

DOCK2 is a Rac activator that regulates motility and polarity during neutrophil chemotaxis

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Neutrophils are highly motile leukocytes, and they play important roles in the innate immune response to invading pathogens. Neutrophil chemotaxis requires Rac activation, yet the Rac activators functioning downstream of chemoattractant receptors remain to be determined. We show that DOCK2, which is a mammalian homologue of *Caenorhabditis elegans* CED-5 and *Drosophila melanogaster* Myoblast City, regulates motility and polarity during neutrophil chemotaxis. Although DOCK2-deficient neutrophils moved toward the chemoattractant source, they exhibited abnormal migratory behavior with a marked reduction in translocation speed.

In DOCK2-deficient neutrophils, chemoattractant-induced activation of both Rac1 and Rac2 were severely impaired, resulting in the loss of polarized accumulation of F-actin and phosphatidylinositol 3,4,5-triphosphate (PIP₃) at the leading edge. On the other hand, we found that DOCK2 associates with PIP₃ and translocates to the leading edge of chemotaxing neutrophils in a phosphatidylinositol 3-kinase (PI3K)-dependent manner. These results indicate that during neutrophil chemotaxis DOCK2 regulates leading edge formation through PIP₃-dependent membrane translocation and Rac activation.

Introduction

Cell migration involves membrane polarization and cytoskeletal dynamics, both of which are regulated by Rho family GTPases (Raftopoulos and Hall, 2004). Among these molecules, Rac is crucial for generating the actin-rich lamellipodial protrusion, which is a principal part of the driving force for movement. Rac is composed of three isoforms, Rac1, Rac2, and Rac3. Rac1 is ubiquitously expressed and Rac3 is highly expressed in the brain, whereas Rac2 expression is largely restricted to hematopoietic cells. The role of Rac in neutrophil functions has been extensively analyzed with knockout mice lacking Rac1 and/or Rac2, and in a human patient with a point mutation in the conserved GTP-binding domain of Rac2. These studies clearly indicate that Rac2 is a major Rac isoform that regulates chemoattractant-induced neutrophil functions, such as chemotaxis and superoxide production (Roberts et al., 1999; Williams

et al., 2000; Gu et al., 2003). However, the defects in chemotaxis and superoxide production of Rac2-deficient neutrophils are significantly augmented by additional loss of Rac1 (Gu et al., 2003). In addition, it has been reported that Rac1 deficiency alone results in an inability of neutrophils to detect and to orient in a chemotactic gradient (Sun et al., 2004), suggesting that Rac1 is also involved in the chemotactic response of murine neutrophils.

Like other Rho family GTPases, Rac cycles between GDP-bound inactive and GTP-bound active states. Because the GTP loading is mediated by guanine nucleotide exchange factors (GEFs), significant efforts have been made to identify a Rac GEF that functions downstream of chemoattractant receptors in neutrophils. P-Rex1 is a phosphatidylinositol 3,4,5-triphosphate (PIP₃)- and Gβγ-regulated Rac GEF that has been purified from neutrophils (Welch et al., 2002). It had been thought that P-Rex1 would be a major Rac activator that regulates neutrophil chemotaxis. Unexpectedly, however, it was recently found that neutrophil chemotaxis is only mildly affected in P-Rex1-deficient (P-Rex1^{-/-}) neutrophils (Dong et al., 2005; Welch et al., 2005). In addition, chemoattractant-induced Rac activation was also reported to occur normally in neutrophils lacking both

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Abbreviations used in this paper: BM, bone marrow; fMLP, N-formyl-methionyl-leucyl-phenylalanine; GEF, guanine nucleotide exchange factor; HEK, human embryonic kidney; PH, pleckstrin homology; PI3K, phosphatidylinositol 3-kinase; PIP₃, phosphatidylinositol 3,4,5-triphosphate; SOD, superoxide dismutase.

The online version of this article contains supplemental material.

Vav1 and Vav3 (Gakidis et al., 2004). Thus, the Rac activator that is critical for neutrophil chemotaxis remains to be determined.

CDM family proteins (*Caenorhabditis elegans* CED-5, mammalian DOCK180, and *Drosophila melanogaster* Myoblast City) are known to regulate the actin cytoskeleton by functioning upstream of Rac (Wu and Horvitz, 1998). DOCK2 is a new member of the CDM family proteins, and is expressed predominantly in hematopoietic cells (Fukui et al., 2001). Although DOCK2 does not contain the Dbl homology domain and the pleckstrin homology (PH) domain that are typically found in GEFs, DOCK2 binds to nucleotide-free Rac and catalyzes GTP loading through its Docker (also known as DHR-2) domain (Brugnera et al., 2002; Côté and Vuori, 2002). We had previously reported that DOCK2 regulates lymphocyte migration and immunological synapse formation through Rac activation (Fukui et al., 2001; Sanui et al., 2003a). However, the role of DOCK2 in neutrophils remains unknown, an issue that was addressed in this study.

Results and discussion

To examine whether DOCK2 functions in neutrophils, we first compared Rac activation between C57BL/6 (B6) and DOCK2^{-/-} mice. When bone marrow (BM) neutrophils from B6 mice were stimulated with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), activated Rac1, Rac2, and Cdc42 were readily detected at 5 s after stimulation (Fig. 1 A). This activation was rapid and transient, and the levels of the GTP-bound Rac1, Rac2, and Cdc42 were substantially decreased at 15 s. In DOCK2^{-/-} neutrophils, Cdc42 was activated to the same extent and at the same kinetics as in B6 neutrophils (Fig. 1 A). However, fMLP-induced activation of both Rac1 and Rac2 were reduced by 70% in DOCK2^{-/-} neutrophils at 5 s after stimulation (Fig. 1 A). Similar defects were observed when DOCK2^{-/-} neutrophils were stimulated with PMA (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200602142/DC1>). These results indicate that DOCK2 plays a major role in chemoattractant- and PMA-induced Rac activation in neutrophils.

