The Guanine Nucleotide Exchange Protein for ADP-ribosylation Factor 6, ARF-GEP100/BRAG2, Regulates Phagocytosis of Monocytic Phagocytes in an ARF6-dependent Process*

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Phagocytosis is a complex multistep process requiring diverse signaling and regulatory molecules. ADP-ribosylation factor 6 (ARF6), a small GTPase, is known to regulate membrane trafficking and the actin cytoskeleton at the plasma membrane and functions as a regulatory molecule of phagocytosis. ARF activity is regulated by cycling between GDP-bound and GTP-bound forms. ARF activation is catalyzed by guanine nucleotide exchange factors (GEFs) that facilitate GTP binding. We had earlier reported a 100-kDa ARF-GEF, termed ARF-guanine nucleotide exchange protein 100, GEP100, that preferentially activates ARF6 and was also described by Dunphy et al. (Dunphy, J. L., Moravec, R., Ly, K., Lasell, T. K., Melancon, P., and Casanova, J. E. (2006) Curr. Biol. 16, 315–320) as brefeldin A-resistant ARF-GEF2 (BRAG2). We have now examined a role for GEP100 in phagocytosis. Stable depletion of GEP100 decreased phagocytosis of serum-treated zymosan and IgG-coated latex.

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GEP100-depleted cells also exhibited reduced F-actin fibers around internalized particles. Attachment of these particles to cells and amounts of C3bi and Fcγ receptors, however, were not affected by GEP100 depletion. On immunofluorescence microscopy, GEP100 and ARF6 were concentrated and partially colocalized around internalized particles. Phagocytosis by GEP100-depleted cells was not further affected by depletion of ARF6.

Phagocytic activity of GEP100-depleted cells was, however, rescued by expression of the constitutively active ARF6Q67N mutant but not by the dominant-negative ARF6T27N mutant. These data are consistent with the conclusion that GEP100 functions in phagocytosis via its role in ARF6-dependent actin remodeling.

Phagocytosis is the process of internalization by a cell of insoluble materials from the external environment (1–3). “Professional” phagocytes, such as neutrophils, macrophages, and dendritic cells, ingest bacteria, microorganisms, and foreign particles via phagocytosis for degradation and antigen presentation (3). Phagocytosis is initiated by interaction of structural molecules on the particle with specific cell-surface receptors. Thereafter, multiple intracellular signaling pathways are activated, initiating particle uptake with pseudopod extension followed by phagosome formation and particle internalization (1, 3, 4). ADP-ribosylation factors (ARFs), 2–20-kDa GTP-binding proteins (small GTPases), are members of the Ras superfamily that regulate vesicular trafficking and intracellular transport (5–9).

One of the six mammalian ARF proteins, ARF6, participates in specifically regulating plasma membrane/endosomal trafficking and remodeling of the peripheral actin cytoskeleton (8, 10). Effects of ARF6 on the activity of lipid-modifying enzymes such as phosphatidylinositol-4-phosphate 5-kinase (11, 12) and phospholipase D (13–16), can alter actin fiber formation and contribute to membrane remodeling (8). Major functions of phagocytic cells, such as migration (17), superoxide production (18), and phagocytosis (19–21), are regulated by ARF6.

Like other small GTPases, ARFs cycle between the active GTP-bound and inactive GDP-bound forms. The replacement of GDP by GTP and hydrolysis of bound GTP are accelerated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively (6, 7, 9, 22). All ARF GEFs contain an ~200 amino acids Sec7 domain that catalyzes the replacement of ARF-bound GDP by GTP (8, 9, 23). The 100-kDa ARF-GEF, initially described as guanine nucleotide exchange protein 100 (GEP100) (24) and also known as brefeldin A-resistant ARF-GEF 2a (BRAG2a) (25, 26), preferentially activated ARF6. It was resistant to inhibition by brefeldin A (BFA), a fungal fatty acid metabolite known to block specific intracellular trafficking pathways by inhibiting the activity of certain ARF-GEFs, e.g. BFA-inhibited GEP 1 and 2 (23, 27, 28). Several functions of GEP100 have been reported. In HeLa cells, GEP100 regulated cell adhesion by controlling endocytosis of β1 integrins (26), whereas in myoblasts and macrophages, GEP100, like its Drosophila homolog “Loner,” was

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2 The abbreviations used are: ARF, ADP-ribosylation factor; GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; GEP, guanine nucleotide-exchange protein; BRAG, brefeldin A-resistant ARF-GEF; BFA, brefeldin A; PMMA, phorbol 12-myristate 13-acetate; dsRNA, double-stranded RNA; TRITC, tetramethylrhodamine B isothiocyanate; PE, phycoerythrin; PFA, paraformaldehyde; DIC, differential interference contrast; ANOVA, analysis of variance.
reported to function in cell-cell fusion (29, 30). GEP100 is believed to be involved also in modification/remodeling of nuclear architecture (31) and was implicated in breast cancer invasion (32). These as well as GEP100 effects cell adhesion and actin cytoskeleton, which involves E-cadherin and α-catenin, depend on its activation of ARF6 (33). Because GEP100 mRNA is enriched in human peripheral blood leukocytes (24), we focused on GEP100 action in those cells. In 2006, we reported that GEP100 was implicated in apoptotic cell death of phorbol 12-myristate 13-acetate (PMA)-differentiated human macrophage-monocyte-like U937 cells (34). Of note, overexpression of a GEP100 mutant lacking the Sec7 domain induced apoptosis, suggesting that the GEP100 apoptosis-inducing activity was independent of ARF activation (34).

