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Negative Charges in the Flexible N-Terminal Domain of Rho GDP-Dissociation Inhibitors (RhoGDIs) Regulate the Targeting of the RhoGDI–Rac1 Complex to Membranes

Takehiko Ueyama,* Jeonghyun Son,* Takeshi Kobayashi,† Takeshi Hamada,* Takashi Nakamura,* Hirofumi Sakaguchi,‡ Toshihiko Shirafuji,* and Naoaki Saito*

Rac is one of the four cytoplasmic activators (p47phox, p40phox, p67phox, and Rac) of the phagocyte (Nox2-based) NADPH oxidase, which produces reactive oxygen species (ROS) in response to various receptor-mediated signaling events (8). These four activators are further categorized as two protein complexes: a Rac complex (Rac–RhoGDI) and PHOX complex (p47phox–p40phox). In resting states, the two protein complexes are inactive in the cytoplasm. During cell activation, the multiprotein Nox2-p22phox–p47phox–p40phox–p67phox–Rac is formed by independent translocation of the two complexes to membrane-spanning Nox2-p22phox (8). Reviews focusing on phagocytes (9, 10) have speculated that the dissociation of Rac from RhoGDI occurs in the cytoplasm. However, no systematic study currently exists that clarifies this mechanism.

The family of RhoGDIs contains three isoforms: RhoGDIα, RhoGDIβ (LyGDI), and RhoGDIγ. RhoGDIα is ubiquitously expressed, whereas RhoGDIβ is predominantly expressed in hematopoietic cells, and RhoGDIγ is expressed primarily in the brain. In resting cells, RhoGDIα and RhoGDIβ are localized to the cytoplasm with RhoGTPases (1). In sharp contrast, RhoGDIγ is localized at the Golgi apparatus through its unique amphipathic N-terminal segment (aa 1–33) (11). The structure of isolated RhoGDIα/β comprises two distinct regions: an N-terminal unstructured arm (aa 1–58 in RhoGDIα, aa 1–55 in RhoGDIβ), and an Ig-like folded domain (aa 59–204 in RhoGDIα, aa 56–201 in RhoGDIβ) containing a hydrophobic pocket that packs the isoprenylated C-terminal tail of Rac (12). The N termini of RhoGDIα (25 residues) and RhoGDIβ (22 residues) possess numerous negatively charged amino acids: 8 in RhoGDIα and 10 in RhoGDIβ. Based on structural studies of the RhoGDIα–Rac1 (12, 13) and RhoGDIβ–Rac2 (14)

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Abbreviations used in this article: DGK, diacylglycerol kinase; GDI, GDP-dissociation inhibitor; mKO, monomeric Kusabira orange; PA, phosphatidic acid; pAb, polyclonal Ab; PB, polybasic; PM, plasma membrane; RhoGDI, Rho GDP-dissociation inhibitor; RhoGTPase, Rho-family small GTPase; ROS, reactive oxygen species.

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complexes, the 25 and 22 N-terminal residues were found to remain flexible even in the RhoGDI–Rac complex. With respect to the translocation of Rac to the phagosomes, other researchers and we (15, 16) have reported an interaction between the polybasic (PB) motif in the C terminus of Rac (K183RKKKRK306) and anionic phospholipids, such as phosphatidic acid (PA) and phosphatidylinerine, is a key determinant. Previous studies established the identification of RhoGDIα as a component of the phagosome protein-associate (17), the accumulation of RhoGDI on phagosomes (18), and phosphorylation of RhoGDIα (5, 19) by phagosome-targeting kinases (18, 20). Based on these observations, we hypothesized that the RhoGDIα–Rac complex translocates to phagosomes, and then Rac is dissociated/activated on the phagosomes. Moreover, we hypothesized that the negatively charged and flexible N terminus (25 residues) of RhoGDIα functions a suppressor for the phagosomal recruitment of the RhoGDIα–Rac complex. In this study, we verified our hypotheses, and they are applicable not only for RhoGDIα but also for RhoGDIβ in FcyR-mediated phagocytosis.

Materials and Methods

Materials

The polyclonal Ab (pAb) against RhoGDIα, mAb against c-Myc(9E10)–conjugated agarose resin, and mAb against GAPDH were purchased from Santa Cruz Biotechnology. The mAb against HA(TANA2)-conjugated magnetic agarose were from MBL International. The mAbs against HA (3F10) and Myc(9E10) were from Roche. The mAb against Rac1 was from Millipore. The pAbs against p47-phox, p67-phox, and p40-phox were described previously (21). The rabbit pAb against GFP was made in-house. The IgG-opsinized 2-μm glass beads (BilG; Duke Scientific) were prepared as described previously (20).

Cell culture

The RAW264.7 macrophages (15) and HEK293 line with stable knockdown of Rac1 (HEK293Rac1KD) were transfected with 1 μg/ml G418 (Wako). For establishing clonally derived HEK293 cell lines with stable knockdown of Rac1 (HEK293Rac1KD), pSUPER-Rac1 (618) (23) was transfected into HEK293 cells using FuGENE6, followed by clone selection in the presence of 1 μg/ml G418 (Wako).