We next examined how DOCK2 deficiency affects neutrophil functions. In the transwell chemotaxis assay, 8–11% of B6 BM neutrophils migrated into the lower chamber in response to 8 μ M fMLP (Fig. 1 B, left). In the case of DOCK2^{-/-} neutrophils, however, the percentage of migrating cells was <2% under the same conditions (Fig. 1 B, left). A similar defect was observed when complement factor 5a (C5a) was used as a chemoattractant (Fig. 1 B, right). When B6 BM neutrophils were stimulated with fMLP, they produced superoxides in a superoxide dismutase (SOD)-inhibitable manner (Fig. 1 C, left). However, the total amount of superoxides produced by DOCK2^{-/-} neutrophils was reduced to <20% of the wild-type level (Fig. 1 C, left). In addition, unlike P-Rex1^{-/-} neutrophils (Dong et al., 2005; Welch et al., 2005), DOCK2^{-/-} neutrophils exhibited a defect in PMA-induced superoxide production (Fig. 1 C, right). These results indicate that DOCK2 is required in neutrophils for chemotaxis and superoxide production.

To determine more precisely the role of DOCK2 in neutrophil chemotaxis, we analyzed BM neutrophils undergoing

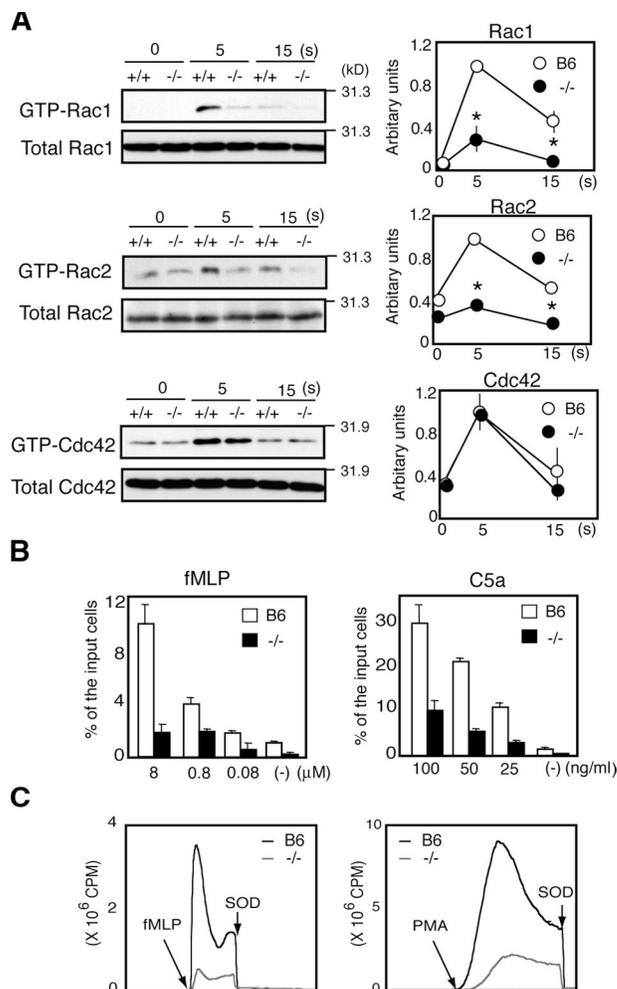


Figure 1. DOCK2 is a Rac activator that regulates chemotaxis and superoxide production in neutrophils. (A) BM neutrophils from B6 (+/+) and DOCK2^{-/-} (-/-) mice were stimulated with 8 μ M fMLP for the indicated times and analyzed for activation of Rac1, Rac2, and Cdc42. Results were quantified by densitometry and are expressed as the ratio of GTP-bound form to total protein after normalization of the 5-s value of B6 neutrophils to an arbitrary value of 1. The results indicate the mean \pm the SD of three separate experiments. *, $P < 0.01$. (B) Chemotactic response of BM neutrophils to fMLP or C5a was compared between B6 and DOCK2^{-/-} mice in a transwell chemotaxis assay. The results are expressed as the percentage of the input cells (mean \pm the SD of triplicate wells), and represent three separate experiments. (C) Superoxide production in response to 8 μ M fMLP or 200 ng/ml PMA was compared between B6 and DOCK2^{-/-} BM neutrophils. The results represent two separate experiments.

chemotaxis in a Zigmond chamber containing the fMLP gradient. At first glance, DOCK2^{-/-} neutrophils were less motile than B6 neutrophils (Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200602142/DC1>). Detailed analysis revealed that the average speed of DOCK2^{-/-} neutrophils was reduced to 55% of the wild-type level (Fig. 2 A). When the final location relative to the initial position was analyzed at 12.5 min, >85% of neutrophils had migrated toward the fMLP source, irrespective of DOCK2 expression (Fig. 2 B). However, although B6 neutrophils moved in relatively straight paths up the fMLP gradient, DOCK2^{-/-} neutrophils often showed indecisive wandering behavior and took a turn in a short period (Video 2). Supporting this, DOCK2 deficiency was found to affect the

