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J Immunol 2013; 191:2560-2569; Prepublished online 5 August 2013; doi: 10.4049/jimmunol.1300209
http://www.jimmunol.org/content/191/5/2560

Supplementary Material  http://www.jimmunol.org/content/suppl/2013/08/06/jimmunol.1300209.DC1

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The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
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*

In its resting state, Rho GDP-dissociation inhibitor (RhoGDI) α forms a soluble cytoplasmic heterodimer with the GDP-bound form of Rac. Upon stimulation, the dissociation of RhoGDα from the RhoGDα–Rac complex is a mandatory step for Rac activation; however, this mechanism is poorly understood. In this study, we examined how the cytoplasm/membrane cycles of the RhoGDI–Rac complex are regulated, as well as where RhoGDI dissociates from the RhoGDI–Rac complex, during FcγR-mediated phagocytosis. The negatively charged and flexible N terminus (25 residues) of RhoGDIα, particularly its second negative amino acid cluster possessing five negatively charged amino acids, was a pivotal regulator in the cytoplasm/membrane cycles of the RhoGDI–Rac complex. We also found that RhoGDIα translocated to the phagosomes as a RhoGDIα–Rac1 complex, and this translocation was mediated by an interaction between the polybasic motif in the C terminus of Rac1 and anionic phospholipids produced on phagosomes, such as phosphatidic acid, that is, by a phagosome-targeting mechanism of Rac1. Thus, we demonstrated that the targeting/accumulation of the RhoGDIα–Rac1 complex to phagosomes is regulated by a balance between three factors: 1) the negatively charged and flexible N-terminal of RhoGDIα, 2) the binding affinity of RhoGDIα for Rac1, and 3) anionic phospholipids produced on phagosomes. Moreover, we demonstrated that the mechanism of targeting/accumulation of the RhoGDIα–Rac1 complex is also applicable for the RhoGDIβ–Rac1 complex. The Journal of Immunology, 2013, 191: 2560–2569.

Small GTPases are cell signaling molecules switching between active (GTP-bound) and inactive (GDP-bound) forms. The cycles of Rho-family and Rab-family small GTPases (RhoGTPases and RabGTPases) are regulated by three major factors: guanine nucleotide exchange factors, GTPase-activating proteins, and GDIs (1, 2). The inactive forms of RhoGTPases are sequestered in the cytoplasm by dimerization with Rho GDP-dissociation inhibitors (RhoGDIs). During their activation, they are released from RhoGDIs to be converted into the GTP-bound form to execute a specific function on membranes (1, 2). The dissociation of RhoGDI from Rac is further regulated by three major factors, known as the RhoGDI dissociation factors (1, 2): RhoGDI-displacement proteins, such as the ERM family proteins (3) and p75NTR (4); kinases (PAK1, Src, and protein kinase Cs) that phosphorylate RhoGDI (1, 5, 6); and phospholipids (1, 2, 7).