Construction of plasmids

Human RhoGDIα, RhoGDIβ, and RhoGDIγ were amplified by PCR using first-strand cDNA (BD Biosciences), cloned into the pEGFP(N1) vector (Invitrogen), and named RhoGDIα-GFP, RhoGDIβ-GFP, and RhoGDIγ-GFP. They were also cloned into the pHK01(MN1) vector (humanized mono- dimeric Kusabira orange; excitation, 548 nm, emission, 561 nm; Amagama) (24) and named RhoGDIα-mKO, RhoGDIβ-mKO, and RhoGDIγ-mKO. The expression plasmids of p47-phox, p67-phox, and p40-phox were described previously (22). The rabbit pAb against GFP was made in-house. The IgG-opsinized 2-μm glass beads (BilG; Duke Scientific) were prepared as described previously (20).

Immunoprecipitation and immunoblotting

The Myc-Rac1 and RhoGDIα-GFP constructs (HA-PLD2 plus GFP-Rac1, or HA-PLD2 plus RhoGDIα-GFP) were cotransfected into HEK293 cells plated on 10-cm dishes using 2.5× volume of FuGENE6. Forty-eight hours after the transfection, the cells were lysed in 250 μl in lysis buffer (25 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 0.25% Triton X-100) by sonication. The total cell lysates were centrifuged at 20,000 × g for 20 min at 4˚C, and the supernatants were incubated with 20 μl c-Myc(9E10) mAb-conjugated agarose (or 10 μl HA(TANA2) mAb-conjugated magnetic agarose) for 2 h at 4˚C. The precipitates were washed three times, and the aliquots of the precipitate were subjected to SDS-PAGE (12.5%) followed by immunoblotting using a GFP pAb (1:1000). The bound Abs were detected with secondary Ab-HRP conjugates using the ECL detection system (GE Healthcare).

Confocal fluorescence imaging of fixed cells or live cells

RAW264.7 cells were seeded onto 35-mm glass-bottom dishes (MatTek chambers) and transfected using 2.5× volume of FuGENE6 HD reagent (Promega). After 48 h, cells were stimulated with BlgG and fixed 5 min later with 4% parasformaldehyde in 0.1 M PB buffer for 30 min at room temperature. The fixed cells were permeabilized using PBS containing 0.3% Triton X-100 for 10 min and stained using IgG primary Ab (μHA: 1:250, μRac1: 1:500, 2 h at room temperature). The primary Abs were visualized on a confocal laser-scanning fluorescence microscope (LSM 700; Carl Zeiss) using Alexa-conjugated anti-IgG (Invitrogen; 1:2000; 0.5 h at room temperature). For live imaging 45–48 h after the transfection, the culture medium was replaced with HBSS(+) (Wako). After BlgG was added to each plate, the images were collected at 5-s intervals for 10 min using a confocal laser-scanning fluorescence microscope with a Woche objective. All imaging experiments were performed in triplicate or more at least three independent transfection experiments (n ≥ 9).

ROS production assay

HEK293N50×2FcyRβ cells in six-well dishes were transfected with various combinations of plasmids using FuGENE6 40–48 h before the assay. The ROS release in response to BlgG from 2.0 × 107 trypsinized cells was measured in HBSS(+) during 15 min by a luminol-enhanced chemiluminescence method using a luminometer (Mithras LB940; Berthold) (22). Consistency in protein expression was confirmed by immunoblotting of total lysate for the same number of cells.

Statistical analysis

The production of ROS is presented as a percentage relative to the control experiment. The phagosomal accumulation of proteins is expressed using the ratio of fluorescence intensity (phagosome/cytoplasm). Immunoprecipitated protein bands were quantified using ImageJ (National Institutes of Health) and is expressed as a ratio relative to RhoGDIα. All data are presented as the means ± SEM. Significant differences between two groups (p < 0.05) were identified using an unpaired two-tailed Student t test by Prism 5.0 (GraphPad Software).

Results

Three isofoms of RhoGDI

The three RhoGDI isofoms were expressed in RAW264.7 cells, with mRNA levels significantly higher for RhoGDIα and RhoGDIβ than those for RhoGDIγ (Supplemental Fig. 1A). Immunolocalization in RAW264.7 cells revealed that RhoGDIα-mKO, whose tag is a red fluorescent protein named as monomeric Kusabira orange with excitation of 548 nm and emission of 561 nm (24), and RhoGDIβ-mKO were localized in the cytoplasm, whereas RhoGDIγ-mKO was confined to the Golgi apparatus (data not shown). In contrast, GFP-Rac1 was localized to the plasma membrane (PM), in addition to the cytoplasm and nucleus (Supplemental Fig. 1B). When coexpressed with RhoGDIα, GFP-Rac1 was not detected at the PM but was colocalized with RhoGDIα-mKO and RhoGDIβ-mKO in the cytoplasm and with RhoGDIγ-mKO in the Golgi (Supplemental Fig. 1B). These data indicated that the subcellular localization of Rac1 is regulated by RhoGDIα.