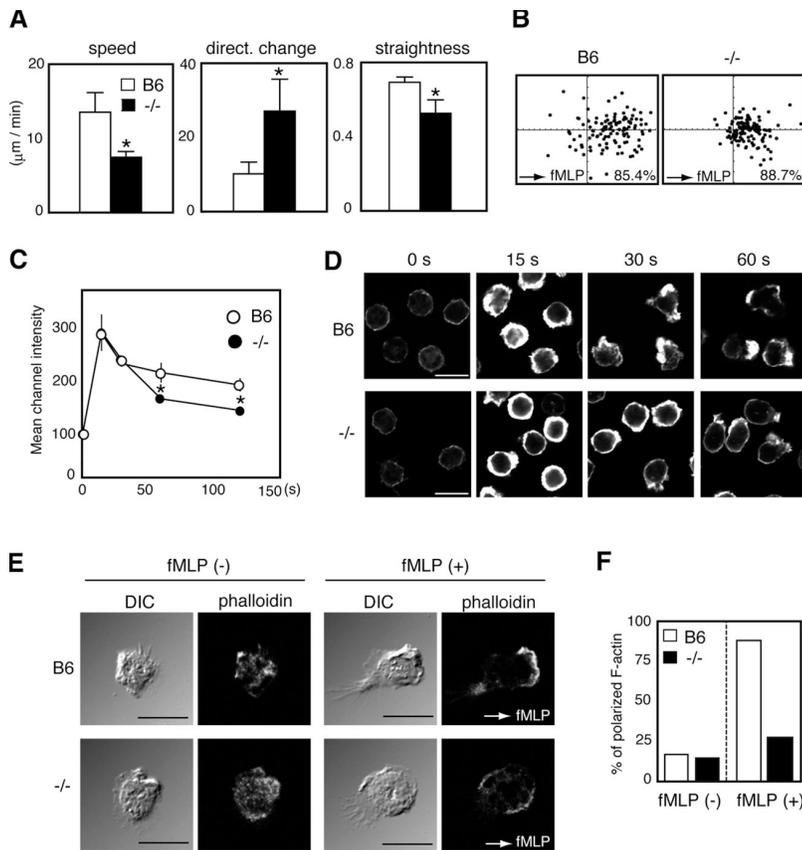


Figure 2. DOCK2 regulates motility and polarity during neutrophil chemotaxis. (A) BM neutrophils chemotaxing under the fMLP gradient were analyzed for speed, directional change, and straightness using time-lapse video microscopy. Data were collected at 15-s intervals for 12.5 min and processed with MetaMorph software. The directional change is a measure of the frequency of turns that a cell makes to move 100 μm in a given direction. Cells are judged to take a turn when the angle between the migration vector and the final direction changes from a positive value to a negative one, and vice versa. The straightness was estimated by dividing the distance from the initial position to the final location by the total path length. The results are expressed as the mean \pm the SEM of seven separate experiments using age-matched mice. *, $P < 0.001$. (B) The final location relative to the initial position is compared between B6 and DOCK2^{-/-} neutrophils ($n = 117$). The crossmarks indicate the origin. (C and D) BM neutrophils were stimulated in suspension with 10 μM fMLP for the indicated times, and analyzed for the content (C) and localization (D) of F-actin by staining the cells with phalloidin. In C, the results are expressed as the mean channel fluorescence (mean \pm the SD of triplicate wells) and represent three separate experiments. *, $P < 0.01$. (E and F) BM neutrophils chemotaxing under the fMLP gradient were stained with phalloidin, and the percentages of neutrophils with polarized F-actin localization were compared ($n = 200$). Cells are judged to be positive when F-actin staining is confined to less than one third of the circumference. Bars, 10 μm .

directional change and the straightness during neutrophil chemotaxis (Fig. 2 A).