We report here that GEP100 and ARF6 were accumulated and colocalized at sites of phagocytosed particles in PMA-differentiated human U937 cells. In cells depleted of GEP100 by the stable transfection of short hairpin RNA (shRNA), phagocytosis of serum-opsonized zymosan and IgG-coated latex beads was inhibited, and F-actin formation surrounding phagocytosed particles was diminished. In contrast, GEP100 depletion did not significantly affect cell-surface levels of complement and IgG receptors involved in the binding of particles to cells, suggesting that GEP100 is involved in phagocytosis via regulation of actin remodeling. Furthermore, these events appear to be ARF6-dependent, as expression of the constitutively active form of ARF6, ARF6Q67L, rescued GEP100-depleted cells.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Zymosan, latex beads (diameter 3 μm), phalloidin-TRITC, and PMA were purchased from Sigma; bovine serum albumin (BSA) was from Nacalai Tesque, Inc. (Kyoto, Japan); phycoerythrin (PE)-conjugated mouse IgG1 and fluorescein isothiocyanate (FITC)-conjugated mouse IgG1 were from eBioscience (San Diego, CA); whole goat serum was from Immunological Laboratories Co., Ltd. (Takasaki, Japan).

**Antibodies**—Rabbit anti-human GEP100 antibody was prepared and purified as described previously (34). Rabbit anti-ARF6 serum against human ARF6 was prepared after immunization with a synthetic peptide corresponding to the ARF6 C-terminal amino acid sequence. Mouse anti-human ARF1 IgG (3F1) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); mouse anti-glycerolaldehyde-3-phosphate (GAPDH) IgG (MAB374) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were from Chemicon International (Temecula, CA); HRP-conjugated goat anti-mouse IgG/IgM was from Jackson ImmunoResearch Laboratories (West Grove, PA); PE-conjugated mouse anti-human CD11b IgG (ICRF44), FITC-conjugated mouse anti-human CD64 IgG (10.1), and PE-conjugated mouse anti-human CD71 IgG (55.7/14) were from eBioscience; mouse anti-bovine α-tubulin IgG (C26–10501), Alexa Fluor 594-conjugated goat anti-rabbit IgG, and Alexa Fluor 488-conjugated goat anti-mouse IgG were from Invitrogen; mouse anti-FLAG IgG M2 and Cy3-conjugated anti-FLAG IgG M2 were from Sigma.

**Cells and Culture**—Human monocyte macrophage U937 cells (American Type Culture Collection, Manassas, VA) were grown at 37 °C with 5% CO2 in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal calf serum (FCS, Cell Culture Technologies, Herndon, VA), penicillin (100 units/ml), and streptomycin (0.1 mg/ml).

We used stably GEP100-depleted cells (34). Briefly, two shRNA expression vectors that targeted different sequences in GEP100 (GEP100 shRNA-1 and -2) were transfected. These sequences are described in Someya et al. (34). Cells were grown in RPMI 1640 medium containing 1 mg/ml G418, with selection of GEP100-depleted cells by repeated dilution (10-fold dilution was repeated 6–8 times). Scrambled shRNA sequences corresponding to GEP100 shRNA-1 and -2, respectively, were stably expressed as specific controls. Cell content of GEP100 was assessed by immunofluorescence and Western blotting.

**GEP100 and ARF6 Expression Plasmids—**RNA interference (RNAi)-resistant plasmids for the expression of wild-type GEP100 and Sec7 domain-deleted GEP100 (GEP100ΔSec7) were constructed by replacing the bases within the target of shRNA without changing the coding amino acids. Sequences of site-directed mutagenesis of RNAi-resistant expression plasmids for two GEP100-depleted cells (GEP100 shRNA-1 and -2) were, respectively, 5’-gaagaactctgccaac-3’ and 5’-atgtaaggacc-3’ (the bold-font bases were changed from the wild-type bases). Specifically, PCR was performed using FLAG-CMV-GEP100WT or FLAG-CMV-GEP100ΔSec7 as a template with the following primers: 5’-gtcatacatacagtccgacgacctc-3’ and 5’-gtatctctctctctgagatc-3’ for GEP100 shRNA-1-resistant cDNA (reFLAG-CMV-GEP100WT-1 or -GEP100ΔSec7-1); 5’-gtcatacattacagctgaagtc-3’ and 5’-taaaggttaaggccgagatccg-3’ for GEP100 shRNA-2-resistant cDNA (reFLAG-CMV-GEP100WT-2 or -GEP100ΔSec7-2).

After treatment with DpnI, PCR products were circularized by incubation with T4 polynucleotide kinase and T4 DNA ligase.