Translocation of the RhoGDIα–Rac1 complex to phagosomes

Because RhoGDIα was reported to accumulate on phagosomes of J744 macrophages (detected by proteomics) and primary neutrophils (detected by immunocytochemistry) during FcyR-mediated phagocytosis (17, 18), we hypothesized that the RhoGDIα–Rac1 complex...
migrates to the phagosome where it releases Rac in proximity to Nox2-based NADPH oxidase. Fig. 1A shows that GFP-Rac1 accumulated on the phagosomes during FcγR-mediated phagocytosis in RAW264.7 cells, as shown previously (15). However, RhoGDId1α-mKO did not accumulate on the phagosomes (Fig. 1B). Moreover, coexpression of RhoGDId1-mKO and GFP-Rac1 in RAW264.7 cells did not lead to their accumulation on phagosomes (Fig. 1C). Our understanding of the discrepancy about accumulation of RhoGDId on previous reports and the current study at that time was that detection of accumulated RhoGDId on phagosomes is influenced by various factors, such as cell type used (primary or immortalized cells, macrophages, or neutrophils) and detection method used (proteomics, immunocytochemistry, or confocal fluorescence imaging). Then, we tested RhoGDId1α-mKO mutants, RhoGDId1α(D45A) and RhoGDId1α(D185A), characterized by weakened interactions with RhoGTPTes due to partial conformational changes in RhoGDId–RhoGTPase complexes (13, 14, 27). Neither mutant expressed in RAW264.7 cells showed any accumulation on the phagosomes (Supplemental Fig. 2). However, they both accumulated on phagosomes during FcγR-mediated phagocytosis (Supplemental Fig. 2). Neither mutant expressed in RAW264.7 cells showed any accumulation on the phagosomes during FcγR-mediated phagocytosis (Supplemental Fig. 2). However, they both accumulated on phagosomes during FcγR-mediated phagocytosis (Supplemental Fig. 2).

We hypothesized that the negatively charged flexible N terminus of RhoGDId plays an important role in the subcellular localization of the RhoGDId–Rac1 complex. First, we constructed N-terminal deletion mutants of RhoGDId: ΔN15-RhoGDId1α-mKO constructs inhibited ROS production to a similar extent (data not shown). RhoGDId-GFP completely inhibited ROS production (1.2 ± 0.2%), whereas RhoGDId1α(D45A)-GFP and RhoGDId1α(D185A)-GFP partially suppressed ROS production (62.7 ± 7.0 and 65.1 ± 12.9%, respectively) (Fig. 1G). These data indicated that the inhibitory effect of RhoGDId on FcγR-mediated ROS production depends on its binding affinity to Rac1.

**FIGURE 1.** Impact of RhoGDId on Rac1 translocation to the phagosomes during phagocytosis and on ROS production. (A–E) GFP-Rac1 was cotransfected with mKO (A), RhoGDId1α-mKO (C), RhoGDId1α(D45A)-mKO (D), or RhoGDId1α(D185A)-mKO (E) into RAW264.7 macrophages, and RhoGDId1α-mKO was cotransfected with GFP (B). The transfected cells were stimulated with 2-μm BlgG and visualized under a confocal microscope. GFP-Rac1 (A) but not RhoGDId1α-mKO (B) accumulates on phagosomes. Neither GFP-Rac1 or RhoGDId1α-mKO accumulates on phagosomes (C). RhoGDId1α(D45A)-mKO (D) and RhoGDId1α(D185A)-mKO (E) coexpressed with GFP-Rac1 accumulate on phagosomes. The arrows and arrowheads indicate phagosomes engulfing BlgG. (F) Representative immunoprecipitation analysis of the interaction between Myc–Rac1 and RhoGDId1α-GFP or its mutants (D45A and D185A). RhoGDId1α(D45A) and RhoGDId1α(D185A) maintain a weak interaction with Rac1. (G) ROS production from HEK293Nox2/FcγRIIa cells transfected with a mock vector, RhoGDId1α-GFP, RhoGDId1α(D45A)-GFP, or RhoGDId1α(D185A)-GFP in combination with Phox proteins and Myc-Rac1. The cells were stimulated with 2-μm BlgG, and ROS release was measured by luminol-enhanced chemiluminescence ($n \geq 5$). RhoGDId1α completely inhibited ROS production, whereas RhoGDId1α(D45A) and RhoGDId1α(D185A) partially suppressed ROS production. The comparable expression of the proteins was confirmed with immunoblotting.
and \(\Delta N25\)-RhoGDI\(a\)-mKO (Fig. 2A). In resting RAW264.7 cells, both mutants were detected in the cytoplasm (Fig. 2B, 2C), but \(\Delta N25\)-RhoGDI\(a\)-mKO also exhibited a weak PM localization (Fig. 2C). During FcyR-mediated phagocytosis, only \(\Delta N25\)-RhoGDI\(a\)-mKO was found accumulating on phagosomes (Fig. 2C). The PM localization of \(\Delta N25\)-RhoGDI\(a\)-mKO was also observed in HEK293 cells (Fig. 3A). It disappeared in HEK293 cells with stable knockdown of Rac1, HEK293\(Rac1KD\) cells (Fig. 3B, 3C) and was restored by introduction of full-length GFP-Rac1 (nucleotides 1–598 from ATG), which is resistant to pSUPER-Rac1(618), into HEK293\(Rac1KD\) cells (Fig. 3D). Rac1-dependent PM localization/recruitment of \(\Delta N25\)-RhoGDI\(a\)-mKO was confirmed by single-molecule imaging of tetramethylrhodamine-conjugated Halo-tagged \(\Delta N25\)-RhoGDI\(a\) (HaloTag; Promega) in HeLa cells based on residence time, frequency of recruitment, and trajectory in PM: residence time and recruitment in PM of \(\Delta N25\)-RhoGDI\(a\)-Halo (tetramethylrhodamine) were significantly extended and increased, respectively, by coexpression of GFP-Rac1 (Supplemental Fig. 3). Immunoprecipitation analysis demonstrated that \(\Delta N15\)-RhoGDI\(a\) and \(\Delta N25\)-RhoGDI\(a\) had strong interactions with Rac1 similar to RhoGDI\(a\) (Fig. 3E). FcyR-mediated ROS production in HEK293\(Nox2/Fc\(RIIa\) cells was completely inhibited by \(\Delta N25\)-RhoGDI\(a\)-GFP or \(\Delta N15\)-RhoGDI\(a\)-GFP, similar to RhoGDI\(a\) (Fig. 3F). These data suggested the following: 1) the negatively charged N terminus of RhoGDI\(a\), particularly the sequence E17NEEDE22, plays a pivotal role in retaining RhoGDI\(a\) in cytoplasm; 2) the membrane (PM and phagosome) localization of RhoGDI\(a\) is dependent on Rac1; and 3) the inhibitory effect of RhoGDI\(a\) on ROS production is probably determined by its binding affinity to Rac1, but not on its localization.

