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A Requirement for Phosphatidylinositol 3-Kinase in Pseudopod Extension*

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Phagocytosis requires actin assembly and pseudopod extension, two cellular events that coincide spatially and temporally. The signal transduction events underlying both processes may be distinct. We tested whether phagocytic signaling resembles that of growth factor receptors, which induce actin polymerization via activation of phosphatidylinositol 3-kinase (PI 3-kinase). Fcγ receptor-mediated phagocytosis was accompanied by a rapid increase in the accumulation of phosphatidylinositol 3,4,5-trisphosphate *in vivo*, and addition of wortmannin (WM) or LY294002, two inhibitors of PI 3-kinase(s), inhibited phagocytosis but not Fcγ receptor-directed actin polymerization. However, both compounds prevented maximal pseudopod extension, suggesting that PI 3-kinase inhibition produced a limitation in membrane required for pseudopod extension. Availability of plasma membrane was not limiting for phagocytosis, because blockade of ingestion in the presence of WM was not overcome by reducing the number of particles adhering to macrophages. However, decreasing bead size, and hence the magnitude of pseudopod extension required for particle engulfment, relieved the inhibition of phagocytosis in the presence of WM or LY294002 by up to 80%. The block in phagocytosis of large particles occurred before phagosomal closure, because both compounds inhibited spreading of macrophages on substrate-bound IgG. Macrophage spreading on IgG was accompanied by exocytic insertion of membrane from an intracellular source, as measured by the dye FM1-43. These results indicate that one or more isoforms of PI 3 kinase are required for maximal pseudopod extension but not phagocytosis per se. We suggest that PI 3-kinase is required for coordinating exocytic membrane insertion and pseudopod extension.

Phagocytosis via Fc_γ receptors in macrophages is accompanied by actin assembly, pseudopod extension, and phagosomal closure (1). $Fc_\gamma R$-directed actin assembly is blocked by tyrosine kinase inhibitors (2) and requires the participation of Rac1 and Cdc42 (3), two members of the Rho family of GTPases. However, it is not known precisely how enhanced protein tyrosine phosphorylation leads to changes in either the cytoskeleton or the membrane. Signaling by Fc_γ receptors shares many elements in common with that of growth factor receptors. For example, both classes of receptors signal directly or indirectly through tyrosine kinases, and ligation of multiple growth factor receptors and $Fc_\gamma Rs$[1] culminates in net actin assembly and plasma membrane-based protrusions (1, 4, 5). Studies of the PDGF receptor indicate a prominent role for PI 3-kinase in the generation of F-actin-rich membrane ruffles. Phosphotyrosine residues within the kinase insert region of the cytosolic domain of the PDGF receptor bind the p85/p110 isoform of PI 3-kinase, and mutation of these residues abolishes membrane ruffling induced by this receptor (6–8). Addition of wortmannin, a fungal metabolite that inhibits PI 3-kinases in the nanomolar range, blocks PDGF receptor-induced membrane ruffling and actin assembly (8, 9). Similarly, addition of PI 3-kinase inhibitors abrogated membrane ruffling and actin polymerization in response to insulin (10–12). Precisely how PI 3-kinases participate in actin assembly is not known, but pharmacological inhibition of PI 3-kinase inhibits GTP loading of Rac1 stimulated by PDGF, and addition of constitutively active forms of Rac1 induces membrane ruffling despite the presence of PI 3-kinase inhibitors (10, 13). These data suggest that PI 3-kinase lies upstream of Rac1. In contrast, recent studies of epithelial cells spreading on collagen suggest that PI 3-kinase, which is required for motility, may lie downstream of Rho family GTPases (14).

The role of PI 3-kinase in actin assembly mediated by other types of receptors is less clear. For G protein-linked receptors, such as the thrombin receptor (15) and the chemotactic peptide receptor (16, 17), inhibition of PI 3-kinase has been reported to have no effect on stimulus-induced actin polymerization. For immunoreceptor tyrosine activation motif-containing receptors, such as $Fc_\epsilon RI$, addition of wortmannin does not inhibit IgE-induced actin polymerization but does block the appearance of well-formed membrane ruffles in response to antigen (18). Several studies have demonstrated a role for PI 3-kinase in $Fc_\gamma R$-mediated phagocytosis (19–21). In one, a role for this enzyme was suggested for the closure of phagosomes (21). Although quantitation of PI 3-kinase activity or F-actin was not performed, addition of wortmannin did not appear to inhibit the formation of "phagocytic cups," as determined by fluorescence micrographs of phalloidin-stained cells interacting with phagocytic targets (21).