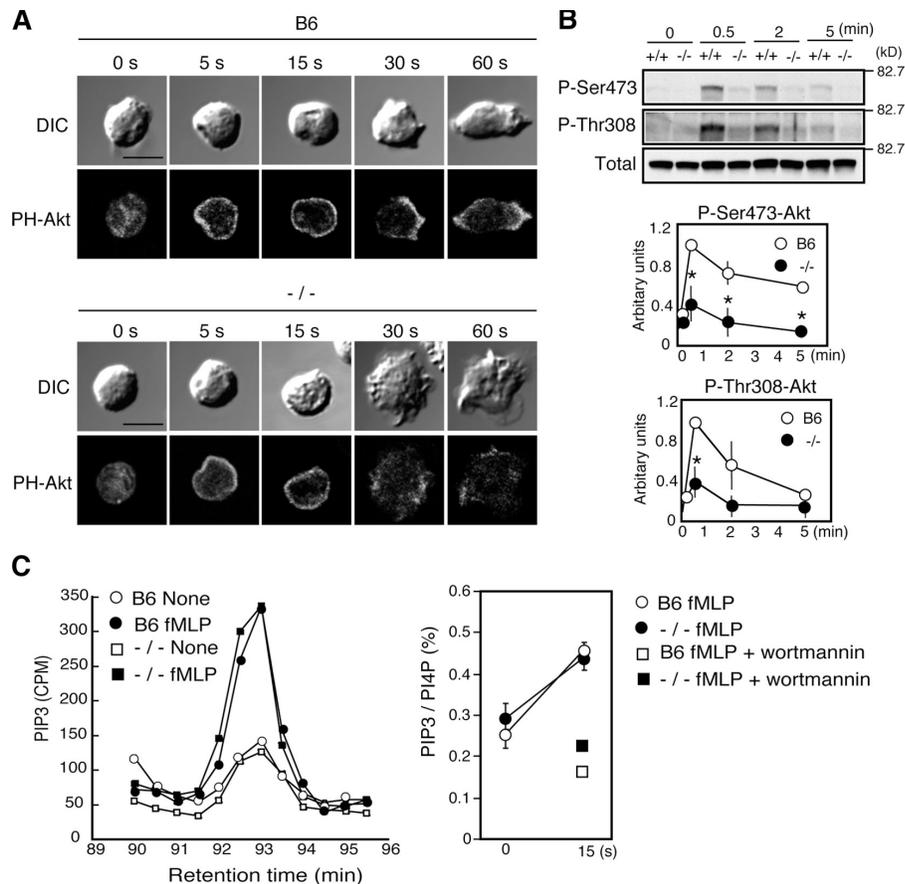
To understand the mechanism by which DOCK2 regulates neutrophil chemotaxis, we first examined actin polymerization in BM neutrophils stimulated in suspension with fMLP. Although fMLP-induced actin polymerization is almost totally abolished in neutrophils lacking Rac1 and Rac2 (Gu et al., 2003), such a drastic effect was not observed in DOCK2^{-/-} neutrophils (Fig. 2 C). This might result from the difference in Cdc42 activation because, unlike DOCK2 deficiency, Rac2 deficiency has been reported to impair fMLP-induced Cdc42 activation in neutrophils (Sun et al., 2004). Microscopic analysis revealed that both B6 and DOCK2^{-/-} neutrophils uniformly accumulated F-actin at 15 s after stimulation (Fig. 2 D). However, although B6 neutrophils exhibited a localized accumulation of F-actin at 30 s, such polarization was scarcely found in DOCK2^{-/-} neutrophils, although there was partial recovery of F-actin polarity at 60 s (Fig. 2 D). Consistent with this finding, the majority of DOCK2^{-/-} neutrophils undergoing chemotaxis exhibited aberrant morphology with poorly focused distribution of F-actin (Fig. 2, E and F). These results indicate that DOCK2 is required for polarized accumulation of F-actin at the leading edge.

In response to chemoattractants, neutrophils accumulate PIP₃, which is a lipid product of phosphatidylinositol 3-kinases (PI3Ks), at the leading edge (Servant et al., 2000). Because this process requires Rac activation and actin polymerization (Wang et al., 2002; Srinivasan et al., 2003), we examined how DOCK2 deficiency affects PIP₃ accumulation by transiently expressing a

GFP-tagged PH domain of Akt (PH-Akt), which is a widely used probe for detecting the spatial distribution of PIP₃. When BM neutrophils were exposed to a uniform concentration of fMLP, PH-Akt rapidly translocated to the plasma membrane in both B6 and DOCK2^{-/-} neutrophils (Fig. 3 A). However, while B6 neutrophils accumulated PH-Akt at the plasma membrane in a highly asymmetric manner at 30 and 60 s after stimulation, the membrane accumulation of PH-Akt was impaired in DOCK2^{-/-} neutrophils at these time points (Fig. 3 A). Similar, but more profound, effects of DOCK2 deficiency were observed when BM neutrophils were stimulated with fMLP supplied by a micropipette (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200602142/DC1>). Consistent with this defect, fMLP-induced Akt phosphorylation was reduced in DOCK2^{-/-} neutrophils (Fig. 3 B). Thus far, Akt phosphorylation has been used as an indirect assay for PIP₃ synthesis. Surprisingly, however, BM neutrophils from B6 and DOCK2^{-/-} mice comparably generated PIP₃ in response to fMLP and C5a (Fig. 3 C and Fig. S3). These results suggest that DOCK2-mediated Rac activation regulates the persistent accumulation of PIP₃ at the leading edge, independently of PI3K activities.

We then asked whether DOCK2 itself is recruited to the leading edge in response to chemoattractants. For this purpose, we developed knock-in mice, where the gene encoding GFP is inserted immediately after the last exon of DOCK2 with a modification of the stop codon. When BM neutrophils of DOCK2-GFP mice were exposed to a uniform concentration of fMLP, DOCK2 rapidly translocated to the plasma membrane (Fig. 4 A, left). However, such translocation was totally

Figure 3. DOCK2 is important in stabilizing the accumulation of PIP₃. (A) After transfection of *PH-Akt-GFP* DNA, BM neutrophils were exposed to a uniform concentration of fMLP (10 μ M), and analyzed for the membrane accumulation of PH-Akt at the indicated times. Bar, 10 μ m. (B) BM neutrophils from B6 (+/+) and *DOCK2*^{-/-} (-/-) mice were stimulated with 10 μ M fMLP for the indicated times and analyzed for Akt phosphorylation at Ser473 and Thr308. Results are expressed as the ratio of phosphorylated Akt to total Akt after normalization of the 30-s value of B6 neutrophils to an arbitrary value of 1. The results indicate mean \pm SD of three separate experiments. *, *P* < 0.01. (C) ³²Pi-labeled BM neutrophils were stimulated with 8 μ M fMLP for 15 s, deacylated, and separated by HPLC. A representative chromatogram showing the peaks corresponding to PIP₃ (left) and the percentage of PIP₃ to PI4P (right; mean \pm the SD of triplicate determination) are indicated. A sample of BM neutrophils treated with wortmannin before stimulation was included as a control.



abolished by pretreating the cells with a PI3K inhibitor wortmannin (Fig. 4 A, right). Although BM neutrophils polarized and accumulated DOCK2 at the leading edge in response to a point source of fMLP, the intracellular DOCK2 dynamics and morphological changes were severely impaired in cells pretreated with the PI3K inhibitors LY294002 and wortmannin (Fig. 4 B and not depicted). These results indicate that DOCK2 translocates to the leading edge in a PI3K-dependent manner.