Rac is one of the four cytoplasmic activators (p47PHOX, p67PHOX, p60CO, 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–p67PHOX, p40CO). In resting states, the two protein complexes are inactive in the cytoplasm. During cell activation, the multiprotein complex Nox2-p22PHOX-p47PHOX-p40CO-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: RhoGDα, RhoGDIβ (LyGDI), and RhoGDIγ. RhoGDα is ubiquitously expressed, whereas RhoGDIβ is predominantly expressed in hematopoietic cells, and RhoGDIγ is expressed primarily in the brain. In resting cells, RhoGDα 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 RhoGDα/β comprises two distinct regions: an N-terminal unstructured arm (aa 1–58 in RhoGDα, aa 1–55 in RhoGDβ), and an Ig-like folded domain (aa 59–204 in RhoGDα, aa 56–201 in RhoGDβ) containing a hydrophobic pocket that packs the isoprenylated C-terminal tail of Rac (12). The N termini of RhoGDα (25 residues) and RhoGDIβ (22 residues) possess numerous negatively charged amino acids: 8 in RhoGDα and 10 in RhoGDIβ. Based on structural studies of the RhoGDα–Rac1 (12, 13) and RhoGDIβ–Rac2 (14)
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 (K^18^KKKK^18^) and anionic phospholipids, such as phosphatidic acid (PA) and phosphatidylserine, as a key determinant. Previous studies established the identification of RhoGDIα as a component of the phagosome protein transesome (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 as 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 RhoGDBβ in FcγR-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) (humanized monoclonal 4G10) was acquired from MBL International. The mAbs against HA and Myc 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-opsonized 2-μm glass beads (BtlG; 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 maintained in Eagle’s MEM (Wako) and in DMEM (Wako Pure Chemical Industries) containing 10% FBS and 100 μM nonessential amino acids (Wako). For clonal derivation HEK293 cell line 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, RhoGDBβ, and RhoGDBγ were amplified by PCR using first-strand cDNA (BD Biosciences), cloned into the pEGFP(N1) vector (Invitrogen), and named RhoGDIα-GFP, RhoGDBβ-GFP, and RhoGDBγ-GFP. They were also cloned into the phmKO1 vector (humanized monoclonal Kusabira orange; excitation, 548 nm, emission, 561 nm; Amalgaam) (24) and named RhoGDIα-mKO, RhoGDBβ-mKO, and RhoGDBγ-mKO. The expression plasmids of p47^phox^, p67^phox^, p40^phox^ (22), Myc-Rac1 (23), GFP-Rac1, and GFP-Rac1(616) (15), whose PB motif in the C terminus is altered by six Ala and is a phagosome-targeting defective mutant (15), were described previously. Mouse HA-PLD2 was a gift from Dr. Frohman (25, 26). Catalytically inactive PLD2 with the H42D mutation (25) was constructed using the QuikChange XL II mutagenesis kit (Stratagene). Various mutants, including RhoGDIα(D45A), RhoGDIα(D185A), RhoGDIα(D45/185A), RhoGDIα(3A), RhoGDIα(5A), RhoGDIα(8A), RhoGDIα(D45/185A), and RhoGDIα(D182A), and RhoGDIα(D182A) both in pEGFP(N1) and phmKO(MN1), were generated using QuikChange, N-terminal deletion mutants, including ΔN15-RhoGDIα, ΔN25-RhoGDIα, and ΔN22-RhoGDIβ both in pEGFP(N1) and phmKO(MN1), were constructed using PCR. The short hairpin RNA expression vectors pSUPER(neo) and pSUPER(gfp-neo) (Oligoengine) containing a Rac1-specific knockdown target sequence (nucleotides 618–636 from ATG), pSUPER-Rac1(618), and pSUPER-Rac1(618)gfp were described previously (23).

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 12,000 × 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 FuGENE HD reagent (Promega). After 48 h, cells were stimulated with BtG and fixed 5 min later with 4% paraformaldehyde 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 (c-HA: 1:250, c-Rac1: 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 BtG 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 heated objective. All imaging experiments were performed in triplicate or more for at least three independent transfection experiments (n ≥ 9).

ROS production assay

HEK293Rac1KD cells in six-well dishes were transfected with various combinations of plasmids using FuGENE6. Forty–48 h before the assay, the ROS release in response to BtG from 2.0 × 10^5 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 phagocosomal 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 isoforms of RhoGDIα