**FIGURE 2.** Membrane (PM and phagosome) localization of RhoGDI\(a\) is dependent on its negatively charged N terminus (25 aa). (A) The amino acid sequence of the N termini in human wild-type (WT) and mutant RhoGDI\(a\) (\(\Delta N15\), \(\Delta N25\)). A red number in parenthesis indicates the total number of negatively charged amino acids in their N termini. (B and C) \(\Delta N15\)-RhoGDI\(a\)-mKO (B) or \(\Delta N25\)-RhoGDI\(a\)-mKO (C) was transfected into RAW264.7 cells. The transfected cells were stimulated with 2-\(\mu\)M BlgG. \(\Delta N25\)-RhoGDI\(a\)-mKO but not \(\Delta N15\)-RhoGDI\(a\)-mKO shows a weak PM localization and accumulation on phagosomes. Arrows and arrowheads indicate PM areas and phagosomes engulfing BlgG, respectively.

**Suppression of the Rac-dependent accumulation of the RhoGDI\(a\)--Rac1 complex on phagosomes by negative charges in the N terminus of RhoGDI\(a\)**

To further investigate the impact of the negative charges located in the N terminus (25 residues) of RhoGDI\(a\), we constructed three mutants with reduced numbers of negatively charged amino acids: RhoGDI\(a\)(3A)-mKO, RhoGDI\(a\)(5A)-mKO, and RhoGDI\(a\)(8A)-mKO (Fig. 4A). Immunoprecipitation analysis showed that all mutants bind Rac1 similar to RhoGDI\(a\), contrary to a faint binding of Rac1 interaction-impaired RhoGDI\(a\)(8A:D45/185A) (Fig. 4B). Confocal microscopy revealed that the mutants RhoGDI\(a\)(3A)-mKO and RhoGDI\(a\)(5A)-mKO were primarily located in the cytoplasm of resting RAW264.7 cells, and they did not accumulate on phagosomes during phagocytosis (Fig. 4C, 4D). In contrast, the RhoGDI\(a\)(8A)-mKO mutant exhibited remarkable PM localization in resting RAW264.7 cells and accumulated on phagosomes during phagocytosis (Fig. 4E), contrary to cytoplasmic RhoGDI\(a\)(8A:D45/185A) (Fig. 4F). Endogenous Rac1 accumulated with RhoGDI\(a\)(8A)-mKO on phagosomes (Supplemental Fig. 4A). These data suggested that membrane (PM and phagosome) localization of RhoGDI\(a\)-mKO is mediated by its binding to Rac1. The coexpression of GFP-Rac1 with RhoGDI\(a\)(5A)-mKO or RhoGDI\(a\)(3A)-mKO stimulated the phagosomal accumulation of RhoGDI\(a\)(5A)-mKO but not RhoGDI\(a\)(3A)-mKO (Supplemental Fig. 4B, 4C). The phagosomal accumulation of RhoGDI\(a\)(8A)-mKO with GFP-Rac1 disappeared when GFP-Rac1(6A), a phagosome-targeting defective mutant (15), was coexpressed with RhoGDI\(a\)(8A)-mKO (Supplemental Fig. 4D, 4E). Because RAW264.7 macrophages express all Rac isoforms (15), we knocked down Rac1 in HEK293\(Nox2/Fc\(RIIa\) cells, in which Rac1 is a predominant Rac isoform, using pSUPER-Rac1(618)gfp (23). The phagosomal accumulation of RhoGDI\(a\)(8A)-mKO in HEK293\(Nox2/Fc\(RIIa\) cells (Fig. 4G) were significantly reduced when Rac1 was knocked down (Fig. 4H, 4I). Moreover, ROS production from HEK293\(Nox2/Fc\(RIIa\) cells increased FcR-mediated ROS production by 5–10-fold compared to HEK293\(Nox2/Fc\(RIIa\) cells, which was completely suppressed by RhoGDI\(a\)-GFP, RhoGDI\(a\)(3A)-GFP, RhoGDI\(a\)(5A)-GFP, and RhoGDI\(a\)(8A)-GFP. However, RhoGDI\(a\)(8A:D45/185A)-GFP caused only a mild suppression (65.9 ± 9.6%) (Fig. 4J). Based on these results, we concluded that all eight, particularly five in the sequence of E17NEEDE22, negatively charged amino acids in the flexible N terminus of RhoGDI\(a\) function as a suppressor for the accumulation of the RhoGDI\(a\)--Rac1 complex on phagosomes and that the inhibitory effect of RhoGDI\(a\) on ROS production is mediated by the binding affinity of RhoGDI\(a\) to Rac1.