For preparation of ARF6 cDNA, total RNA from 2 × 10^6 U937 cells was purified using RNeasy Plus Mini Kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized with total RNA (1 μg) and oligo(dT) primer by reverse transcription, and ARF6 cDNA was amplified by PCR using primers 5’-ggtaggtggcctctgtaatcctgtaattc-3’ and 5’-ttgtaaacccggcgcagactggt-3’ (the underlined and bold font sequences are an EcoRI restriction site and initiation codon, respectively) and 5’-ggagaaccttggccgccgagagagttatgagtt-3’ (the underlined sequence is a KpnI restriction site). To construct an expression vector of C-terminal FLAG-tagged ARF6, the PCR product was subcloned into pCR-Blunt II-TOPO vector (Invitrogen), from which it was excised with EcoRI and KpnI and ligated into the pFLAG-CMV-5b vector (Sigma) to produce pFLAG-CMV-ARF6. The Q67L and T27N mutants of ARF6 were synthesized by PCR using the following primers: ARF6Q67L, 5’-ggatggtggtctggctctcaac-3’ and 5’-ccggtggtggtctggctctcaac-3’; ARF6T27N, 5’-ggactggtcggctctcaac-3’ and 5’-ccggtggtcggctctcaac-3’ (the bold-font bases were changed from the wild-type bases).

**Opsonization of Zymosan Particles and Latex Beads—**Zymosan was opsonized with complement C3bi by incubation at 37 °C for 30 min in fresh human serum (2 × 10^8 particles/ml) prepared from the blood of healthy volunteers. After washing twice with phosphate-buffered saline (PBS), opsonized zymo-
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san was dispersed \((6.4 \times 10^6 \text{ particles/ml})\) in serum/antibiotic-free RPMI 1640 (35). For opsonization with IgG, latex beads \((3 \times 10^7 \text{ particles/ml})\) in 0.1 M glycine-NaOH buffer \((pH 8.2)\) with BSA \((5 \text{ mg/ml})\) were stirred for 2 h at room temperature, washed twice with PBS, and stirred for 3 h at room temperature with human IgG \((1 \text{ mg/ml}, \text{ Sigma})\) and N-cyclohexyl-N'-\((2\text{-}\text{morpholinoethyl})\) carbodiimide metho-p-toluenesulfonyl \((60 \text{ mg/ml}, \text{ Sigma})\) to cross-link IgG covalently to BSA. IgG-opsonized latex beads (IgG beads) were then washed twice with PBS and suspended \((6.4 \times 10^6 \text{ particles/ml})\) in serum/antibiotic-free RPMI 1640 (36).

Measurement of Phagocytosis—Cells were incubated in the 0.5 ml of RPMI 1640 medium containing 10% FCS and antibiotics with 10 nM PMA for 30 h to induce differentiation to monocyte-macrophage-like cells on 12-mm round glass coverslips (Fisher) in 24-well plates \((4 \times 10^5 \text{ cells/well}, 40 \text{–}50\% \text{ confluent})\) (34). After washing with serum-/antibiotic-free RPMI 1640, differentiated cells were incubated with opsonized zymosan or IgG beads \((3.2 \times 10^5 \text{ particles/well}, 1:8)\) for 40 or 70 min, respectively, in 0.5 ml of the same medium and fixed with 4% paraformaldehyde (PFA) in PBS. Because rates of uptake of opsonized zymosan and IgG beads by PMA-differentiated U937 cells were almost constant for 60 and 90 min, respectively, phagocytosis was assayed by incubating cells with opsonized zymosan for 40 or 70 min, respectively, and incubated with blocking buffer. After washing with PBS, cells were then washed twice with PBS containing 1% BSA and 0.05% NaN₃ (PBS-BSA), washed with PBS, and blocked by incubation (1 h, at room temperature) with blocking buffer. After washing with PBS, cells were incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG \((4 \mu g/ml)\) and Alexa Fluor 488-conjugated goat anti-mouse IgG \((4 \mu g/ml)\), washed with PBS, and prepared for immunofluorescence microscopy.

Detection of F-actin—PMADifferentiated cells were incubated with opsonized zymosan or IgG beads and fixed with 4% PFA-PBS as described under “Measurement of phagocytosis” above. After permeabilization with 0.05% Triton X-100 in PBS and incubation with phalloidin-TRITC, 50 ng/ml in diluted (1/10) blocking buffer. After washing with PBS, cells were fixed with 1 h with Alexa Fluor 594-conjugated goat anti-rabbit IgG \((4 \mu g/ml)\) or Alexa Fluor 488-conjugated goat anti-mouse IgG \((4 \mu g/ml)\), washed with PBS, and prepared for immunofluorescence microscopy. To quantify particle-associated F-actin, particles were identified by DIC images \((>100 \text{ cells with internalized or bound particles were chosen})\), and particle-associated F-actin structures in the corresponding phalloidin-TRITC images were counted. Accumulation of F-actin was defined as the percentage of total number of particles that are associated with F-actin (referred to as accumulation index).