A study of $Fc_\epsilon RI$ in mast cells suggests that stimulation of actin polymerization may not necessarily lead to membrane

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[1] The abbreviations used are: $Fc_\gamma R$, receptor for the Fc portion of IgG; EIgG, sheep erythrocytes opsonized with rabbit IgG; PI 3-kinase, phosphatidylinositol 3-kinase; thio-macrophages, mouse macrophages elicited after intraperitoneal injection of thioglycollate broth; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PDGF, platelet-derived growth factor; BSA, bovine serum albumin; PLA_2, phospholipase A_2; GTPγS, guanosine 5′-3- *O*-(thio)triphosphate.

ruffles (18). Similarly, in DT40 lymphocytes expressing chimeric receptors encoding CD16 and the γ subunit of Fc receptors, addition of IgG-coated targets resulted in localized actin assembly and rudimentary plasma membrane protrusions, but phagocytosis did not occur (22). This suggests that actin polymerization at the plasma membrane is not always coupled to pseudopod extension; distinct signals may be required for this function. Interestingly, a recent study suggested that pseudopod extension by $Fc_\gamma RI$ expressed in COS cells occurred in the absence of net actin assembly (23). Collectively, these studies suggest that actin assembly and pseudopod extension, two cellular events that normally coincide spatially and temporally, may be regulated by distinct signal transduction cascades.

PI 3 kinases have been implicated in multiple aspects of membrane trafficking, including endocytosis, exocytosis, and membrane recycling (for review, see Ref. 24). During phagocytosis, significant amounts of plasma membrane are internalized in the form of phagocytic vacuoles. However, this is accompanied by no apparent decrease in membrane surface area (25), suggesting that surface membrane is replenished from an intracellular source. To define the role of PI 3-kinase in phagocytosis, we used a variety of approaches to identify the stage in phagocytosis that was blocked during PI 3-kinase inhibition. These studies indicate that the block occurs during pseudopod extension, not during the very early phases (*i.e.* F-actin accumulation) or late phases (*i.e.* phagosomal closure) of ingestion and could be bypassed when requirements for pseudopod extension were minimized. The block in pseudopod extension coincided with a decrease in exocytic insertion of membrane, suggesting that PI 3-kinases are required for coordinating membrane insertion events and pseudopod extension.

EXPERIMENTAL PROCEDURES

Cells and Reagents—RAW LacR/FMLP.2 cells were derived from RAW 264.7 cells as described previously (3). Primary macrophages were isolated from the peritoneal cavities of C57Bl6 mice 3–5 days after the intraperitoneal injection of thioglycollate (thio-macrophages) as described previously (26). Cells were maintained in RPMI 1640 medium containing 10% fetal calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin. Wortmannin and sulfhydral-modified BSA were from Calbiochem (La Jolla CA). LY294002 was from Biomol (Plymouth Meeting, PA). IgG against sheep erythrocytes was from Diamedix (Miami, FL). A monoclonal antibody directed against the p85 subunit of phosphatidylinositol 3-kinase was from Transduction Laboratories (Lexington, KY). [^{32}P]Orthophosphate and [γ-^{32}P]ATP were from Dupont NEN. Aluminum-backed Silica Gel 60 thin layer chromatography plates were from EM Separations (Gibbstown, NJ). Rabbit serum against BSA was from Dako Corp. (Carpinteria, CA). Fluorescein isothiocyanate- and rhodamine-conjugated $F(ab')_2$ fragments of anti-rabbit IgG were from Jackson ImmunoResearch (West Grove, PA). Rhodamine-phalloidin, YO-PRO 1 iodide, and FM1-43 were from Molecular Probes (Eugene, OR). Carboxylate-modified latex beads of various sizes were from Bangs Laboratories (Fishers, IN).