The three RhoGDIα isoforms were expressed in RAW264.7 cells, with mRNA levels significantly higher for RhoGDIα and RhoGDBγ than those for RhoGDBβ (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 RhoGDBβ-mKO were localized in the cytoplasm, whereas RhoGDBγ-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 RhoGDBβ-mKO in the cytoplasm and with RhoGDBγ-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 immunocytchemistry) during FcγR-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, RhoGDIα-mKO did not accumulate on the phagosomes (Fig. 1B). Moreover, coexpression of RhoGDIα-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 RhoGDIα between previous reports and the current study at that time was that detection of accumulated RhoGDIα 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 RhoGDIα-mKO mutants, RhoGDIα(D45A) and RhoGDIα(D185A), characterized by weakened interactions with RhoGTPases due to partial conformational changes in RhoGDI–RhoGTPase complexes (13, 14, 27). 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 when coexpressed with GFP-Rac1 (Fig. 1D, 1E). Immunoprecipitation analysis showed that RhoGDIα(D45A) and RhoGDIα(D185A) maintained a weak interaction with Rac1 (Fig. 1F), which is consistent with a previous report (27). Thus, these results suggested that RhoGDIα and Rac1 accumulate on phagosomes as a complex during FcγR-mediated phagocytosis using the interaction between RhoGDIα and Rac1 after conformational changes in the complex.

Next, we examined the effects of RhoGDIα, RhoGDIα(D45A), and RhoGDIα(D185A) on ROS production using HEK293Nox2/FcγRIIα cells. Untagged and C-terminally GFP- or mKO-tagged RhoGDIα constructs inhibited ROS production to a similar extent (data not shown). RhoGDIα-GFP completely inhibited ROS production (1.2 ± 0.2%), whereas RhoGDIα(D45A)-GFP and RhoGDIα(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 RhoGDIα on FcγR-mediated ROS production depends on its binding affinity to Rac1.

Negatively charged N terminus of RhoGDIα suppresses Rac-dependent membrane (PM and phagosome) targeting of RhoGDIα

We hypothesized that the negatively charged flexible N terminus (25 residues) of RhoGDIα plays an important role in the subcellular localization of the RhoGDIα–Rac1 complex. First, we constructed N-terminal deletion mutants of RhoGDIα: ΔN15-RhoGDIα-mKO and RhoGDIα-mKO (Δ45A and Δ185A) are expressed in RAW264.7 cells, macrophages, or neutrophils) and detection method used (proteomics, immunocytochemistry, or confocal fluorescence imaging). Then, we tested RhoGDIα-mKO mutants, RhoGDIα(D45A) and RhoGDIα(D185A), characterized by weakened interactions with RhoGTPases due to partial conformational changes in RhoGDI–RhoGTPase complexes (13, 14, 27). 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 when coexpressed with GFP-Rac1 (Fig. 1D, 1E). Immunoprecipitation analysis showed that RhoGDIα(D45A) and RhoGDIα(D185A) maintained a weak interaction with Rac1 (Fig. 1F), which is consistent with a previous report (27). Thus, these results suggested that RhoGDIα and Rac1 accumulate on phagosomes as a complex during FcγR-mediated phagocytosis using the interaction between RhoGDIα and Rac1 after conformational changes in the complex.