Transfection with Small Interfering RNA (siRNA)—Cells were transiently transfected with double-stranded RNA \((300 \text{ ng of dsRNA/1.3} \times 10^6 \text{ cells/100}\mu\text{ reaction mixture})\) using an Amaza Nucleofector with Cell Line Nucleofector Kit C. Nucleotide sequences used were: ARF1 dsRNA-1 5’-AACAGCUUCUGCACCAACCCUCU-3’; ARF1 dsRNA-2 5’-UGACAGAGAGGCUGUGUAGAC-3’; ARF6 dsRNA-1 5’-GCACCCGAUUAUCAAUGACCG-3’; ARF6 dsRNA-2 5’-CAAGUUCACGUAUGGGAU-3’. Nontargeting dsRNA sequence (negative control) was 5’-GUACCGACGUACUUGUAUC-3’. The dsRNAs were chemically synthesized by RNAi Co., Ltd. (Tokyo, Japan). After growth for 3 h in RPMI 1640 supplemented with 10% FCS and antibiotics, transfected cells \((4 \times 10^6 \text{ cells/12-mm round glass coverslip in well})\) were treated with PMA for 30 h in the same medium in a 24-well plate. PMA-differentiated cells were incubated with opsonized zymosan or IgG beads and fixed with 4%PFA-PBS. Phagocytosis was evaluated using DIC microscopy.

Transfection of GEP100-depleted Cells with Expression Plasmids—GEP100-depleted cells were transiently transfected with RNAi-resistant GEP100 or GEP100Sec7 expression plasmid. After treatment with PMA for 7 h in RPMI 1640 with 10% FCS and antibiotics, GEP100-depleted cells were transfected with plasmids (reFLAG-CMV-GEP100WT-1 or -GEP100Sec7-1 to GEP100Sec7-2 to
GEP100 shRNA-2 cells, 8 μg of plasmid/1.3 × 10^6 cells/100 μl reaction mixture) using an Amaxa Nucleofector. Then transfected cells were incubated in an 8-well Lab-Tek Chamber Slide System (1.5 × 10^5 cells/well, 40–50% confluent; Nunc, Rochester, NY) with PMA for 11 h in RPMI 1640 supplemented with 10% FCS and antibiotics. For transfection of ARF6 and its mutant expression plasmids, cells were transiently transfected with plasmids (3 μg plasmid/1.3 × 10^6 cells/100 μl reaction mixture) using an Amaxa Nucleofector and were incubated in an 8-well Lab-Tek Chamber Slide with PMA for 22 h.

PMA-differentiated transfected cells were incubated with opsonized zymosan or IgG beads (1.2 × 10^6 particles/well, cell-to-particle ratio 1:8) for 40 or 70 min, respectively, and phagocytosis was evaluated using immunofluorescence microscopy.

**Western Blot Analysis**—Cells suspended in SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.005% bromphenol blue, and 5% 2-mercaptoethanol) were disrupted by sonication on ice. After heating (3 min, 100 °C), cell lysates (20 μl, 8 × 10^6 cell equivalents) were subjected to SDS-PAGE in 8 or 12% gels, and separated proteins were electroblotted onto PVDF membranes (Immobilon-P; Millipore, Bedford, MA). Blots were incubated (1 h, 37 °C) with BlockAce (Dainippon Pharmaceutical Co. Ltd. Osaka, Japan) and then overnight at 4 °C with Cy3-conjugated anti-FLAG M2 IgG (0.5 μg/ml). After mounting, cells stained with Cy3-conjugated anti-FLAG IgG were selected, and phagocytosis was evaluated using immunofluorescence microscopy.

**Statistical Analysis**—Results are expressed as the means ± S.E. Statistical analyses were performed using one-way ANOVA (and non-parametric), and statistical significance was accepted at p < 0.05.

**RESULTS**

Effect of GEP100 Suppression on Phagocytic Activity—Human monocyte macrophage-like U937 cells were used to examine a potential role for GEP100 in phagocytosis of opsonized zymosan or IgG beads, which were bound predominantly to complement (C3bi receptor; CR3, CD11b/18 integrin) and Fcγ receptors, respectively. The ability of undifferentiated U937 cells to ingest particles was low (phagocytic indices: 0.37 ± 0.18 for opsonized zymosan, 0.68 ± 0.19 for IgG beads; means ± S.E., n = 3). However, phagocytic activity of U937 cells for opsonized zymosan (Fig. 1A) or IgG beads (Fig. 1B) was enhanced significantly after their differentiation into macrophage-like cells by incubation with PMA. Under differentiating conditions, phagocytic indices for uptake of opsonized zymosan and IgG beads were, respectively, 22.5 ± 5.7 (n = 11) and 24.2 ± 9.1 (n = 7) (means ± S.E.). In contrast, untreated zymosan and uncoated latex beads were ingested poorly by PMA-differentiated U937 (data not shown), consistent with earlier findings that internalization of the opsonized ligands occurs via complement and Fcγ receptors.