**PLD2 promotes the translocation of the RhoGDI\(a\)--Rac1 complex to phagosomes and enhances ROS production**

The interaction between the PB motif in the C terminus of Rac1 (K\(^{183}\)KRRKK\(^{188}\) and PA is important for Rac1 accumulation on the phagosomes and for ROS production during FcyR-mediated phagocytosis (15); besides, we reported the accumulation of the PA-producing enzyme PLD2 on phagosomes (26). Coexpression of PLD2, but not the catalytically inactive mutant PLD2(\(H442D\)), induced the translocation of RhoGDI\(a\)-mKO and GFP-Rac1 to the phagosomes of RAW264.7 cells (Fig. 5A, 5B). This response by PLD2 disappeared when GFP-Rac1 was replaced by the phagosome-targeting defective mutant GFP-Rac1(6A), despite PLD2 accumulation on phagosomes (Fig. 5C). No interaction between PLD2 and Rac1 or RhoGDI\(a\) was confirmed by immunoprecipitation analysis (Fig. 5D). Transfection of PLD2 in HEK293\(Nox2/Fc\(RIIa\) cells increased FcR-mediated ROS production by 3.5-fold (340.5 ± 40.1%), whereas the PLD2(\(H442D\)) mutant did not enhance ROS production (70.2 ± 7.4%) (Fig. 5E). These data indi-
cated that RhoGDIα accumulates on phagosomes as a RhoGDIα–Rac1 complex, and the accumulation is mediated by interaction between the PB motif of Rac1 and the anionic phospholipid PA produced on phagosomes, that is, by a phagosome-targeting mechanism of Rac1.

Negatively charged N terminus of RhoGDIβ suppresses the Rac-dependent phagosomal targeting of RhoGDIβ

Finally, we tested the impact of the negatively charged N terminus (22 residues) of RhoGDIβ as a Rac1–RhoGDIβ complex, and the accumulation is mediated by interaction between the PB motif of Rac1 and the anionic phospholipid PA produced on phagosomes, that is, by a phagosome-targeting mechanism of Rac1.

Negatively charged N terminus of RhoGDIβ suppresses the Rac-dependent phagosomal targeting of RhoGDIβ

FIGURE 3. Membrane localization of RhoGDIα and its inhibitory effect on ROS production are dependent on Rac1. (A) GFP and ΔN25-RhoGDIα-mKO were co transfected in HEK293 cells. ΔN25-RhoGDIα-mKO is localized at PM (arrows). (B) Stable knockdown of Rac1 in HEK293Rac1KD cells was confirmed by immunoblotting. (C and D) GFP plus ΔN25-RhoGDIα-mKO (C) or GFP-Rac1 plus ΔN25-RhoGDIα-mKO (D) was cotransfected into HEK293Rac1KD cells. The PM localization of ΔN25-RhoGDIα-mKO disappeared (C) and was restored by GFP-Rac1 (D). (E) Representative immunoprecipitation data (n ≥ 3) showing strong interaction between Myc-Rac1 and ΔN15-RhoGDIα-GFP or ΔN25-RhoGDIα-GFP, similar to WT RhoGDIα. (F) HEK293Rac1KD cells were transfected with a mock vector, RhoGDIα-GFP, ΔN15-RhoGDIα-GFP, or ΔN25-RhoGDIα-GFP in combination with Phox proteins and Myc-Rac1. The cells were stimulated with BigG, and ROS release was measured (n ≥ 5). ΔN15-RhoGDIα and ΔN25-RhoGDIα as well as RhoGDIα completely inhibited ROS production. The comparable expression of proteins was confirmed by immunoblotting. Scale bars, 10 μm.

The negatively charged and flexible N-terminal domain of RhoGDIα (25 residues) and RhoGDIβ (22 residues) contains two highly conserved clusters of negatively charged amino acids (Fig. 7A). The first cluster consists of three and two negative amino acids in human RhoGDIα and RhoGDIβ, respectively. The second cluster consists of five and eight negative amino acids in human RhoGDIα and RhoGDIβ, respectively. In the present study, we demonstrated that previously reported on RhoGDIβ showing a 10- to 20-fold lower affinity for Cdc42 (28). Although ΔN22-RhoGDIβ maintained an interaction with Rac1, RhoGDIβ(D42A) demonstrated no interaction (Fig. 6E). The ROS production assay in HEK293Nox2/FcγRIIa cells revealed a moderate suppression by RhoGDIβ (32.2 ± 6.0%) or RhoGDIβ(D182A) (85.0 ± 7.8%), which is a homologous mutant of RhoGDIα (D185A), in contrast to the complete suppression by RhoGDIα (Fig. 6F). There was no statistical difference between RhoGDIβ and RhoGDIβ(D182A) with regard to binding affinity for Rac1 or inhibitory effect on ROS production.

