A Requirement for ARF6 in Fcγ Receptor-mediated Phagocytosis in Macrophages*

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Phagocytosis requires extension of F-actin-rich pseudopods and is accompanied by membrane fusion events. Members of the ARF family of GTPases are essential for many aspects of membrane trafficking. To test a role for this family of proteins in Fcγ receptor-mediated phagocytosis, we utilized the fungal metabolite brefeldin A (BFA). The addition of 100 μM BFA to a subclone of RAW 264.7 macrophages disrupted the appearance and function of the Golgi apparatus as indicated by altered immunofluorescent distribution of β-COP and reduced efflux of BODIPY C₅-ceramide, a phospholipid that normally accumulates in the Golgi apparatus. In contrast, BFA had no effect on phagocytosis of IgG-coated erythrocytes. These results suggested that activation of BFA-sensitive ARFs is not required for phagocytosis. ARF6 is unique among members of the ARF family in that its membrane association is unaffected by BFA. Expression of ARF6 mutants defective in either GTP hydrolysis (Q67L) or binding (T27N) inhibited phagocytosis of IgG-coated erythrocytes and attenuated the focal accumulation of F-actin beneath the test particles. These results indicate a requirement for ARF6 in Fcγ receptor-mediated phagocytosis and suggest that ARF6 is an important mediator of cytoskeletal alterations after Fcγ receptor activation.

During phagocytosis, foci of F-actin accumulate beneath bound particles, conforming to the cytoarchitecture of pseudopods that engulf the particles (for review, see Ref. 1). Macrophages and other phagocytically competent cells are capable of ingesting many particles without an apparent reduction in surface area, suggesting that F-actin-based membrane protrusive events are coupled to membrane recruitment. The exact relationship between actin assembly and membrane protrusion and recruitment is not clear.

Members of the ARF family of GTPases have been implicated in numerous membrane trafficking events in eukaryotic cells (for review, see Refs. 2 and 3). ARF proteins contain consensus amino acid sequences for GTP binding as well as NH₂-terminal glycline-containing consensus sequences required for myristoylation and membrane targeting. ARF1, the best characterized member of this family, cycles between cytosol and membrane depending on its nucleotide status. ARF1 binding of the activating nucleotide, GTP, results in a conformational change of the protein and enhanced affinity for membranes. Once membrane-bound, the ARF1 participates in recruitment of coatamer protein that is required for budding and fission of membrane vesicles (4–7).

Many insights into the process of membrane trafficking came from the use of the fungal metabolite brefeldin A (BFA),¹ a substance that impairs the association of ARF proteins with membranes and therefore blocks early steps in the budding of vesicles. Although the exact mechanism of action of BFA is unknown, it appears to inhibit membrane-catalyzed guanine nucleotide exchange activity on ARFs (8–10). More recently, several BFA-sensitive guanine nucleotide exchange factors for ARFs have been identified (11, 12). ARF6 is unique among the ARFs in that its association with membranes is not sensitive to BFA (13–15). Immunolocalization studies indicate that ARF6 resides primarily in the plasma membrane, and studies using various mutant alleles of ARF6 suggest that ARF6 cycles between an intracellular compartment and the plasma membrane, depending on its nucleotide status. Expression of a GTP hydrolysis-deficient ARF6 mutant, Q67L, leads to accumulation of the protein in the plasma membrane and the induction of plasma membrane invaginations, whereas expression of the GTP binding-defective mutant, T27N, results in accumulation of the protein in an internal tubulovesicular compartment (13, 16, 17). A recent report suggests that ARF6 is also found in the cytosol (18), raising the possibility that its function may also be regulated by its subcellular distribution. Although the function of ARF6 is less well understood than that of ARF1, recent studies using mutant alleles of ARF6 suggest a prominent role for ARF6 in endocytosis (19), membrane recycling (16, 17), and regulated exocytosis (20). In addition, expression of the Q67 allele of ARF6 results in F-actin-rich plasma membrane protrusions in HeLa cells (15) and accumulation of F-actin at the cell periphery in Chinese hamster ovary cells (21). In the latter study, D’Souza-Schorey et al. (21) showed that ARF6 was capable of interacting with POR1, a Rac1-binding protein implicated in membrane ruffling (22). Interestingly, these workers suggested that Rac1 and ARF6 functioned on separate signaling pathways.