To evaluate the effect of GEP100 on phagocytosis, GEP100-depleted U937 cell lines were established by stable transfection of shRNA expression plasmids (GEP100 shRNA-1 or shRNA-2), which were directed at different GEP100 sequences (34). Immunofluorescence microscopy (Fig. 2A) and Western blotting (Fig. 2B) showed that the amounts of GEP100 in cells expressing the respective GEP100 shRNAs were less than those of cells with the relevant scrambled shRNA-1 or -2. Amounts of immunoreactive GEP100 in cells expressing GEP100 shRNA-1 or -2 were, respectively, <33 and <40% that of control cells expressing the corresponding scrambled shRNA-1 or -2, whereas ARF6 levels in the same cells were not affected (Fig. 2C). Uptake of opsonized zymosan (Fig. 2D) or IgG beads (Fig. 2E) was significantly less in GEP100-depleted cells (GEP100 shRNA-1 and shRNA-2) than in respective control cells (Scrambled shRNA-1 or shRNA-2), consistent with a role for GEP100 in both complement receptor- and Fcγ receptor-mediated phagocytosis. Binding of opsonized zymosan (Fig. 2F) and IgG beads (Fig. 2G) to GEP100-depleted cells, however, was not significantly different from that of control cells.

Next, we examined the effect of recombinant GEP100 and Sec7 domain-deleted GEP100 mutant (GEP100ΔSec7) on phagocytosis by GEP100-depleted cells using RNAi-resistant GEP100 and GEP100ΔSec7 expression plasmids. The Sec7 domain of ARF-GEPs is necessary for the exchange of GDP with GTP on ARFs. Our previous report showed that expression of recombinant GEP100- or GEP100ΔSec7-induced apoptotic cell death (34). In a preliminary experiment, cells over-expressing GEP100- or GEP100ΔSec7 underwent cell death 12–13 h after transfection (data not shown). Therefore, in this experiment, phagocytic activity was evaluated 11 h after transfection of GEP100 or GEP100ΔSec7 expression plasmid.
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FIGURE 2. Effect of GEP100 depletion on phagocytosis by U937 cells. Untreated (U937), control (Scrambled, shRNA-1 and shRNA-2), and stably depleted cells (GEP100, shRNA-1 and shRNA-2) were incubated with PMA for 30 h. A, fixed cells were incubated with rabbit anti-GFP100 antibody followed by Alexa 594-labeled anti-rabbit IgG. GEP100 immunofluorescence (upper) and DIC (lower) images are shown. All fluorescence images (upper) were recorded in the same detection sensitivity, so fluorescent intensity correlates to the amount of GEP100. Scale bars, 30 μm. B, PMA-treated cells were lysed, and GEP100, ARF6, tubulin, and GAPDH in total cell lysates were analyzed by Western blotting. C, proteins were quantified by densitometry amounts expressed relative to that of U937 in the same experiments. *P < 0.05, **P < 0.01, ***P < 0.001. D–G, U937, control (Scrambled, shRNA-1 and shRNA-2) and depleted cells (GEP100, shRNA-1 and shRNA-2) were incubated with opsonized zymosan (D and F) or IgG beads (E and G). After fixation, phagocytosed and attached particles were counted (DIC images). Phagocytic (D and F) and attachment indices (E and G) were calculated and expressed relative to that of untreated cells (U937) in the same experiment. *P < 0.05, **P < 0.01 for the indicated differences.

though GEP100-FLAG and GEP100ΔSec7-FLAG were expressed in GEP100-depleted cells (Fig. 3A, FLAG-protein), the expression levels of these proteins were low compared with endogenous GEP100 (Fig. 3A, GEP100). Probably, in addition to the short incubation time after transfection (11 h), the presence of only a small percentage of transfected cells (8–20% of total cells) was responsible for the low level of GEP100-FLAG and GEP100ΔSec7-FLAG compared with endogenous GEP100 as detected by Western blotting. Therefore, to assess the effect of GEP100 overexpression on phagocytosis of GEP100-depleted cells, cells reactive with anti-FLAG antibody were selected, and ingested particles in transfected cells were counted. The low phagocytic activity of GEP100-depleted cells (GEP100 shRNA-1 and shRNA-2), based on uptake of opsonized zymosan (Fig. 3B) or IgG beads (Fig. 3C), was rescued by transfection of GEP100-FLAG expression plasmids (WT). In contrast, GEP100ΔSec7-FLAG did not restore phagocytic activity (Fig. 3, B and C, ΔSec7). Therefore, GEP100 appears to regulate uptake of opsonized zymosan or IgG beads through control of ARF activation.

Distribution of Internalized GEP100—We had observed that endogenous GEP100 was scattered in punctate clusters throughout the cytoplasm in human T98G glioblastoma cells and HepG2 liver carcinoma cells, with concentration in the perinuclear region (24, 33). In PMA-differentiated U937 cells, GEP100 was concentrated in punctate collections in the cytoplasm and perinuclear region, similar to the distributions already reported in other cell types (Figs. 4, Resting, and 2A, U937). GEP100 surrounded the internalized vesicles containing opsonized zymosan or IgG beads (Fig. 4).