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and ΔN25-RhoGDIα-mKO (Fig. 2A). In resting RAW264.7 cells, both mutants were detected in the cytoplasm (Fig. 2B, 2C), but ΔN25-RhoGDIα-mKO also exhibited a weak PM localization (Fig. 2C). During FcyR-mediated phagocytosis, only ΔN25-RhoGDIα-mKO was found accumulating on phagosomes (Fig. 2C). The PM localization of ΔN25-RhoGDIα-mKO was also observed in HEK293 cells (Fig. 3A). It disappeared in HEK293 cells with stable knockdown of Rac1, HEK293Rac1KD 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(1618), into HEK293Rac1KD cells (Fig. 3D). Rac1-dependent PM localization/ recruitment of ΔN25-RhoGDIα-mKO was confirmed by single-molecule imaging of tetramethylrhodamine-conjugated Halo-tagged ΔN25-RhoGDIα (HaloTag; Promega) in HeLa cells based on residence time, frequency of recruitment, and trajectory in PM: residence time and recruitment in PM of ΔN25-RhoGDIα-Halo (tetramethylrhodamine) were significantly extended and increased, respectively, by coexpression of GFP-Rac1 (Supplemental Fig. 3). Immunoprecipitation analysis demonstrated that ΔN15-RhoGDIα and ΔN25-RhoGDIα had strong interactions with Rac1 similar to RhoGDIα (Fig. 3E). FcyR-mediated ROS production in HEK293Nox2/FcγRIIa cells was completely inhibited by ΔN25-RhoGDIα-GFP or ΔN15-RhoGDIα-GFP, similar to RhoGDIα (Fig. 3F). These data suggested the following: 1) the negatively charged N terminus of RhoGDIα plays a pivotal role in retaining RhoGDIα in the cytoplasm; 2) the membrane (PM and phagosome) localization of RhoGDIα is dependent on Rac1; and 3) the inhibitory effect of RhoGDIα 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 RhoGDIα 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 RhoGDIα (ΔN15, ΔN25). A red number in parenthesis indicates the total number of negatively charged amino acids in their N termini. (B and C) ΔN15-RhoGDIα-mKO (B) or ΔN25-RhoGDIα-mKO (C) was transfected into RAW264.7 cells. The transfected cells were stimulated with 2-μm BlgG. ΔN25-RhoGDIα-mKO but not ΔN15-RhoGDIα-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 RhoGDIα–Rac1 complex on phagosomes by negative charges in the N terminus of RhoGDIα

To further investigate the impact of the negative charges located in the N terminus (25 residues) of RhoGDIα, we constructed three mutants with reduced numbers of negatively charged amino acids: RhoGDIα(3A)-mKO, RhoGDIα(5A)-mKO, and RhoGDIα(8A)-mKO (Fig. 4A). Immunoprecipitation analysis showed that all mutants bind Rac1 similar to RhoGDIα, contrary to a faint binding of Rac1 interaction-impaired RhoGDIα(8A:D45/185A) (Fig. 4B). Confocal microscopy revealed that the mutants RhoGDIα(3A)-mKO and RhoGDIα(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 RhoGDIα(8A)-mKO mutant exhibited remarkable PM localization in resting RAW264.7 cells and accumulated on phagosomes during phagocytosis (Fig. 4E), contrary to cytoplasmic RhoGDIα(8A:D45/185A) (Fig. 4F). Endogenous Rac1 accumulated with RhoGDIα(8A)-mKO on phagosomes (Supplemental Fig. 4A). These data suggested that membrane (PM and phagosome) localization of RhoGDIα(8A)-mKO is mediated by its binding to Rac1. The coexpression of GFP-Rac1 with RhoGDIα (5A)-mKO or RhoGDIα(3A)-mKO stimulated the phagosomal accumulation of RhoGDIα(5A)-mKO but not RhoGDIα(3A)-mKO (Supplemental Fig. 4B, 4C). The phagosomal accumulation of RhoGDIα(8A)-mKO with GFP-Rac1 disappeared when GFP-Rac1(6A), a phagosome-targeting defective mutant (15), was coexpressed with RhoGDIα(8A)-mKO (Supplemental Fig. 4D, 4E). Because RAW264.7 macrophages express all Rac isoforms (15), we knocked down Rac1 in HEK293N25/FcγRIIa cells, in which Rac1 is a predominant Rac isoform, using pSUPER-Rac1(1618)gfp (23). The phagosomal accumulation of RhoGDIα(8A)-mKO in HEK293N25/FcγRIIa cells (Fig. 4G) were significantly reduced when RhoGDIα was knocked down (Fig. 3H, 3I). Moreover, ROS production from HEK293N25/FcγRIIa cells was completely suppressed by RhoGDIα-GFP, RhoGDIα(3A)-GFP, RhoGDIα(5A)-GFP, and RhoGDIα(8A)-GFP. However, RhoGDIα(8A:D45/185A)-GFP caused only a mild suppression (65.9 ± 9.6%) (Fig. 3J). These data indicated that all eight, particularly the sequence E17NEEDE22, plays a pivotal role in retaining RhoGDIα in cyttoplasm; the membrane (PM and phagosome) localization of RhoGDIα is dependent on Rac1; and the inhibitory effect of RhoGDIα on ROS production is probably determined by its binding affinity to Rac1, but not on its localization.