To elucidate the mechanism for PI3K-dependent intracellular DOCK2 dynamics, we examined whether DOCK2 binds to PIP₃ by expressing DOCK2 in human embryonic kidney (HEK) 293T cells with or without ELMO1, which is known to function cooperatively with DOCK2 in lymphocytes (Sanui et al., 2003b). When cell extracts expressing DOCK2 alone were precipitated with PIP₃-coated beads, only a weak association was found (Fig. 5 A). However, the association of DOCK2 with PIP₃ was significantly augmented by coexpression with ELMO1 (Fig. 5 A). DOCK2 was associated with PIP₃, but not PI(3,4)P₂ and PI(4,5)P₂ (Fig. 5 B). This association seemed to be specific because the PIP₃ binding was inhibited when cell extracts were preincubated with PIP₃-containing liposomes (Fig. 5 C). Recently, DHR-1, which is an evolutionarily conserved domain among CDM family proteins, has been shown to play an important role in PIP₃ binding of DOCK180 (Côté et al., 2005). When a DOCK2 mutant lacking DHR-1 (DHR1del) was expressed in HEK293T cells with ELMO1, PIP₃ binding was hardly detected (Fig. 5 D). This does not result from an inability

to bind to ELMO1 because the deletion of DHR-1 does not affect DOCK2 binding to ELMO1 (unpublished data). Collectively, these results indicate that DOCK2 associates with PIP₃ through DHR-1, and that this association is indirectly regulated by ELMO1.

Several lines of evidence indicate that a PIP₃- and Rac-mediated positive-feedback loop is required for neutrophil chemotaxis by amplifying chemoattractant signals at the leading edge (Weiner et al., 2002; Wang et al., 2002; Srinivasan et al., 2003). We have shown that whereas DOCK2 translocates to the leading edge in a PI3K-dependent manner, DOCK2 activates Rac and stabilizes the accumulation of PIP₃ at the leading edge. These results suggest that DOCK2, in some sense, regulates leading edge formation by functioning in a PIP₃- and Rac-mediated feedback loop. However, it is unlikely that DOCK2-Rac signaling affects the catalytic activities of PI3Ks because BM neutrophils from B6 and *DOCK2*^{-/-} mice comparably generated PIP₃ in response to chemoattractants. Thus far, it has been reported that an inhibitor of actin polymerization reduces insulin-mediated Akt phosphorylation and PH-Akt translocation, without affecting PI3K activity, in cells other than neutrophils (Peyrollier et al., 2000). Similar findings have been reported in neutrophil-like HL-60 cells stimulated with fMLP (Wang et al., 2002). On the other hand, we found that the persistence and polarized distribution of PIP₃ is correlated with the localized F-actin assembly in fMLP-treated BM neutrophils. Although the precise meaning of this correlation remains unknown at this stage, the localized F-actin assembly might prevent PIP₃ diffusion away

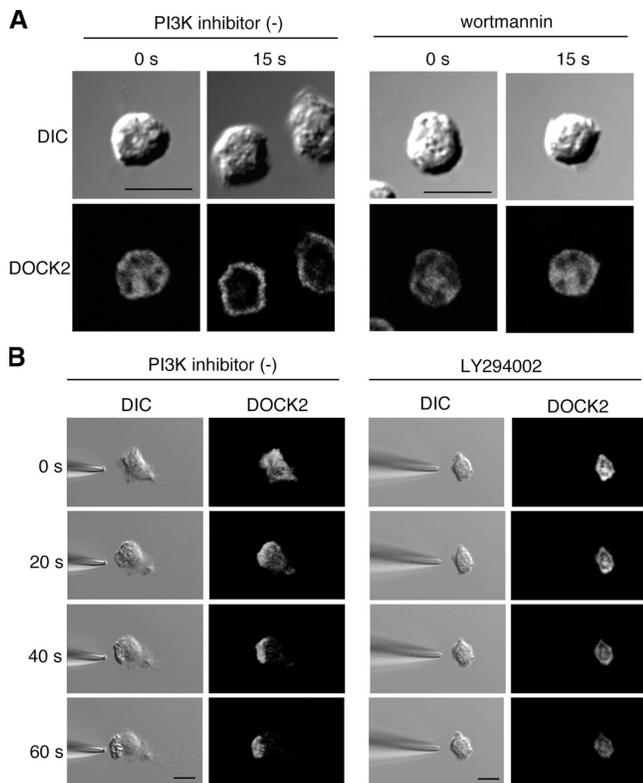


Figure 4. DOCK2 translocates to the leading edge in a PI3K-dependent manner. (A) BM neutrophils from DOCK2-GFP mice were exposed to a uniform concentration of fMLP (10 μ M), with or without pretreatment of 200 nM wortmannin, and analyzed for the membrane translocation of DOCK2 at 15 s. (B) BM neutrophils from DOCK2-GFP mice were stimulated with a micropipette containing 10 μ M fMLP, in the presence or absence of pretreatment of 400 μ M LY294002, and analyzed for intracellular DOCK2 dynamics at 5-s intervals with time-lapse video microscopy. Bars, 10 μ m.

from the leading edge and regulate activation of PIP₃-binding proteins by facilitating the effective protein-protein or protein-lipid interaction.