Cell-surface Expression of Phagocytic Receptors on GEP100-suppressed Cells—Phagocytosis is initiated by interaction of a particle with a specific receptor on the cell surface. GEP100/BRAG2a was reported to regulate amounts of β1 integrin (CD29) on the HeLa cell surface (26). We quantified CD11b (C3bi receptor subunit) and CD64 (high affinity Fcγ receptor) on GEP100-depleted cells. As shown in Fig. 5A, amounts of CD11b in GEP100-depleted cells were similar to those of control cells. The expression level of CD64 (Fig. 5B) in one of two GEP100-suppressed cells (GEP100 shRNA-1) was decreased significantly (GEP100 shRNA-1 versus Scrambled shRNA-1; P < 0.001). In contrast, in the other GEP100-depleted cells (GEP100 shRNA-2), CD64 expression was the same as that in control cells. We also evaluated expression of GEP100 by flow cytometry using Alexa488-labeled anti-GEP100 antibody or anti-GEP100 primary antibody/Alexa 488-labeled secondary anti-rabbit IgG and did not detect GEP100 (data not shown). Therefore, GEP100 may not exist on the cell surface with endocytosed and recycled to the cell surface, did not differ in GEP100-depleted and control cells (Fig. 5C). Although decreased CD64 expression in GEP100 shRNA-1 cells (one of two GEP100-depleted cell populations) was observed (Fig. 5B), attachment of opsonized zymosan (Fig. 2F) and IgG beads (Fig. 2G) to GEP100 shRNA-1 cells appeared similar to attachment to scrambled shRNA-1 cells. Altogether, reduction of phagocytic activity of GEP100-depleted cells seems not to be due to the effects on the recognition/attachment phase of phagocytosis.
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**FIGURE 3.** Effect of recombinant GEP100 on phagocytosis by GEP100-depleted cells. Stably GEP100-depleted cells (GEP100-shRNA-1 and shRNA-2) were transfected with plasmids encoding either RNAi-resistant GEP100 (WT), GEP100ΔSec7 (ΔSec7) or empty (−) plasmid. U937 and control (Scrambled, shRNA-1 and shRNA-2) cells were transfected with empty plasmid. Transfected cells were incubated for 11 h with PMA, A, a representative Western blot from one of six experiments shows GEP100, FLAG-tagged proteins (FLAG-protein), ARF6, tubulin, and GAPDH in total cell lysates. On the blot that was reacted with anti-GEP100 antibody, short (Short) and long (Long) time exposure images were shown. B and C, PMA-differentiated U937, control (Scrambled, shRNA-1 and shRNA-2), and depleted cells (GEP100, shRNA-1 and shRNA-2) were incubated with opsonized zymosan (B) or IgG beads (C). After fixation, cells were reacted with Cy3-conjugated mouse anti-FLAG antibody. Cells reacted with anti-FLAG antibody were selected, and phagocytosed particles were counted using immunofluorescence microscopy. Phagocytic indices were calculated and expressed relative to that of U937 cells in the same experiment, = 1.0. Data are the means ± S.E. of values from five to six experiments. *, p < 0.05 for the indicated differences.

**FIGURE 4.** Distribution of GEP100 during phagocytosis. PMA-differentiated U937 cells were incubated without (Resting) or with opsonized zymosan or IgG beads as in Fig. 2. After fixation, cells were reacted with rabbit anti-GEP100 antibody followed by Alexa 594-labeled anti-rabbit IgG. GEP100 (upper) and DIC (lower) images are shown. Resting reflects the distribution of GEP100 in the absence of particles. Arrows indicate the phagocytosed particles. The experiment was repeated six times with a similar result. Scale bars, 6 μm.

**Effect of GEP100 Depletion on F-actin Formation during Phagocytosis**—Internalization of a cell-bound particle has been generally considered to depend on remodeling the actin cytoskeleton including polymerization and concentration of actin filaments. F-actin formation was also known to be important for the uptake process (1, 3). ARF6 is also reported to be involved in actin remodeling (8, 10, 37).

To determine whether GEP100 depletion affects actin polymerization and concentration during phagocytosis, F-actin was evaluated by fluorescence microscopy. After phagocytosis of opsonized zymosan or IgG-coated beads, F-actin appeared around phagocytosed particles and at sites of particle attachment (Fig. 6, A, C, D, and F; Scrambled); however, there was much less F-actin around particles in GEP100-depleted than control cells (Fig. 6, A, C, D, and F). Accumulation indices for GEP100-depleted cells were only about 50% (GEP100 shRNA-1 and shRNA-2) that of control cells (Scrambled shRNA-1 and shRNA-2), consistent with a role for GEP100 in actin dynamics during phagocytosis (Fig. 6, B and E).

**Effects of ARF1 and ARF6 siRNA on Phagocytosis**—Among ARF family proteins, ARF6 is recognized as a regulator of phagocytosis (19–21), although in a more recent report Fc receptor-mediated phagocytosis was seemingly controlled by ARF1 (38), and involvement of both ARF1 and ARF6 in the control of phagocytosis was described in 2006 (21). To examine the roles of ARF1 and ARF6 in phagocytosis by PMA-differentiated U937 cells, we investigated the effects of ARF1 and ARF6 depletion on phagocytosis using transient transfection of siRNA for ARF1 and ARF6, respectively (Fig. 7, A and B). Of note, phagocytosis of both opsonized zymosan (Fig. 7C) and IgG beads (Fig. 7D) was diminished by knockout of ARF6 but not ARF1. Therefore, under these conditions, ARF6, rather than ARF1, was important for phagocytosis.