Discussion

The negatively charged and flexible N-terminal domain of RhoGDIα (25 residues) and RhoGDIβ (22 residues) contains two highly conserved clusters of negatively charged amino acids (Fig. 7A). The first cluster consists of three and two negative amino acids in human RhoGDIα and RhoGDIβ, respectively. The second cluster consists of five and eight negative amino acids in human RhoGDIα and RhoGDIβ, respectively. In the present study, we demonstrated that...
all eight N-terminal negative amino acids of RhoGDIα (particularly five in the second cluster) function as a suppressor for the membrane targeting of the RhoGDIα–Rac1 complex and for RhoGDIα itself. The Rac proteins possess two membrane-targeting motifs (15): 1) the isoprenylated tail at C-terminal Cys189 position, which interacts with a hydrophobic pocket of RhoGDIα (12, 13), and 2) the PB motif (K183KRKRK188 in Rac1, R183QQKRA188 in Rac2) next to the isoprenylated tail. Whereas the C-terminal of Rac1 encompassing FIGURE 4. Negatively charged amino acids in the N terminus of RhoGDIα suppress Rac1-dependent translocation of the RhoGDIα–Rac1 complex to phagosomes. (A) Amino acid sequence of the N termini in human wild-type (WT) and mutant RhoGDIα (3A, 5A, and 8A). A red number in parenthesis indicates the total number of negatively charged amino acids in their N termini. The blue number and blue A indicate the total number and position of mutated Ala, respectively. (B) Representative immunoprecipitation data (n = 5) showing strong interaction between Myc-Rac1 and RhoGDIα-GFP (WT, 3A, 5A, 8A) and a faint interaction between Myc-Rac1 and RhoGDIα(8A,D45/185A)-GFP. (C-F) RhoGDIα(3A)-mKO (C), RhoGDIα(5A)-mKO (D), RhoGDIα(8A)-mKO (E), or RhoGDIα(8A,D45/185A)-mKO (F) was transfected into RAW264.7 cells. The transfected cells were stimulated with 2-μm BlgG. RhoGDIα(8A)-mKO (E), but not RhoGDIα(8A,D45/185A)-mKO (F), shows PM localization and accumulates on phagosomes. The arrow and arrowheads indicate PM and phagosomes engulfing BlgG, respectively. (G and H) RhoGDIα-mKO(8A) was cotransfected with pSUPER(gfp-neo) (G) or pSUPER-Rac1(681)gfp (H) in HEK293Nox2/FcRIIa cells. Cotransfection of pSuper-Rac1(681)gfp was confirmed by the expression of GFP (small panel in middle). The arrowheads indicate the phagosomes engulfing BlgG. Right graphs show fluorescence intensity profile of RhoGDIα(8A)-mKO detected along the arrows. (I) Quantification of fluorescence intensity ratio (phagosome/cytoplasm) of RhoGDIα(8A)-mKO in (G) and (H). Studies included n ≥ 30 from at least four individual experiments (≥12 dishes; *p < 0.01). (J) ROS production from HEK293Nox2/FcRIIa cells transfected with a mock vector, RhoGDIα-GFP, RhoGDIα(3A)-GFP, RhoGDIα(5A)-GFP, RhoGDIα(8A)-GFP, or RhoGDIα(8A,D45/185A)-GFP in combination with Phox proteins and Myc-Rac1. The cells were stimulated with BlgG, and ROS release was measured (n = 5). RhoGDIα(8A) shows complete suppression, but RhoGDIα(8A,D45/185A) shows only a mild suppression. The comparable expression of proteins was confirmed by immunoblotting.
residues 180–189 (Rac2 encompassing residues 182–189), but not the isoprenylated tail, is poorly defined in the crystal structure of the RhoGDIα–Rac1 (RhoGDIβ–Rac2) complex, the negatively charged N-terminal of RhoGDIα/b and the PB motif in C-terminal of Rac1/2 are expected to be in close proximity around the exit of the hydrophobic pocket of RhoGDIα/b (13, 14). It was reported that a peptide of Glu5-Glu20 from human RhoGDIα, which contains five negatively charged amino acids (Fig. 6A), inhibited Rac1 in a cell-free NADPH oxidase assay system using the membrane fraction of neutrophils as oxidase assembly (29). However, a peptide of Thr7-Ile14 from human RhoGDIα, which contains only one negatively charged amino acid, was less effective (29). These data are consistent with our previous report showing the dependence of Rac accumulation on phagosomes and ROS production on the number of positively charged amino acids in the PB motif of Rac (15). Thus, the inhibitory effects of the negative charges found in the flexible N-terminal of RhoGDIα and RhoGDIβ are likely mediated by masking of the PB motif in the C terminus of Rac, as suggested in a recent review (30).

We previously demonstrated that an interaction between the PB motif of Rac and anionic phospholipids (particularly PA and phosphatidylinositol 3,4,5-triphosphate) is a key determinant in Rac accumulation on phagosomes (15). Afterward, a study described the constant presence of phosphatidylerine, an anionic phospholipid, on phagosomes and its significant contribution to targeting and retaining proteins containing a PB cluster (16). In the present study, we demonstrated that RhoGDIα translocates to phagosomes as a RhoGDIα–Rac1 complex through the phagosome-targeting mechanism of Rac1 (Rac1-dependent mechanism) by the following experiments: 1) using a Rac1 interaction–impaired mutant RhoGDIα(D45/185A) (Fig. 4F), 2) using HEK293Nox2/FcRIIa cells with Rac1 knockdown (Fig. 4G–I), 3) using a phagosome-targeting impaired mutant Rac1(6A) (Fig. 5C, Supplemental Fig. 4E), and 2) using RhoGDIα(5A) with coexpression of Rac1 (Supplemental Fig. 4C). Furthermore, based on our previous report of the accumulation of PA-producing PLD2 on phagosomes (26), we demonstrated that both RhoGDIα-mKO and GFP-Rac1 accumulate on the phagosome when PLD2 but not inactive PLD2(H442D) was overexpressed, and that PLD2 does not bind to Rac1 or RhoGDIα. Aside from the Rac1-PA interaction on RhoGDIα–Rac1 translocation, another mechanism that uses PA may function. During hepatocyte growth factor–mediated membrane ruffling in Madin–Darby canine kidney cells, diacylglycerol kinase (DGK)α promoted the translocation of atypical protein kinase Cζ/ζ, stably associated with the RhoGDIα–Rac1 complex, to the PM through PA production (31).
Additionally, it was reported that DGKζ, stably associated with Pak1 and RhoGDIα–Rac1, promoted the release of RhoGDIα from Rac1 through DGKζ→PA→Pak1–mediated RhoGDIα phosphorylation at platelet-derived growth factor–induced membrane ruffling in fibroblasts (32). These studies support an involvement of PA production during phagocytosis.