Phagocytosis Assays—5×10^6 sheep erythrocytes opsonized with rabbit IgG (EIgG) were added to adherent cells for 30 min as described previously (27). For opsonization of latex beads, carboxylate-modified latex beads ranging in size from 1 to 6 μm were incubated with 4 mg/ml sulhydryl-modified BSA dissolved in 0.05 M 2-[*N*-morpholino]ethanesulfonic acid, pH 5.5, and 1 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, followed by washing and incubation in various dilutions of rabbit antiserum against BSA for 1 h at 25 °C. After washing three times in phosphate-buffered saline, aliquots of beads were removed for immunoblotting to determine the quantity of IgG deposited on the beads. The density of IgG/μm^2 was determined based on the calculated surface areas of the different bead size. Batches were discarded if the density of IgG deposited varied >15% of the mean, which was 5000 molecules/μm^2. For phagocytosis assays, cells were incubated with beads for 45 min at 37 °C. Excess beads were washed away and the extent of binding and ingestion of the beads was determined. To detect attached, but uningested beads, cells were incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG for 45 min at 4 °C, followed by fixation in 3.7% formaldehyde. Cells were subsequently permeabilized with 0.2% Triton X-100, and total cell-associated beads were stained with rhodamine-conjugated anti-rabbit IgG. Phagocytosis was quantified as the total number of beads per cell (*i.e.* rhodamine-stained) minus the number of bound but uningested beads (*i.e.* those that were accessible to staining with fluorescein isothiocyanate-conjugated anti-rabbit IgG). 5–10 high power fields were scored for attached and ingested particles, and phagocytosis assays were performed in duplicate.

Determination of PIP_3 Content of RAW 264.7 Cells—Adherent RAW LacR/FMLP.2 cells were grown to 90% confluence in 6-cm dishes and were washed and incubated in minimal essential medium minus phosphates (Life Technologies, Inc.) containing 1% BSA and 37 MBq/ml [^{32}P]orthophosphate acid for 2 h at 37 °C. Cells were washed and incubated further with 8×10^7 EIgG for varying times followed by phospholipid extraction and thin layer chromatography as described in (17). Samples were run with a PIP_3 standard obtained by incubation of purified phosphatidylinositol-4,5-bisphosphate with [γ-^{32}P]ATP and anti-phosphatidylinositol 3-kinase immunoprecipitates derived from RAW LacR/FMLP.2 cells.

Fluorescence Microscopy—Adherent RAW LacR/FMLP.2 cells on coverslips were incubated with 3×10^6 EIgG on ice for 30 min to allow for particle binding, and after washing with ice-cold buffer, cells were incubated for a further 5 min at 37 °C before fixation in 3.7% formaldehyde and staining with 0.33 μM rhodamine-phalloidin. Cells were imaged using a confocal scanning system (Bio-Rad MRC 600) as described previously (22).

Quantitation of F-actin—Total cellular F-actin was quantitated as described (22) with the following modifications: RAW LacR/FMLP.2 cells were plated at 2×10^4 cells/well in 96 well tissue culture plates and treated with vehicle (dimethyl sulfoxide), 100 nM wortmannin, or 100 μM LY294002 at 37 °C for 30 min. 2×10^6 EIgG were added at 37 °C for 5 min, and cells were stained for F-actin with rhodamine-phalloidin as described above. Rhodamine fluorescence (excitation, 540 nm; emission, 590 nm) was measured using a fluorescence plate reader (CytoFluor II; Millipore) and normalized to cell number by dividing by a subsequent measurement of fluorescence (excitation, 485 nm; emission, 530 nm) after addition of 5 μM YO-PRO. Experiments were performed in triplicate.

Spreading of Macrophages on Human IgG—Thio-macrophages were pretreated with vehicle (dimethyl sulfoxide), 100 nM wortmannin, or 100 μM LY294002 for 30 min at 37 °C and then applied to 13-mm^2 round coverslips previously coated with 1 mg/ml human IgG. Cells were allowed to spread for varying intervals at 37 °C followed by fixation and staining for F-actin (to facilitate delineation of cell margins). The cell diameter in close contact with the coverslip was measured, and the apparent adherent surface area was calculated. The adherent surface areas of ~30 cells/coverslip were measured, and experiments were performed in duplicate.