We showed recently that Rac1 and Cdc42 are required for FcγR-mediated phagocytosis (23). Given the likelihood that extensive membrane remodeling is required for phagocytosis of multiple particles and the findings that phagocytosis requires

* This work was supported in part by a grant from the American Cancer Society and National Institutes of Health grants supporting the Herbert Irving Cancer Center Confocal Microscopy Facility. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: BFA, brefeldin A; ElgG, sheep erythrocytes opsonized with rabbit anti-sheep IgG; FcγR, receptor for the Fc portion of IgG; GA, Golgi apparatus; G2A, ARF6 bearing an alanine substitution for glycine 2; TGN, trans-Golgi network; WT, wild-type ARF6.
actin assembly at the plasma membrane (24, 25), we wondered whether one or more members of the ARF family participated in the process of particle engulfment. We tested the sensitivity of phagocytosis to BFA and probed the role of ARF6 in FcyR-mediated phagocytosis.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—RAW LacR/FMLPR.2 cells, a clone derived from the RAW 264.7 cell line (American Type Culture Collection) was maintained in RPMI medium containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin at 37 °C in a CO2 incubator. Rabbit anti-ARF6 serum was derived as described (15). Rabbit anti-sheep erythrocyte IgG was from Diamedix (Miami, FL). Rhodamine-phalloidin and BODIPY C5-ceramide were from Molecular Probes (Eugene, OR). Fluorescein isothiocyanate-, AMCA-, and rhodamine-conjugated anti-rabbit IgG were from Jackson ImmunoResearch (West Grove, PA). Superfect transfection reagent was from QIAGEN (Santa Clarita, CA).

**Efflux of BODIPY FL C5-Ceramide**—RAW LacR/FMLPR.2 cells were plated overnight in 96-well plates and were incubated with 1 μM BODIPY C5-ceramide (Molecular Probes, Eugene, OR) in a buffer containing 125 mM NaCl, 5 mM KCl, 1 mM KH2PO4, 5 mM glucose, 10 mM NaHCO3, 1 mM MgCl2, 1 mM CaCl2, 20 mM HEPES, pH 7.4, and 1 mg/ml defatted bovine serum albumin for 30 min at 4 °C. The cells were washed four times and incubated in RPMI containing 10% fetal bovine serum with or without 100 μM BFA at 37 °C. At varying time intervals, cells were washed, fixed in 3.7% formaldehyde, and the cell-associated fluorescence was measured in a fluorescence plate reader (Cytofluor II, Millipore; excitation, 485 nm; emission, 530). To normalize for the cell number present in the excitation beam, 1 μM ethidium bromide in the presence of 0.2% Triton X-100 was added, and fluorescence (excitation, 530 nm; emission, 590) was recorded. Data are reported as the ratio of BODIPY C5-ceramide/ethidium bromide fluorescence intensities.

**Fluorescence Microscopy and Immunoblotting**—Fluorescence microscopy using a confocal scanning system (Zeiss) was performed as described (24). For visualization of submembranous accumulations of F-actin ("phagocytic cups"), transfected cells were washed once and incubated with 2 × 105 ElG at 37 °C for 7 min before fixation. Bound ElG was stained with fluorescein isothiocyanate-conjugated anti-rabbit IgG. Cells were permeabilized at this stage and stained for F-actin with rhodamine phalloidin and for ARF6 expression with rabbit anti-ARF6 followed by AMCA-conjugated anti-rabbit IgG. Immunoblotting was performed as described previously (26).

**Phagocytosis Assays**—All transfections were done using Superfect according to the manufacturer’s recommendations. RAW LacR/FMLPR.2 cells were transfected overnight with the indicated constructs subcloned into pKS (27) and replated onto 19-mm round coverslips in RPMI medium containing 10% fetal bovine serum. Experiments were carried out 3–5 h after replating. 2 × 105 ElG were added for 30 min at 37 °C followed by washing and hypotonic lysis. Bound, uningested ElG were detected by staining with fluorescein isothiocyanate anti-rabbit IgG for 30 min at 4 °C. Cells were fixed in 3.7% formaldehyde, permeabilized, and stained for expression of ARF6 proteins using anti-ARF6 serum. After the addition of rhodamine anti-rabbit IgG, to detect both the presence of ARF6 proteins and ElG, cells were examined by fluorescence microscopy. Ingested ElG were identified as fluorescein isothiocyanate-negative rhodamine-positive phagocytic vacuoles. 50 cells expressing the indicated ARF constructs and 50 nonexpressing control cells on the same coverslip were scored for internalized or bound ElG. The phagocytic index was calculated as the average number of ElG ingested per cell, and the association index was calculated as the sum of bound plus ingested ElG per cell. Data represent an average of five to seven experiments performed in duplicate. A one-tailed Student’s t test was performed on the phagocytosis data.