In contrast to no detection of RhoGDIα-mKO on phagosomes without coexpression of Rac1 and PLD2 under our confocal fluorescence imaging, ∆N25-RhoGDIα-mKO and RhoGDIα(8A)-mKO accumulated on phagosomes even without coexpression of Rac1; nevertheless, ∆N25-RhoGDIα and RhoGDIα(8A) maintained their abilities to bind Rac1 and to inhibit ROS production similar to RhoGDIα. Although we cannot exclude the possibility that ∆25-RhoGDIα and RhoGDIα(8A) are more stable with Rac1 on phagosomes owing to an undefined mechanism, the discrepancy may be at least partially explained by the electrostatic repulsion between the negatively charged N terminus of RhoGDIα and the negative charge of the phagosome, which is enhanced by anionic phospholipids produced during phagocytosis. ∆N25-RhoGDIα and RhoGDIα(8A) have much weaker electrostatic repulsion potency than does RhoGDIα; as a result, these proteins with the similar ability to bind Rac1 may remain longer on phagosomes with Rac1 and may keep the inhibitory effect on ROS production (Fig. 7B, 7C).

In support of this proposal, our single molecule imaging showed prolonged residence time in the PM of ∆25-RhoGDIα compared with RhoGDIα (Supplemental Fig. 3D). Single molecule imaging also showed that the frequency of RhoGDIα recruitment to the PM was slightly increased by Rac1 coexpression but was apparently less than that of ∆N25-RhoGDIα (Supplemental Fig. 3D). The residence time of RhoGDIα in the PM was not increased by Rac1. These results are consistent with the observed lack of phagosomal RhoGDIα accumulation under confocal fluorescence imaging, even with Rac1 coexpression (Fig. 1C). Thus, a phagosomal accumulation enhancing factor for Rac1, such as PLD2, or neutrophils, which shows 10-fold or more ROS production and stronger phagosomal accumulation of Rac than RAW264.7 cells (15), is probably required to detect accumulation of RhoGDIα on phagosomes under confocal fluorescence imaging.

The RhoGDIβ–Rac1 complex is expected to be more steadily retained in the cytoplasm due to the larger number of negatively charged amino acids in the N terminus of RhoGDIβ compared with RhoGDIα. However, the inhibitory effects of RhoGDIβ on Rac1 translocation to phagosomes and ROS production were weaker than those of RhoGDIα. Our imaging studies using RAW264.7 cells revealed no apparent accumulation of RhoGDIβ-mKO on phagosomes, whereas the N-terminal deletion mutant ∆22-RhoGDIβ-mKO accumulated on phagosomes along with GFP-Rac1. A study using the fluorescence resonance energy transfer technique in RAW264.7 cells showed that RhoGDIβ accumulates on phagosomes during the phagocytosis of Listeria monocytogenes or Escherichia coli (33). These data suggest that RhoGDIβ also accumulates on phagosomes as a RhoGDIβ–Rac1 complex similar to RhoGDIα, but the accumulation of the RhoGDIβ is much weaker than that of RhoGDIα. The weaker accumulation of RhoGDIβ on phagosomes is likely due to its weaker binding affinity for Rac1 and its stronger electrostatic repulsion on phagosomes than RhoGDIα.
In summary, the translocation/accumulation of RhoGDIα/β-Rac1 to phagosomes and the inhibitory effect of RhoGDIα/β on ROS production in Fcγ-mediated phagocytosis may be regulated by a balance of the following three factors: 1) the negatively charged N-terminal of RhoGDIα, 2) the binding affinity of RhoGDIα/β for Rac1, and 3) anionic phospholipids produced on phagosomes (Fig. 7B). After the translocation of the RhoGDIα/β–Rac1 complex to phagosomes, Rac1 must be released for its activation on phagosomes, which is likely induced by RhoGDI dissociation factors (1, 2) (Fig. 7C).

Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure Legends

**Fig. S1: Immunolocalization of the RhoGDI isoforms in RAW264.7 macrophages**

A, RT reaction was performed with 1 µg of total RNA from RAW264.7 macrophages and SuperScript III (Invitrogen). The cDNA product was amplified by PCR using specific primer pairs for RhoGDIα (5’-agcatggcagaaggaaccact-3’ and 5’-tcagtcctccacctcttttgtaggg-3’), RhoGDIβ (5’-aagatgacggagaaggatgcacagc-3’ and 5’-tcattctgtcaatcctttaatggcc-3’) and RhoGDIγ (5’-acctctgctgcctgacgctgt-3’ and 5’-tcagtcctccagctgacgatg-3’). A negative control experiment was performed without the cDNA obtained from the RT reaction. The PCR cycle numbers were ×25, ×30, and ×35. B, GFP-Rac1 was co-transfected with mKO (empty vector), RhoGDIα-mKO, RhoGDIβ-mKO, or RhoGDIγ-mKO in RAW264.7 cells. The co-transfected cells were visualized by confocal microscopy. The PM localization of GFP-Rac1 is disappeared by co-expression of RhoGDIs. Bar: 10 µm.