Materials and methods

Mice

DOCK2^{-/-} mice were backcrossed with B6 mice for more than eight generations before use. For development of DOCK2-GFP mice, a targeting vector was designed to insert the gene encoding GFP and a floxed-neomycin-resistant cassette (Neo) immediately after the last exon of DOCK2 and was introduced into embryonic stem cells by electroporation. Correctly targeted embryonic stem clones were microinjected into B6 blastocysts, and the male chimeras obtained were crossed with female B6 mice. Heterozygous mutant mice were crossed with Ella Cre mice to remove Neo, and Neo-deleted heterozygous mutant mice were intercrossed to develop homozygous mutants expressing the DOCK2-GFP chimeric molecule. All experiments were done in accordance with the guidelines of the committee of Ethics of Animal Experiments, Faculty of Medical Sciences, Kyushu University.

Isolation of BM neutrophils

BM cells were isolated from femurs and tibias of mice and layered onto the discontinuous Percoll (Sigma-Aldrich) gradient. After centrifugation, cells at the 81/62% interface were recovered and incubated with anti-B220-coated magnetic beads (Miltenyi Biotec) to remove B cells. More than 90% of the remaining cells were Gr-1⁺Mac-1⁺ mature neutrophils.

Chemotaxis assay

Transwell chemotaxis assays were performed as previously described (Fukui et al., 2001), using fMLP (Nacal Tesque) or C5a (Sigma-Aldrich)

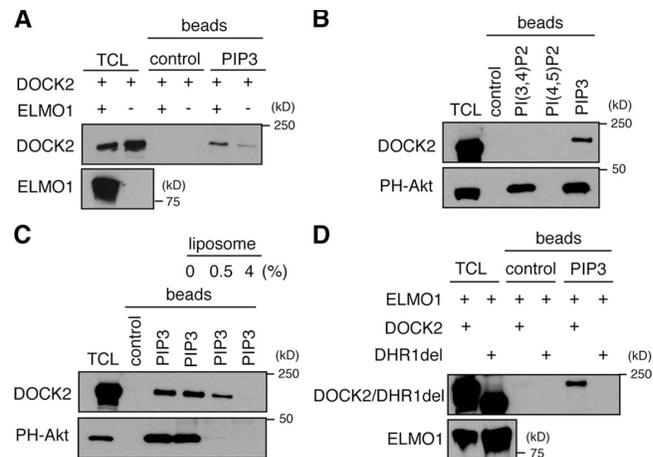


Figure 5. DOCK2 associates with PIP₃ through DHR-1. (A) After HA-tagged DOCK2 was expressed in HEK293T cells with or without ELMO1, cell extracts were incubated with PIP₃-coated or control beads, and the bound proteins were analyzed with an anti-HA antibody. The expression of DOCK2 and ELMO1 in total cell lysate (TCL) is shown on the left. (B and C) After HA-tagged DOCK2 and ELMO1 were coexpressed in HEK293T cells, cell extracts were incubated with (C) or without (B) phosphatidylinositol liposomes containing various concentrations of PIP₃ before a pull-down experiment using the indicated beads. As a control, PH-Akt-GFP was expressed in HEK293T cells and similarly analyzed with anti-GFP antibody. (D) After either Flag-tagged DOCK2 or DHR1del was expressed in HEK293T cells with ELMO1, cell extracts were incubated with PIP₃-coated or control beads, and the bound proteins were analyzed with anti-Flag antibody.

as chemoattractants. After a 3-h incubation at 37°C, cells migrating to the lower chamber were collected and stained with anti-Gr-1 (BD Biosciences) and anti-F4/80 (Invitrogen) mAbs. The percentage of migrating neutrophils was calculated by dividing the number of Gr-1⁺F4/80⁻ cells in the lower chamber by that of the input cells. Analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson).

For the chamber chemotaxis assay, BM neutrophils suspended in HBSS containing a 1:10 dilution of 10% gelatin were allowed to adhere to glass coverslips for 15 min at 37°C. The coverslips were rinsed and placed on a Zigmond chamber (Neuroprobes). Aliquots of HBSS containing gelatin were added to one side of the chamber and the same solution containing 10 μ M fMLP was added to the other side. Time-lapse video microscopy was used to examine neutrophil movement in Zigmond chambers placed on a heated stage (37°C). A microscope (model IX70; Olympus) was equipped with differential interference contrast optics, and a 20 \times or 40 \times objective. Images were captured with a camera (CoolSNAP HQ/OL; Photometrics) and processed with MetaMorph software (Universal Imaging Corp.).

Superoxide production

BM neutrophils (1×10^5) suspended in HEPES-buffered saline containing 0.03% BSA were stimulated at 37°C with 8 μ M fMLP or 200 ng/ml PMA, and the reaction was terminated by addition of 50 μ g/ml SOD. The chemiluminescence was counted with an enhancer-containing, luminol-based detection system (National Diagnostics) using a luminometer (Auto Lumat LB953; Berthold).