**RESULTS**

Brefeldin A Disrupts the Golgi Apparatus (GA) in RAW LacR/FMLPR.2 Cells but Has No Effect on FcyR-mediated Phagocytosis—To test the effects of BFA on phagocytosis in RAW LacR/FMLPR.2 cells, we first verified that this fungal toxin disrupted the GA. We examined the distribution of β-COP, a coat protein that accumulates in the GA in a BFA-sensitive fashion (28–30). Similar to results in other cell types, the addition of BFA resulted in the redistribution of β-COP (Fig. 1), consistent with disruption of the integrity of the GA. To confirm that the addition of BFA resulted in functional disruption of the GA, we determined the effect of the toxin on the efflux of BODIPY C5-ceramide, a fluorescent lipid analog that accumulates in GA (31, 32). The presence of BFA resulted in a 73% inhibition in efflux rate (15 min to 4 h) of BODIPY C5-ceramide (Fig. 2). Thus, the effects of BFA on the GA in this macrophage-like cell line were similar to those observed in other cell types. Despite disruption of the GA, concentrations up to 100 μM BFA had no effect on the rate or extent of phagocytosis of ElG (Fig. 3). These results are consistent with a lack of requirement for an intact GA in FcyR-mediated phagocytosis and suggest that BFA-sensitive ARFs are dispensable for phagocytosis.

Expression of GTP Hydrolysis-defective or GTP Binding-defective Alleles of ARF6 Inhibits FcyR-mediated Phagocytosis—Although the experiments described above suggested that BFA-sensitive ARFs do not play a role in phagocytosis of ElG, they did not test the requirement for ARF6 in phagocytosis. To test if the BFA-insensitive ARF6 plays a role in phagocytosis, we transiently expressed wild type (WT), GTP hydrolysis-deficient (Q67L), or GTP binding-deficient (T27N) mutant ARF6 proteins in RAW LacR/FMLPR.2 cells. These cells were chosen because they consistently demonstrated enhanced transfection efficiency compared with the parental RAW 264.7 strain (not shown). Expression of ARF6 protein was assessed by immunofluorescence (not shown) and immunoblotting. Endogenous ARF6 protein was detected in mock-transfected cells, and higher levels of ARF6 were detected in transfected cells (Fig. 4). Expression of WT or Q67L was always greater that that of T27N, although on a per cell basis, it was comparable because they consistently demonstrated enhanced transfection efficiency compared with the parental RAW 264.7 strain (not shown). Expression of ARF6 protein was assessed by immunofluorescence (not shown) and immunoblotting. Endogenous ARF6 protein was detected in mock-transfected cells, and higher levels of ARF6 were detected in transfected cells (Fig. 4). Expression of WT or Q67L was always greater that that of T27N, although on a per cell basis, it was comparable because transfection of RAW LacR/FMLPR.2 cells with T27N led to 2.8-fold fewer T27N-expressing cells compared with transfection of this cell line with Q67L. Expression of all three constructs led to markedly enhanced staining for ARF6 throughout the cytoplasm and, in the case of WT and Q67L, the plasma membrane; similar results were obtained using anti-hemagglutinin monocular antibodies with hemagglutinin-tagged versions of the proteins (not shown). To test if ARF6 is required for FcyR-mediated phagocytosis, we performed phagocytosis assays on RAW LacR/FMLPR.2 cell transfecants. Expression of various ARF6 constructs had a moderate effect on the total number of ElG associated with the macrophages (ranging from a 24% reduction in cells overexpressing WT ARF6 to a 47% reduction in cells expressing ARF6 Q67L; Fig. 5A). Although expression of WT ARF6 led to a proportionate decrease...
in ingestion, expression of either Q67L or T27N led to a disproportionate decrease in phagocytosis (Fig. 5B). When the efficiency of ingestion was expressed as the percentage of total cell-associated (i.e. bound and ingested) IgG ingested (Fig. 5C), expression of Q67L and T27N led to a 71% and 93% inhibition, respectively, in the phagocytosis of IgG. Expression of WT ARF6 did not affect significantly the percent of total cell-associated IgG ingested compared with controls (p = 0.17). The inhibitory effects of the T27N mutant required membrane association because a nonmyristoylated double mutant (ARF6 G2A/T27N) did not inhibit phagocytosis (113 ± 7.5% of control, n = 4). These results indicate that functional ARF6 is required for FcγR-mediated phagocytosis.

**DISCUSSION**

The data presented here demonstrate a requirement for ARF6 in FcγR-mediated phagocytosis. Because expression of either GTP-binding or GTPase-deficient alleles of ARF6 inhibited phagocytosis, it is possible that cycling of ARF6 between GDP- and GTP-bound forms is required for the ingestion process. Alternatively, because expression of Q67L produced alterations in the distribution of F-actin even in the absence of added IgG, it is possible that expression of this allele results in a phenotype that does not support stimulated actin assembly at the plasma membrane. This implies that GTP hydrolysis by ARF6 may not actually be a prerequisite for phagocytosis, a possibility that would be difficult to verify. In either case, the requirement for ARF6 in the accumulation of submembranous F-actin induced by IgG-opsonized particles suggests that ARF6 plays a role in the regulation of the cytoskeletal changes that underlie FcγR-mediated phagocytosis.

