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

Etsuko Kiyokawa,1 Yuko Hashimoto,1 Shin Kobayashi,3 Haruhiko Sugimura,2 Takeshi Kurata,1 and Michiyuki Matsuda1,3,4

1Department of Pathology, National Institute of Infectious Diseases, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan; 2First Department of Pathology, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka 431-3192, Japan; 3Department of Pathology, Research Institute, International Medical Center of Japan, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan

DOCK180 is involved in integrin signaling through CrkII-p130Cas 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 p130Cas 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 (Ceri and Zheng 1996). It has been also reported that PIP2 sequences with the Dbl-oncogene product; thus, this domain is designated as the Dbl-homology domain (Ceri and Zheng 1996). It has been also reported that PIP2

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*Corresponding author.
E-MAIL mmatsuda@rimcj.go.jp; FAX 81-3-3205-1236.
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 ~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 ~15% with or without expression of DOCK180. We also tried to measure the GTP/GDP ratio on Cdc42Hs by the same method; however, 32P-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, 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...
of the guanine nucleotide exchange reaction by DOCK180 was specific to Rac1.

We further examined the effect of CrkII and p130Cas on the activation of Rac1, because expression of the CrkII-p130Cas complex recruits DOCK180 to focal adhesions (Kiyokawa et al. 1998). As shown in Figure 3D, expression of CrkII and p130Cas 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 Mg2+ 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-γS- 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 p130Cas 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 focal adhesions (Fig. 5, second row). Therefore, we examined the role of Rac1 on the membrane spreading induced by the DOCK180–CrkII–p130Cas complex by use of RacN17. Expression of RacN17 significantly suppressed both cellular spreading and recruitment of CrkII, p130Cas, 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 exchange 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
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, (Crespo et al. 1997). Expression of Vav increased the amount of $[^{32}\text{P}]\text{GDP}$-Rac1, which was thought to reflect the rapid turnover from $[^{32}\text{P}]\text{GTP}$-Rac1 to $[^{32}\text{P}]\text{GDP}$-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 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 4](image-url)  

**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 GTPyS-bound GST–Rac1 (GTP). Proteins bound to beads were analyzed by immunoblotting with anti-His antibody.

![Figure 5](image-url)  

**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 μm.
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<sup>C</sup>-cs 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<sup>C</sup>-cs 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<sup>C</sup>-cs 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<sup>C</sup>-cs 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 pCA–DOCK180 and pCA–DOCK-F.

#### Purification of DOCK180 from 293T cells

Purification of DOCK180 from 293T cells included the following steps: (1) DOCK180 was cotransfected with pCA–Flag–DOCK180 and lysed in the buffer described above. 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 MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.5% Triton X-100) for 2 hr and lysed in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 5% Triton X-100, 5 µg/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–Rac1 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 for 2 hr. Immune complexes were washed several times, separated by SDS-PAGE, and analyzed by immunoblotting with anti-DOCK180 antibody and anti-HA antibody.

#### Antibodies

Antibodies against DOCK180, CrkII, and GST were developed in our laboratory (Hasegawa et al. 1996). Anti-p130<sup>C</sup>-cs 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 confluenty on fibronectin-coated glass dishes (Mat Tek). After 16 hr, cells were fixed with PBS containing 4% paraformaldehyde and 0.33 mM 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, pDNA3–Cdc42Q, pZM16–RacV12, or empty vector. After 36 hr, cells were serum-starved for 12 hr, washed three times with ice-cold B5-V buffer (20 mM Tris-HCl [pH 7.5]), 150 mM NaCl, 0.5 mM Na<sub>2</sub>VO<sub>4</sub>, 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 Na<sub>2</sub>VO<sub>4</sub>, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 10% glycerol, 5 µg/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 MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>) and incubated in 10 µl of kinase buffer containing 2 µg of substrate (myelin basic protein for ERK2 or GST–c-Jun for JNK). 100 µCi ATP, and 10 µCi of [γ-<sup>32</sup>P]ATP (6000 mCi/mmol, NEN) for 15 min at 30°C. The kinase reaction was stopped by addition of 10 µl of 2x 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<sup>5</sup> 293T cells in a 35-mm dish with or without pCA–FLAG–DOCK180. Forty-eight hours after transfection, cells were labeled with 0.05 µCi of [γ-<sup>32</sup>P]GTPγS (5000 Ci/mmol, NEN) in 0.5 ml of phosphatase-free MEM (GIBCO–BRL) for 2 hr and lysed in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 20 mM MgCl<sub>2</sub>, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 0.5% Triton X-100, 5 µg/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 Na<sub>2</sub>VO<sub>4</sub>, 5 µg/ml aprotinin, 1 mM PMSF) for Mb gbuffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 10 mM NaF, 0.5 mM Na<sub>2</sub>VO<sub>4</sub>, 5 µg/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–Rac1 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.
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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 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. Beads were again collected by centrifugation and resuspended in 500 µl 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 µM GDP or 20 µM GTP-γ-S for 20 min at 25°C. Beads were again collected by centrifugation and resuspended in 500 µl of 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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