**PLD2 promotes the translocation of the RhoGDIα–Rac1 complex to phagosomes and enhances ROS production**

The interaction between the PB motif in the C terminus of Rac1 (K188KRKK188) 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 RhoGDIα-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 RhoGDIα was confirmed by immunoprecipitation analysis (Fig. 5D). Transfection of PLD2 in HEK293N25/FcγRIIa cells increased FcyR-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 RhoGDI\(\alpha\) accumulates on phagosomes as a RhoGDI\(\alpha\)–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 RhoGDI\(\beta\) suppresses the Rac-dependent phagosomal targeting of RhoGDI\(\beta\)

Finally, we tested the impact of the negatively charged N terminus (22 residues) of RhoGDI\(\beta\) (Fig. 6A) on phagocytosis and ROS production. When GFP-Rac1 was coexpressed with RhoGDI\(\beta\)-mKO in RAW264.7 cells, GFP-Rac1 (but not RhoGDI\(\beta\)-mKO) accumulated on phagosomes during Fc\(\gamma\)R-mediated phagocytosis (Fig. 6B), in sharp contrast with GFP-Rac1 plus RhoGDI\(\alpha\)-mKO (Fig. 1C). Even RhoGDI\(\beta\)(D42A)-mKO, which is a homologous mutant of RhoGDI\(\alpha\)(D45A), did not accumulate on phagosomes, despite significant GFP-Rac1 accumulation (Fig. 6C). However, deletion of the N terminus (ΔN22-RhoGDI\(\beta\)-mKO) allowed RhoGDI\(\beta\) to accumulate with GFP-Rac1 on phagosomes (Fig. 6D). Immunoprecipitation analysis indicated that RhoGDI\(\beta\) has a much weaker interaction with Rac1 than RhoGDI\(\alpha\) (Fig. 6E). This finding is consistent with a previous report on RhoGDI\(\beta\) showing a 10- to 20-fold lower affinity for Cdc42 (28). Although ΔN22-RhoGDI\(\beta\) maintained an interaction with Rac1, RhoGDI\(\beta\)(D42A) demonstrated no interaction (Fig. 6E). The ROS production assay in HEK293 Nox2/Fc\(\gamma\)RIIa cells revealed a moderate suppression by RhoGDI\(\beta\) (32.2 ± 6.0%) or ΔN22-RhoGDI\(\beta\) (35.8 ± 5.5%) and a faint suppression by RhoGDI\(\beta\)(D182A) (85.0 ± 7.81%), which is a homologous mutant of RhoGDI\(\alpha\)(D185A), in contrast to the complete suppression by RhoGDI\(\alpha\) (Fig. 6F). There was no statistical difference between RhoGDI\(\beta\) and ΔN22-RhoGDI\(\beta\) with regard to binding affinity for Rac1 or inhibitory effect on ROS production.

Discussion

The negatively charged and flexible N-terminal domain of RhoGDI\(\alpha\) (25 residues) and RhoGDI\(\beta\) (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 RhoGDI\(\alpha\) and RhoGDI\(\beta\), respectively. The second cluster consists of five and eight negative amino acids in human RhoGDI\(\alpha\) and RhoGDI\(\beta\), 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) RhoGDIα(8A)-mKO was cotransfected with pSUPER(gfp-neo) (G) or pSUPER-Rac1(681)gfp (H) in HEK293 Nox2/FcRIIα 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 HEK293 Nox2/FcRIIα 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 phosphatidylserine, 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/ι, stably associated with the RhoGDIα–Rac1 complex, to the PM through PA production (31).