**Co-localization of GEP100 and ARF6 during Phagocytosis**—Because ARF6 had been reported to accumulate at the phagocytic cup regions adjacent to IgG-opsonized particles, we looked for colocalization of GEP100 and ARF6 during uptake of opsonized zymosan and IgG beads. After initial attempts to evaluate endogenous ARF6 using a mouse monoclonal or affinity-purified rabbit anti-ARF6 antibodies failed due to lack of sufficient sensitivity, we overexpressed C-terminal FLAG-tagged ARF6 in the cells and examined the localization of ARF6-FLAG and endogenous GEP100 during phagocytosis by immunofluorescence microscopy. As shown in Fig. 8A, ARF6-FLAG in resting cells was distributed in punctate collections throughout the cytoplasm, a pattern very similar to that of...
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endogenous GEP100 in both ARF6-overexpressing and non-transfected cells (Fig. 4, Resting). After cells ingested opsonized zymosan (Fig. 8B) or IgG beads (Fig. 8C), both ARF6-FLAG and GEP100 were partially colocalized around phagocytosed particles.

Percentage of GEP100- and ARF6-FLAG-associated particles in the total number of phagocytosed particles was calculated, and 20.3 and 15.4% of phagocytosed opsonized zymosan and IgG beads were, respectively, associated with GEP100 and ARF6-FLAG. These results suggest that association of GEP100 and ARF6 around phagocytosed particles occurs for only a brief period during the phagocytic process.

**Role of ARF6 on Phagocytosis in GEP100-suppressed Cells**—Finally, to assess whether the decreased phagocytic activity resulting from GEP100 depletion was linked to ARF6, we examined the effect of ARF6 siRNA on phagocytosis in GEP100-depleted cells. As shown in Figs. 9, A and B, ARF6 content of both untreated U937 and GEP100-depleted U937 cells was markedly decreased by transfection of ARF6 siRNA duplexes (siRNA-1 and siRNA-2). Knockdown of ARF6 in control U937 cells decreased phagocytosis of opsonized zymosan and IgG latex beads (Fig. 9, C and D) ~50%. The low phagocytic activity of cells stably depleted of GEP100 was, however, not additionally affected by ARF6 knockdown (Fig. 9, C and D). Phagocytic activity of ARF6-depleted U937 cells was similar to that of GEP100-depleted cells (Fig. 9, C and D). To confirm further whether low phagocytic activity of GEP100-depleted cells is due to decreased ARF6 activation, GEP100-depleted cells were transfected with expression plasmids of ARF6 and its mutants. C3bi receptor (Fig. 9F) and Fcγ receptor-mediated phagocytosis of GEP100-depleted cells (Fig. 9G) was significantly enhanced by the transfection of ARF6Q67L-FLAG, a GTP hydrolysis-defective constitutively active mutant. In contrast, expression of GTP binding-defective ARF6T27N-FLAG mutant did not restore the activity. These results led us to conclude that GEP100 is a regulator of ARF6 activation in the signaling cascade involved in complement receptor- and Fcγ receptor-mediated phagocytosis.

**DISCUSSION**

GEP100 is ARF6-specific ARF-GEF that was originally recognized in a data base search using the DNA sequence of the cytohesin-1 Sec7 domain (24). Among the reported functions of GEP100 are cell-cell fusion (30, 32), cell adhesion (26, 29), and cancer cell invasion (32). ARF6, an in vitro substrate for GEP100, is important in several leukocyte functions including phagocytosis, leading to speculation of a possible role for
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FIGURE 7. Effect of ARF1 or ARF6 depletion on phagocytosis. U937 cells were transiently transfected with nontargeting (Control), ARF6-specific (ARF6 siRNA-1 and siRNA-2), or ARF1-specific siRNA duplexes (ARF1 siRNA-1 and siRNA-2) and then differentiated by PMA treatment. ARF1, ARF6, GEP100, tubulin, and GAPDH in cell lysates were subjected to Western blotting (A) and quantified by densitometry (B). For analysis of data from seven experiments, densitometric values for each experiment were expressed relative to that of the same protein in control. Relative values are shown by normalizing the value obtained for control cells to 1.0. The cells after incubation with opsonized zymosan (C) or IgG beads (D) were fixed, and the number of internalized particles was counted (DIC images) for calculation of phagocytic indices. Data are reported as the means ± S.E. (n = 7). **, p < 0.01; ***, p < 0.001 for the indicated differences from control.

GEP100 in this activity if in vitro activation of ARF6 by GEP100 accurately reflected in vivo behavior/function.

