, J. Groffen, T. Kurata, S. Hattori, and M. Matsuda. 1997. Enhancement of guanine-nucleotide exchange activity of C3G for Rap1 by the expression of Crk, CrkL, and Grb2. *J. Biol. Chem.* **272**: 22215–22220.
- Keely, P.J., J.K. Westwick, I.P. Whitehead, C.J. Der, and L.V. Parise. 1997. Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature* **390**: 632–636.
- Kiyokawa, E., N. Mochizuki, T. Kurata, and M. Matsuda. 1997. Role of Crk oncogene product in physiologic signaling. *Crit. Rev. Oncog.* **8**: 329–342.
- Kiyokawa, E., Y. Hashimoto, T. Kurata, H. Sugimura, and M. Matsuda. 1998. Evidence that DOCK180 upregulates signals from CrkII-p130^{Cas} complex. *J. Biol. Chem.* **273**: 24479–24484.
- Klemke, R.L., J. Leng, R. Molander, P.C. Brooks, K. Vuori, and D.A. Cheresh. 1998. CAS/Crk coupling serves as a 'molecular switch' for induction of cell migration. *J. Cell Biol.* **140**: 961–972.
- Lamarche, N. and A. Hall. 1994. GAPs for rho-related GTPases. *Trends Genet.* **10**: 436–440.
- Matsuda, M. and T. Kurata. 1996. Emerging components of the Crk oncogene product: The first identified adaptor protein. *Cell. Signalling* **8**: 335–340.
- Matsuda, M., S. Tanaka, S. Nagata, A. Kojima, T. Kurata, and M. Shibuya. 1992. Two species of human CRK cDNA encode proteins with distinct biological activities. *Mol. Cell. Biol.* **12**: 3482–3489.
- Michiels, F., G.G. Habets, J.C. Stam, R.A. van der Kammen, and J.G. Collard. 1995. A role for Rac in Tiam1-induced membrane ruffling and invasion. *Nature* **375**: 338–340.
- Miki, T. 1995. Interaction of ect2 and Dbl with Rho-related GTPases. *Method. Enzymol.* **256**: 90–98.
- Miki, T., C.L. Smith, J.E. Long, A. Eva, and T.P. Fleming. 1993. Oncogene ect2 is related to regulators of small GTP-binding proteins. *Nature* **362**: 462–465.
- Minden, A., A.N. Lin, F.X. Claret, A. Abo, and M. Karin. 1995. Selective activation of the JNK signaling cascade and c-jun transcriptional activity by the small GTPases rac and Cdc42Hs. *Cell* **81**: 1147–1157.
- Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**: 193–200.
- Nobes, C. and A. Hall. 1994. Regulation and function of the Rho subfamily of small GTPases. *Curr. Opin. Genet. Dev.* **4**: 77–81.
- . 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**: 53–62.
- Ota, S., S. Kizaka-Kondoh, Y. Hashimoto, H. Nishihara, K. Nagashima, T. Kurata, H. Okayama, and M. Matsuda. 1998. Signaling from CrkII-23 mutant that inhibits transformation of NRK cells by EGF and TGF- β . *Cell. Signalling* **10**: 283–290.
- Sasaki, T. and Y. Takai. 1998. The Rho small G protein family-Rho GDI system as a temporal and spatial determinant for cytoskeletal control. *Biochem. Biophys. Res. Commun.* **245**: 641–645.
- Sasaki, T., M. Kato, and Y. Takai. 1993. Consequences of weak interaction of rho GDI with the GTP-bound forms of rho p21 and rac p21. *J. Biol. Chem.* **268**: 23959–23963.
- Smith, D.B. and K.S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67**: 31–40.
- Tollas, K.F., L.C. Cantley, and C.L. Carpenter. 1995. Rho family GTPases bind to phosphoinositide kinases. *J. Biol. Chem.* **270**: 17656–17659.
- Tollas, K.F., A.D. Couvillon, L.C. Cantley, and C.L. Carpenter. 1998. Characterization of a Rac1 and RhoGDI-associated lipid kinase signaling complex. *Mol. Cell. Biol.* **18**: 762–770.
- Vuori, K., H. Hirai, S. Aizawa, and E. Ruoslahti. 1996. Introduction of p130cas signaling complex formation upon integrin-mediated cell adhesion: A role for Src family kinases. *Mol. Cell. Biol.* **16**: 2606–2613.
- Wu, Y.C. and H.R. Horvitz. 1998. *C. elegans* phagocytosis and cell-migration protein CED-5 is similar to human DOCK180. *Nature* **392**: 501–504.
- Zheng, Y., J.A. Glaven, W.J. Wu, and R.A. Cerione. 1996. Phosphatidylinositol 4,5-bisphosphate provides an alternative to guanine nucleotide exchange factors by stimulating the dissociation of GDP from Cdc42Hs. *J. Biol. Chem.* **271**: 23815–23819.

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