Actin polymerization and F-actin localization

BM neutrophils were stimulated with 10 μ M fMLP for the specified times, and then fixed with 4% paraformaldehyde and 0.1% BSA in PBS. Cells were treated with 0.1% Triton X-100, soaked in PBS containing 3% BSA, stained with Alexa Fluor 488-conjugated phalloidin (Invitrogen), and analyzed with a FACSCalibur flow cytometer.

For visualization of F-actin localization, BM neutrophils stimulated in suspension with 10 μ M fMLP or those chemotaxing under the 10 μ M fMLP gradient were fixed with 4% paraformaldehyde and treated with 0.2% Triton X-100 in PBS. Cells were then soaked in PBS containing 3% BSA and stained with Alexa Fluor 546-conjugated phalloidin (Invitrogen). All images were taken with a laser scanning confocal microscope (FLUOVIEW FV500; Olympus).

Membrane translocation of PH-Akt and DOCK2

BM neutrophils (5×10^6) were electroporated with 2.5 μ g PH-Akt-GFP DNA construct by using the Human Monocyte Nucleofector kit (Amaxa Biosystems) following protocol Y001, and then placed on a glass-bottom microwell dish. At 2 h after transfection, BM neutrophils were exposed to a uniform concentration of fMLP or stimulated on a heated stage (37°C) with a micropipette containing 10 μ M fMLP. Images were taken from either a laser scanning confocal microscope (FLUOVIEW FV500; Olympus) or a microscope (Axiovert 200M; Carl Zeiss MicroImaging, Inc.) with differential interference contrast and fluorescence image capability. The intracellular DOCK2 dynamics were similarly analyzed using freshly isolated BM neutrophils from DOCK2-GFP mice with or without pretreatment of PI3K inhibitors.

Metabolic cell labeling and lipid extraction

Neutrophils ($6\text{--}7.5 \times 10^7$) were labeled with [32 P]orthophosphate in a labeling buffer (136 mM NaCl, 4.9 mM KCl, 5.5 mM glucose, 0.1% BSA [fatty acid-free], and 10 mM HEPES-NaOH, pH 7.4) for 1.5 h at 37°C. Cells were stimulated with 8 μ M fMLP or 25 nM C5a for the specified times. Treatments were quenched by the addition of chloroform/methanol/8% HClO₄ (5:10:4). After vigorous vortexing, chloroform/HClO₄ (1:1) was added to isolate the organic phase, which was washed twice in chloroform-saturated 1% HClO₄ before drying. Dried lipids were resolved in chloroform/methanol (95:5) and separated by thin-layer chromatography, or they were deacylated and analyzed by HPLC as previously described (Serunian et al., 1991), using a Partisphere SAX column (Whatman).

Pull-down assay and immunoblotting

Aliquots of the cell extracts were kept for total lysate controls, and the remaining extracts were incubated with the GST-fusion, Cdc42/Rac-binding domain of PAK1 at 4°C for 60 min. The bound proteins and the same amounts of total lysates were analyzed by SDS-PAGE, and blots were probed with a mAb 23A8, which preferentially reacts with Rac1 (Upstate Biotechnology), Rac2-specific antibody (Santa Cruz Biotechnology, Inc.), or Cdc42-specific antibody (Upstate Biotechnology). Activation of Akt was assessed with a phosphorylation-specific antibody against Ser473 or Thr308 (Cell Signaling Technology).

For lipid-binding assays, HEK293T cells were transfected with the specified plasmid DNAs and suspended in 450 μ l of lipid-binding buffer (20 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA, pH 7.5). The cells were passed 10 times through a G25 needle and sonicated on ice. After insoluble debris was removed by ultracentrifugation at 100,000 g for 30 min at 4°C, a 20- μ l slurry of PIP beads (Echelon Biosciences) and 1% NP-40 solution (at a final concentration 0.25%) were added to the tube and incubated for 3 h at 4°C under rotary agitation. The beads were washed with lipid-binding buffer supplemented with 0.25% NP-40, and the bound proteins were subjected to immunoblotting. For competitive inhibition with liposomes, cell extracts were incubated on ice with 200 μ M phosphatidylserine/PIP₃ liposomes for 2 h before addition of PIP₃ beads and NP-40. Liposome was prepared as follows: phospholipids (Avanti Polar Lipids) dissolved in chloroform were mixed and dried down under nitrogen. The lipid film was then resuspended in lipid-binding buffer and sonicated on ice.

Online supplemental material

Fig. S1 shows that DOCK2 deficiency impairs PMA-induced Rac activation. Fig. S2 shows that lamellipod formation and membrane accumulation of PH-Akt are impaired in DOCK2^{-/-} neutrophils stimulated by a point source of fMLP. Fig. S3 shows that BM neutrophils from B6 and DOCK2^{-/-} mice comparably generate PIP₃ in response to C5a. Video 1 shows a low magnification image for B6 and DOCK2^{-/-} neutrophils chemotaxing under the fMLP gradient. Video 2 shows the migratory behavior of B6 and DOCK2^{-/-} neutrophils chemotaxing under the fMLP gradient. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200602142/DC1>.