The addition of aluminum fluoride to HeLa cells overexpressing ARF6 Q67L or T27N Inhibits Submembranous Accumulations of F-actin Beneath Attached IgG in RAW LacR/FMLPR.2 Cells—To test whether inhibition of phagocytosis by ARF6 mutants correlated with alterations in FcγR-directed actin assembly, we tested the effects of expressing various ARF6 constructs on phagocytic cup formation. 7 min after the onset of phagocytosis, well formed phagocytic cups were evident in WT-expressing cells (Fig. 6). In cells expressing ARF6 Q67L or T27N, however, focal accumulation of F-actin beneath most attached IgG was diminished (Fig. 6, right panel), despite the presence of surface-bound IgG (Fig. 6, left panel). In those instances where focal accumulations of F-actin were apparent, they were poorly formed or attenuated.
Although our study did not address whether ARF6 in macro-
trophically overlap with a recycling endosomal compartment (17). Ferrin receptors, indicating that this compartment may par-
electron micrographic studies of Chinese hamster ovary cells,
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class I proteins and the interleukin-2 targeting sequences, such as major histocompatibility complex
in water-soluble pinocytic tracers; however, it is accessible to
transferrin-positive endosomes, and is not readily accessible to
containing compartment appears tubular, is distinct from
intracellular compartment. In HeLa cells, the intracellular ARF6-
recycling pathway between the plasma membrane and a jux-
turnuclear compartment. In HeLa cells, the intracellular ARF6-
functions in addition to regulating the cytoskeleton. For ex-
example, it is possible that GTP “cycling” by ARF6 participates in
the delivery of components necessary for remodeling of the cell
surface during phagocytosis. The Fcγ receptors themselves are
unlikely to be one of these components because, unlike the
major histocompatibility complex class I and Tac, they contain
residues that confer localization to coated pits (33). Further-
more, expression of Q67L and T27N inhibited, but did not abol-
ish, binding of IgG. In mouse macrophages, phagocytosis
proceeds in direct proportion to particle binding even when
FcγR surface expression is reduced by more than 90% (34). The
disproportionate reduction in phagocytosis compared with
binding of IgG in cells expressing either Q67L or T27N sug-
gests a more profound defect in the phagocytic apparatus. It is
possible that during phagocytosis, ARF6 mediates the delivery
to the plasma membrane of additional protein effectors or
membrane components required for phagocytosis, although the
nature of these effectors/components remains to be defined.

From the above discussion, it is not clear whether it is
possible to distinguish how ARF6 functions in membrane traf-
ficking and cytoskeletal remodeling. Because the two events
may be coupled in vivo, especially during events such as
pseudodop extension and membrane ruffling, ARF6 may, in fact,
function in the coupling of these events. The lack of an inhib-
itory effect of BFA on phagocytosis suggests that other ARFs and
the GA itself may not be critical for phagocytosis and
pseudodop extension. However, we cannot discount the partic-
ipation of the TGN in phagocytosis because BFA may not
necessarily affect this component of the GA, and studies using
antiserum against TGN-38, a marker of the TGN, did not
reveal uniform alteration in the distribution of TGN-38 in
macrophages incubated with BFA (not shown).

The lack of inhibition of phagocytosis using ARF6 G2A/
T27N, a nonmyristoylated mutant of ARF6 T27N, indicates that
myristoylation and/or membrane association of ARF6 T27N is
required for effective inhibition of phagocytosis by this GTP
binding-deficient allele of ARF6. This is consistent with find-
ings that myristoylation of ARF1 is required for guanine nu-
cleotide exchange activity catalyzed by ARNO (35) and an
unknown component of a retinal extract (36). It is possible that
myristoylation is also required for efficient binding of ARF6 to
its exchange factors. Therefore, nonmyristoylated alleles of
ARF6, such as ARF6 G2A/T27N, may be incapable of interact-
ing with ARF6 exchange factor(s) and cannot compete with
endogenous ARF6 for their binding. Thus, they could not act in
a “dominant-negative” fashion to inhibit ARF6 function.

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Fig. 6. Phagocytic cup formation is inhibited by ARF6 Q67L or T27N. RAW LacR/FMLPR.2 cells transfected with the indicated con-
structs were incubated with EIgG for 7 min at 37 °C and were fixed and
stained for the presence of EIgG (left) and for F-actin (right). Expression
of the indicated constructs was confirmed by staining with anti-ARF6
serum followed by AMCA-conjugated anti-rabbit IgG (not shown).
Bar = 10 μm.
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