**FIGURE 5.** PA produced by PLD2 promotes the translocation of the RhoGDIα–Rac1 complex to phagosomes and enhances ROS production. (A–C) RhoGDIα-mKO was cotransfected with GFP-Rac1 and HA-PLD2 (A), GFP-Rac1 and HA-PLD2(H442D) (B), or GFP-Rac1(6A) and HA-PLD2 (C) into RAW264.7 macrophages. The transfected cells were stimulated with 2-μm BigG. After 5 min, fixed cells were stained with a hemagglutinin (HA) mAb and visualized with a confocal microscope using an Alexa647-conjugated anti-IgG. Accumulated PLD2 (A), but not PLD2(H442D) (B), induces the translocation of RhoGDIα-mKO and GFP-Rac1 to phagosomes. Accumulated PLD2 induces no translocation of RhoGDIα-mKO when GFP-Rac1(6A), a phagosome-targeting defective mutant of GFP-Rac1, was used. The arrows, arrowheads, and double arrows indicate phagosomes engulfing BigG. Scale bar, 10 μm. (D) Representative immunoprecipitation data (n = 3) showing no interaction between HA-PLD2 and GFP-Rac1 or RhoGDIα-GFP. (E) HEK293Nox2/FcRIIa cells were transfected with a mock vector, HA-PLD2 or HA-PLD2(H442D), in combination with Phox proteins. The cells were stimulated with BigG, and ROS release was measured (n = 5). PLD2 but not PLD2(H442D) enhances ROS production. The comparable expression of proteins was confirmed by immunoblotting.
expression of proteins was confirmed by immunoblotting. GFP, or ImageJ. (phagosomes (tion between Myc-Rac1 and RhoGDI
the PM was slightly increased by Rac1 coexpression but was ap-
figuration also showed that the frequency of RhoGDI
with Rac1 and may keep the inhibitory effect on ROS production
replurston between the negatively charged N terminus of RhoGDI
crepancy may be at least partially explained by the electrostatic
Rac1 on phagosomes owing to an undefined mechanism, the dis-
PAK1–mediated RhoGDI
suppresses the Rac-dependent phagosomal targeting of RhoGDI
RAW264.7 cells showed that RhoGDI
accumulates on phagosomes during the phagocytosis of
Listeria monocytogenes (33). These data suggest that RhoGDI
and Rosechichia coli (33). These data suggest that RhoGDI
accumulates on phagosomes as a RhoGDI
EDA
-EDG
-EDA

FIGURE 6. The negatively charged N terminus of RhoGDIβ suppresses the Rac-dependent phagosomal targeting of RhoGDIβ and ROS production. (A) Comparison of the amino acid sequence of human RhoGDIX and RhoGDIβ. (B–D) GFP-Rac1 was cotransfected with RhoGDIX-mKO (B), RhoGDIX (D42A)-mKO (C), or ΔN22-RhoGDIXβ-mKO (D) in RAW264.7 cells. The transfected cells were stimulated with 2-μM BlgG. Only GFP-Rac1, but not RhoGDIX-mKO (B) or RhoGDIXβ(D42A)-mKO (C), accumulates on phagosomes. In sharp contrast, ΔN22-RhoGDIXβ-mKO accumulates with GFP-Rac1 on phagosomes (D). The arrows and arrowheads indicate phagosomes engulfing BlgG. (E) Representative immunoprecipitation data (n = 5) for the interaction between Myc-Rac1 and RhoGDIXβ-GFP, its mutants, or RhoGDIXα-GFP. Right panel shows quantification of immunoprecipitated protein bands using ImageJ. (F) HEK293N22/RhoGDIXα cells were transfected with a mock vector, RhoGDIXα-GFP, RhoGDIXβ-GFP, RhoGDIXβ(D42A)-GFP, RhoGDIXβ(D182A)-GFP, or ΔN22-RhoGDIXβ-GFP in combination with Phox proteins and Myc-Rac1. The cells were stimulated with BlgG, and ROS release was measured (n = 5). RhoGDIXβ and ΔN22-RhoGDIXβ show moderate suppression and RhoGDIXβ(D42A) and RhoGDIXβ(D182A) show faint suppression. The comparable expression of proteins was confirmed by immunoblotting.