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References

- Brugnera, E., L. Haney, C. Grimsley, M. Lu, S.F. Walk, A.C. Tosello-Tramont, I.G. Macara, H. Madhani, G.R. Fink, and K.S. Ravichandran. 2002. Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex. *Nat. Cell Biol.* 4:574–582.
- Côté, J.-F., and K. Vuori. 2002. Identification of an evolutionarily conserved superfamily of DOCK180-related proteins with guanine nucleotide exchange activity. *J. Cell Sci.* 115:4901–4913.
- Côté, J.-F., A.B. Motoyama, J.A. Bush, and K. Vuori. 2005. A novel and evolutionarily conserved PtdIns(3,4,5)P₃-binding domain is necessary for DOCK180 signalling. *Nat. Cell Biol.* 7:797–807.
- Dong, X., Z. Mo, G. Bokoch, C. Guo, Z. Li, and D. Wu. 2005. P-Rex1 is a primary Rac2 guanine nucleotide exchange factor in mouse neutrophils. *Curr. Biol.* 15:1874–1879.
- Fukui, Y., O. Hashimoto, T. Sanui, T. Oono, H. Koga, M. Abe, A. Inayoshi, M. Noda, M. Oike, T. Shirai, and T. Sasazuki. 2001. Haematopoietic cell-specific CDM family protein DOCK2 is essential for lymphocyte migration. *Nature.* 412:826–831.
- Gakidis, M.A., X. Cullere, T. Olson, J.L. Wilsbacher, B. Zhang, S.L. Moores, K. Ley, W. Swat, T. Mayadas, and J.S. Brugge. 2004. Vav GEFs are required for β_2 integrin-dependent functions of neutrophils. *J. Cell Biol.* 166:273–282.
- Gu, Y., M.D. Filippi, J.A. Cancelas, J.E. Siefring, E.P. Williams, A.C. Jasti, C.E. Harris, A.W. Lee, R. Prabhakar, S.J. Atkinson, et al. 2003. Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases. *Science.* 302:445–449.
- Peyrollier, K., E. Hajdich, A. Gray, G.J. Litherland, A.R. Prescott, N.R. Leslie, and H.S. Hundal. 2000. A role for the actin cytoskeleton in the hormonal and growth-factor-mediated activation of protein kinase B. *Biochem. J.* 352:617–622.
- Raftopoulos, M., and A. Hall. 2004. Cell migration: Rho GTPases lead the way. *Dev. Biol.* 265:23–32.
- Roberts, A.W., C. Kim, L. Zhen, J.B. Lowe, R. Kapur, B. Petryniak, A. Spaetti, J.D. Pollock, J.B. Borneo, G.B. Bradford, et al. 1999. Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense. *Immunity.* 10:183–196.
- Sanui, T., A. Inayoshi, M. Noda, E. Iwata, M. Oike, T. Sasazuki, and Y. Fukui. 2003a. DOCK2 is essential for antigen-induced translocation of TCR and lipid rafts, but not PKC- θ and LFA-1, in T cells. *Immunity.* 19:119–129.
- Sanui, T., A. Inayoshi, M. Noda, E. Iwata, J.V. Stein, T. Sasazuki, and Y. Fukui. 2003b. DOCK2 regulates Rac activation and cytoskeletal reorganization through interaction with ELMO1. *Blood.* 102:2948–2950.
- Serunian, L.A., K.R. Auger, and L.C. Cantley. 1991. Identification and quantification of polyphosphoinositides produced in response to platelet-derived growth factor stimulation. *Methods Enzymol.* 198:78–87.
- Servant, G., O.D. Weiner, P. Herzmark, T. Balla, J.W. Sedat, and H.R. Bourne. 2000. Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science.* 287:1037–1040.
- Srinivasan, S., F. Wang, S. Glavas, A. Ott, F. Hofmann, K. Aktories, D. Kalman, and H.R. Bourne. 2003. Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P₃ and polarity during neutrophil chemotaxis. *J. Cell Biol.* 160:375–385.
- Sun, C.X., G.P. Downey, F. Zhu, A.L. Koh, H. Thang, and M. Glogauer. 2004. Rac1 is the small GTPase responsible for regulating the neutrophil chemotaxis compass. *Blood.* 104:3758–3765.
- Wang, F., P. Herzmark, O.D. Weiner, S. Srinivasan, G. Servant, and H.R. Bourne. 2002. Lipid products of PI(3)Ks maintain persistent cell polarity and directed motility in neutrophils. *Nat. Cell Biol.* 4:513–518.
- Weiner, O.D., P.O. Neilsen, G.D. Prestwich, M.W. Kirschner, L.C. Cantley, and H.R. Bourne. 2002. A PtdInsP₃- and Rho GTPase-mediated positive feedback loop regulates neutrophil polarity. *Nat. Cell Biol.* 4:509–512.
- Welch, H.C., W.J. Coadwell, C.D. Ellson, G.J. Ferguson, S.R. Andrews, H. Erdjument-Bromage, P. Tempst, P.T. Hawkins, and L.R. Stephens. 2002. P-Rex1, a PtdIns(3,4,5)P₃- and G β -regulated guanine-nucleotide exchange factor for Rac. *Cell.* 108:809–821.
- Welch, H.C., A.M. Condliffe, L.J. Milne, G.J. Ferguson, K. Hill, L.M. Webb, K. Okkenhaug, W.J. Coadwell, S.R. Andrews, M. Thelen, et al. 2005. P-Rex1 regulates neutrophil function. *Curr. Biol.* 15:1867–1873.
- Williams, D.A., W. Tao, F. Yang, C. Kim, Y. Gu, P. Mansfield, J.E. Levine, B. Petryniak, C.W. Derrow, C. Harris, et al. 2000. Dominant negative mutation of the hematopoietic-specific Rho GTPase, Rac2, is associated with a human phagocyte immunodeficiency. *Blood.* 96:1646–1654.
- 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.