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 (Raftopoulou 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 isofrom 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; PI, 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 μM 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 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
revealed that both B6 and DOCK2
vation in neutrophils (Sun et al., 2004). Microscopic analysis
iciency has been reported to impair fMLP-induced Cdc42 acti-
Cdc42 activation because, unlike DOCK2 defi-
deneutrophils (Fig. 2 C). This might result from the difference in
the majority of DOCK2
F-actin polarity at 60 s (Fig. 2 D). Consistent with this fi nding,
inserted immediately after the last exon of
we developed knock-in mice, where the gene encoding GFP is
leading edge in response to chemoattractants. For this purpose,
we then asked whether DOCK2 itself is recruited to the
leading edge, independently of PI3K activities.

To understand the mechanism by which DOCK2 regu-
lates neutrophil chemotaxis, we first examined actin polymer-
ization 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 defi ciency, Rac2 de-
fi ciency has been reported to impair fMLP-induced Cdc42 acti-
allocation of 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 distribu-
tion 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
(P13Ks), 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 defi ciency were observed when
BM neutrophils were stimulated with fMLP supplied by a mi-
cropipette (Fig. S2, available at http://www.jcb.org/cgi/content/ full/jcb.200602142/DC1). Consistent with this defec-
t, 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 gen-
erated 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 P13K 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
abolished by pretreating the cells with a PI3K inhibitor wortmannin (Fig. 4A, 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. 4B 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. 5A). However, the association of DOCK2 with PIP3 was significantly augmented by coexpression with ELMO1 (Fig. 5A). DOCK2 was associated with PIP3, but not PI(3,4)P2 and PI(4,5)P2 (Fig. 5B). This association seemed to be specific because the PIP3 binding was inhibited when cell extracts were preincubated with PIP3-containing liposomes (Fig. 5C). 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. 5D). 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<sup>−/−</sup> 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 from the leading edge.
pretreatment of 400 nM wortmannin, and analyzed for the membrane translocation of a uniform concentration of fMLP (10 μM), 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 10 μM fMLP, in the presence or absence of pretreatment of 400 μM LY294002, and analyzed for intracellular DOCK2 dynamics at 5-s intervals with time-lapse video microscopy. Bars, 10 μm.

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 0.2% Triton X-100, soaked in PBS containing 3% BSA, and then fixed with 4% paraformaldehyde and 0.1% BSA in PBS. Cells were treated with 0.1% Triton X-100, stained with Alexa Fluor 488–conjugated phalloidin (Invitrogen), and the coverslips were rinsed and placed on a Zigmold chamber (Neuroprobes). Aliquots of HBSS containing gelatin were added to one side of the chamber and the same solution containing 10 μM IMPL was added to the other side. Time-lapse video microscopy was used to examine neutrophil movement in Zigmold chambers placed on a heated stage (37°C). A microscope (model IX70; Olympus) was equipped with differential interference contrast optics, and a 20× or 40× objective. Images were captured with a camera (CoolSNAP HQ/OI; Photometrics) and processed with MetaMorph software (Universal Imaging Corp.).

Superoxide production

BM neutrophils (1 × 10^6) suspended in Heps-buffered saline containing 0.03% BSA were stimulated at 37°C with 8 μM IMPL or 200 ng/ml PMA, and the reaction was terminated by addition of 50 μg/ml SOD. The chemiluminescence was counted with an enhancer-containing, luminol-based detection system [National Diagnostics) using a luminometer (Auto Lumat LB953; Berthold).
Membrane translocation of PH-Akt and DOCK2

BM neutrophils (5 × 10^6) were electroporated with 2.5 μg 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 IMPL or stimulated on a heated stage (37°C) with a micropipette containing 10 μM IMPL. Images were taken from either a laser-scanning confocal microscope (FUJOUVIEW FV500; Olympus) or a microscope (Axiovert 200M; Carl Zeiss Microlmaging, 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 (6–7.5 × 10^6) were labeled with [32P]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 μM IMPL or 25 mM CsA 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 decayed 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 Biochemistry), or Cdc42-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).

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