**Fig. S2: No phagosomal accumulation of RhoGDIα-mKO mutants without GFP-Rac1 co-expression**

The mutants RhoGDIα(D45A)-mKO (A) or RhoGDIα(D185A)-mKO (B) was transfected in RAW264.7 cells without co-expression of GFP-Rac1. FcγR-mediated phagocytosis was induced with BlG (2 µm). No accumulation of RhoGDIα(D45A)-mKO (A) or RhoGDIα(D185A)-mKO on phagosome is observed under a confocal microscope. The arrowheads indicate the phagosomes engulfing BlG.

**Fig. S3: Phagosomal accumulation of RhoGDIα(8A)-mKO with endogenous Rac1, of RhoGDIα-mKOs (5A or 8A) with GFP-Rac1 co-expression and no accumulation of RhoGDIα(8A)-mKO with GFP-Rac1(6A) co-expression**

A, RAW264.7 cells transfected with RhoGDIα(8A)-mKO were stimulated by BlG (2 µm) for 5 min. Fixed cells were stained with a Rac1 mAb, and visualized with a confocal microscope using an Alexa488-conjugated anti-IgG. Note accumulation of RhoGDIα(8A)-mKO and endogenous Rac1 on phagosomes. B-E, Cells transfected RhoGDIα(3A)-mKO + GFP-Rac1 (B), RhoGDIα(5A)-mKO + GFP-Rac1 (C), RhoGDIα(8A)-mKO + GFP-Rac1 (D), or RhoGDIα(8A)-mKO + GFP-Rac1(6A) (E) were stimulated by BlG (2 µm), and visualized by a confocal microscope. RhoGDIα(5A)-mKO, but not RhoGDIα(3A)-mKO, becomes accumulated by co-expression of GFP-Rac1. No apparent accumulation of RhoGDIα(8A)-mKO is observed when GFP-Rac1(6A) was co-expressed. The arrows and arrowheads indicate the phagosomes engulfing BlG.
Fig. S4: Rac1-dependent PM targeting of ΔN25-RhoGDIα by single-molecule imaging

The C-terminally Halo-tagged RhoGDIα (RhoGDIα-Halo) or its mutant (ΔN25-RhoGDIα-Halo or ΔN25-RhoGDIα(D45/185A)-Halo) was co-expressed in HeLa cells with RhoGDIα-GFP (A, C) or GFP-Rac1 (B, D). Halo-tagged proteins were fluorescently labeled by incubating the cells with 1 nM tetramethylrhodamine (TMR)-conjugated HaloTag® Ligand (Promega) for 20 min, then observed using an objective-type total internal reflection fluorescence microscope (IX-81, Olympus). The TMR-labeled RhoGDIα-Halo molecules were detected at 561 nm using a DPSS laser (85-YCA-010, Melles Griot) and a 100× objective (PlanApo, NA = 1.45; Olympus). The cells expressing GFP-tagged proteins were selected for observation. The trajectory of fluorescent RhoGDIα-Halo(TMR) spots was recorded at a rate of 30 fps and tracked to determine their residence time on PM. We considered that each spot represents a single molecule of RhoGDIα-Halo(TMR), as judged from the distribution of fluorescence intensity (1). A and B, Trajectories of RhoGDIα-Halo(TMR) molecules in PM observed during 2 s by single molecule imaging. Bar: 2 μm. C and D, Distributions of the residence time of individual RhoGDIα-Halo(TMR) molecules in PM. The histograms were fitted to single exponential functions (solid lines). The τ values indicate the time constants of the fit function. Decay due to photobleaching (dash lines) was estimated by observation of Halo-tagged membrane-spanning molecules under identical conditions. In the presence of RhoGDIα-GFP, WT or mutant RhoGDIα-Halo(TMR) molecules were predominantly localized to the cytoplasm, and a small fraction was detected in PM (A). The WT and mutant RhoGDIα-Halo(TMR) molecules were almost immobilized in PM, and their residence time in PM was very short (0.11–0.12 s) (A, C). The WT RhoGDIα-Halo(TMR) molecules exhibited similar behaviors, even when GFP-Rac1 was co-expressed to improve Rac1-binding to the RhoGDIα-Halo(TMR) molecules (B, D top). In contrast, the recruitment of ΔN25 molecules to PM was markedly increased by co-expression of GFP-Rac1 (B, D middle). The ΔN25 molecules became mobile in PM (B middle), and residence time in PM was significantly extended (0.17 s, p < 0.05; D middle). Furthermore, Rac1 interaction-impaired mutations (D45/185A) completely abolished the Rac1-dependent responses of the ΔN25 molecules (B, D bottom). These results indicate that the membrane localization of ΔN25-RhoGDIα requires an interaction between RhoGDIα and Rac1.

Supplemental figure 2

A  
Before  + BlgG

RhoGDIα (D45A)

B  
Before  + BlgG

RhoGDIα (D185A)
Supplemental figure 3

A. + BlgG
  - endogenous Rac1
  - RhoGDlz(5A)

B. Before + BlgG
  - Rac1
  - RhoGDlz(5A)

C. Rac1
  - RhoGDlz(5A)

D. Rac1(5A)
  - RhoGDlz(5A)
Supplemental figure 4

A

WT

ΔN25

ΔN25(D45/185A)

+ RhoGDIα-GFP

B

WT

ΔN25

ΔN25(D45/185A)

+ GFP-Rac1

C

WT

ΔN25

ΔN25(D45/185A)

+ RhoGDIα-GFP

D

WT

ΔN25

ΔN25(D45/185A)

+ GFP-Rac1

Residence Time: 0.11 sec

Residence Time: 0.12 sec

Residence Time: 0.17 sec

Residence Time: 0.12 sec