Additionally, it was reported that DGKζ stably associated with
PAK1 and RhoGDIXα-Rac1, promoted the release of RhoGDIXα from
Rac1 through DGKζ→PA→PAK1–mediated RhoGDIXα phosphory-
lation at platelet-derived growth factor–induced membrane ruffling
in fibroblasts (32). These studies support an involvement of PA pro-
duced from DGK in regulation of RhoGDIXα–Rac1 translocation to
membranes and dissociation on membranes.

In contrast to no detection of RhoGDIXα-mKO on phagosomes
without coexpression of Rac1 and PLD2 under our confocal fluo-
rescence imaging, ΔN25-RhoGDIXα-mKO and RhoGDIXα(8A)-
mKO accumulated on phagosomes even without coexpression of
Rac1; nevertheless, ΔN25-RhoGDIXα and RhoGDIXα(8A) maintained
their abilities to bind Rac1 and to inhibit ROS produc-
tion similar to RhoGDIXα. Although we cannot exclude the pos-
sibility that ΔN25-RhoGDIXα and RhoGDIXα(8A) are more stable with
Rac1 on phagosomes owing to an undefined mechanism, the discrep-
ancy may be at least partially explained by the electrostatic repulsion
between the negatively charged N terminus of RhoGDIXα and the
negative charge of the phagosome, which is enhanced by
anionic phospholipids produced during phagocytosis. ΔN25-
RhoGDIXα and RhoGDIXα(8A) have much weaker electrostatic repu-
sion potency than does RhoGDIXα; 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 im-
ing showed prolonged residence time in the PM of ΔN25-RhoGDIXα
compared with RhoGDIXα (Supplemental Fig. 3D). Single molecule
imaging also showed that the frequency of RhoGDIXα recruitment to
the PM was slightly increased by Rac1 coexpression but was ap-
parently less than that of ΔN25-RhoGDIXα (Supplemental Fig. 3D).
The residence time of RhoGDIXα in the PM was not increased by
Rac1. These results are consistent with the observed lack of phago-
somal RhoGDIXα accumulation under confocal fluorescence imag-
ing, even with Rac1 coexpression (Fig. 1C). Thus, a phagosomal
accumulation enhancing factor for Rac1, such as PLD2, or neu-
rophils, 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 RhoGDIXα
on phagosomes under confocal fluorescence imaging.

The RhoGDIXβ–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 RhoGDIXβ compared with
RhoGDIXα. However, the inhibitory effects of RhoGDIXβ on Rac1
translocation to phagosomes and ROS production were weaker than
those of RhoGDIXα. Our imaging studies using RAW264.7 cells
revealed no apparent accumulation of RhoGDIXβ-mKO on phago-
somes, whereas the N-terminal deletion mutant Δ22-RhoGDIXβ-
mKO accumulated on phagosomes along with GFP-Rac1. A study using the fluorescence resonance energy transfer technique in
RAW264.7 cells showed that RhoGDIXβ accumulates on phago-
somes during the phagocytosis of Listeria monocytogenes or
Escherichia coli (33). These data suggest that RhoGDIXβ also
accumulates on phagosomes as a RhoGDIXβ–Rac1 complex similar
to RhoGDIXα, but the accumulation of the RhoGDIXβ is much
weaker than that of RhoGDIXα. The weaker accumulation of
RhoGDIXβ on phagosomes is likely due to its weaker binding af-
finity for Rac1 and its stronger electrostatic repulsion on phago-
somes than RhoGDIXα.
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/β, 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