Several investigators reported that ARF6 controls Fc receptor-mediated phagocytosis (19–21). Paxillin-associated protein with ARFGAP activity 3 (PAG3), a GAP for ARFs (also called ARF GAP containing SH3 domain, ankyrin repeat, and PH domain 2 (ASAP2)) according to the 2007 nomenclature (39)) was reported to regulate ARF6 location during phagocytosis (40). On the other hand, BFA, a fungal fatty acid metabolite and inhibitor of several ARF-GEF activities, did not affect phagocytosis, suggesting that BFA-insensitive ARF-GEFs were involved (19). Here, we show that the BFA-insensitive ARF1 (24) regulates complement receptor- and Fc receptor-mediated phagocytosis.

Phagocytic activity of GEP100-depleted cells was not rescued by transfection of the GEP100ΔSec7 mutant, which is predicted to be defective in ARF activation. Overexpression of wild-type GEP100, however, enhanced phagocytic activity, suggesting that GEP100 regulated phagocytic activity through the control of ARF activity.

ARF1 as well as ARF6 regulates Fc receptor-mediated phagocytosis by mouse macrophage-like RAW264.7 cells (21, 38). We had initially reported relatively weak activation of ARF1 by GEP100 in vitro, about one-sixth that of ARF6 (24), consistent with participation of ARF1 in GEP100-dependent phagocytic activity. Data presented here, however, provide no evidence of ARF1 involvement in complement receptor- and Fcγ receptor-mediated phagocytosis by PMA-differentiated U937 cells. Immunofluorescence microscopy revealed the accumulation and partial colocalization of GEP100 with ARF6-FLAG surrounding internalized particles. In addition, phagocytic activity of GEP100-depleted cells was not further affected by ARF6 knockdown. Furthermore, phagocytic activity of GEP100-depleted cells was rescued by transfection of constitutively active ARF6ΔQ67L mutant, although GTP binding-defective ARF6ΔT27N mutant did not restore activity. All observations are consistent with the notion that GEP100 regulates complement receptor- and Fcγ receptor-mediated phagocytosis through its control of ARF6 activation.

In contrast to phagocytosis, binding of opsonized zymosan or IgG beads to cells was not significantly altered by GEP100 depletion. Amounts of CD11b and CD64, the cell surface receptors for C3bi and Fcγ, respectively, were not correlated with decreased phagocytic activity in GEP100-depleted cells. F-actin concentrations around the attached and internalized particles were, however, significantly lower in those cells than in control cells, suggesting that GEP100 contributes to the process of particle internalization and the regulation of F-actin formation. GEP100-activated ARF6 might participate also through its activation of lipid-modifying enzymes such as phosphatidylinositol-4-phosphate 5-kinase and phospholipase D (11, 16, 19, 41). Indeed, contributions of another small GTPase, Rac, in ARF6-

FIGURE 8. Intracellular co-localization of endogenous GEP100 and overexpressed ARF6-FLAG during phagocytosis. U937 cells transiently overexpressing ARF6-FLAG were incubated with PMA for 30 h, before incubation without (A), or with opsonized zymosan (B) or IgG beads (C) as in Fig. 2. After fixation of cells, endogenous GEP100 and overexpressed ARF6-FLAG were reacted, respectively, with rabbit anti-GEP100 antibody or mouse anti-FLAG antibody. DIC images are on the right. Arrows indicate phagocytosed particles. The experiment was repeated nine times with a similar result. Scale bars, 5 μm.
tidylinositol-4-phosphate 5-kinase, phospholipase D, and Rac in phagocytosis regulated by GEP100, however, remain to be defined.

We had reported previously a role for GEP100 in the apoptotic death of PMA-differentiated U937 cells, which was independent of ARF6 activity (34), although we believe that GEP100 effects on phagocytosis is dependent on its activation of ARF6. Thus, GEP100 seems, not surprisingly, to be another multifunctional protein that regulates cellular activities via both ARF6-dependent and -independent mechanisms. To elucidate the mechanisms of GEP100 function in these processes, its intracellular binding partners and interacting molecules need to be identified and their roles clarified.

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FIGURE 9. Effect of ARF6 knockdown or overexpression on phagocytosis by GEP100-depleted cells. A–D, untreated (U937) or stably depleted cells (GEP100 KD) were transiently transfected with plasmids encoding wild-type ARF6 (WT), ARF6Q67L (Q67L), or ARF6T27N (T27N) or empty (−) plasmid. Untreated (U937) or control (Scrambled) cells were transiently transfected with empty plasmid. E, a representative Western blot from one of six experiments shows ARF6, FLAG-tagged proteins (FLAG-protein), GEP100, tubulin, and GAPDH in total cell lysates. PMA-differentiated U937, control (Scrambled), and GEP100-depleted cells (GEP100 KD) were transiently transfected with plasmids encoding with opsonized zymosan (F) or IgG-coated beads (G), and ingested particles in cells reacted with anti-FLAG antibody were counted for determination of phagocytic indices. Phagocytic indices were calculated and expressed relative to that of U937 cells in the same experiment. *, p < 0.05; **, p < 0.01; ***, p < 0.001 for the indicated differences from U937 control.

dependent actin remodeling (41–47) are well known, including those in membrane ruffling and lamellipodia formation via actin cytoskeleton reorganization (48). The roles of phospha-
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