Quantitation of Exocytosis—Thio-macrophages were preincubated with various concentrations of FM1-43 for 3 min at 25 °C and added to 96-well plates that had been previously coated with 1 mg/ml human IgG. Using a fluorescence plate reader (CytoFluor II), fluorescence (excitation, 485 nm; emission, 530 nm) was monitored in the continual presence of dye for varying time intervals at 25 °C. Cells and dye alone gave negligible fluorescence readings. FM1-43 fluorescence values were normalized to cell number after a subsequent fluorescence measurement (excitation, 360; emission, 460) after addition of 2 μM 4,6-diamidino-2-phenylindole.

RESULTS

Wortmannin and LY294002 Inhibit Fc_γ-mediated Phagocytosis—PI 3 kinase has been shown to be activated by most receptors capable of mediating actin polymerization. To test the role of this family of enzymes in phagocytosis, we determined the effects of two structurally unrelated PI 3-kinase inhibitors, wortmannin and LY294002, on $Fc_\gamma R$-mediated phagocytosis. Addition of either wortmannin or LY294002 produced a concentration-dependent inhibition of phagocytosis with an IC_{50} of ~4.5 nM and 3 μM, respectively (Fig. 1). These results are similar to those reported for phagocytosis in guinea pig neutrophils (using wortmannin) and bone marrow-derived macrophages (19, 21). The IC_{50} values in this study were similar to those reported for inhibition of PI 3-kinases, but not for a PI 4-kinase (28) and myosin light chain kinase (29). Because mTOR is another target of wortmannin (30), we tested the effects of rapamycin on phagocytosis; however, 10 μM rapamycin had no effect on phagocytosis (not shown). These results

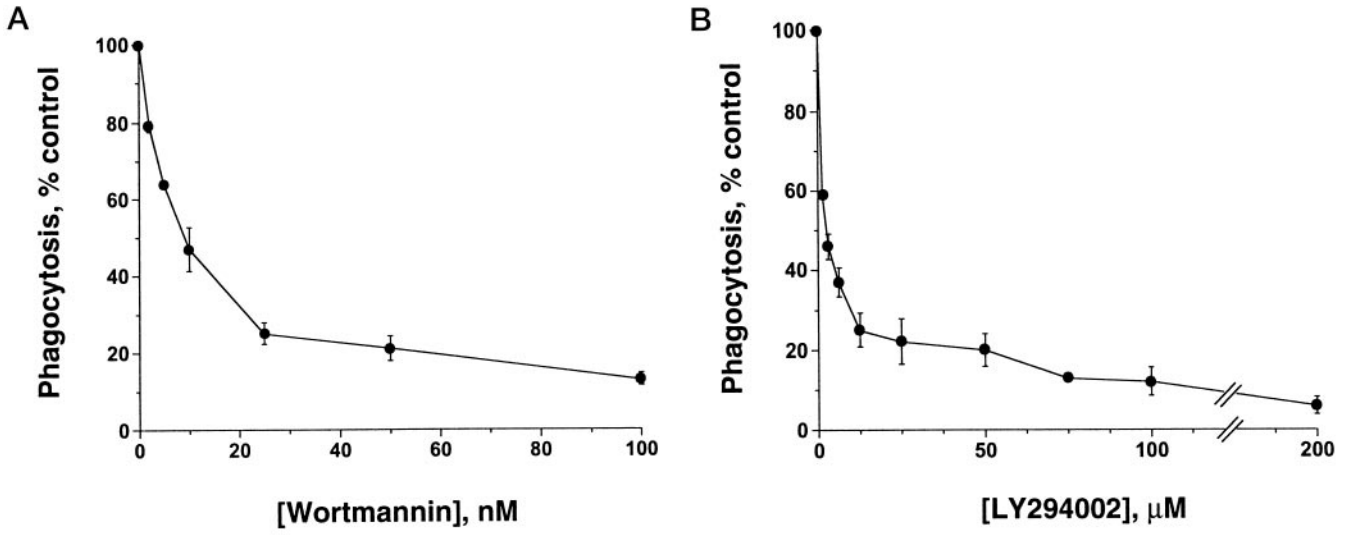

FIG. 1. **Wortmannin or LY294002 inhibits phagocytosis by macrophages in a concentration-dependent manner.** Phagocytosis assays were performed for RAW 264.7 cells incubated with EIgG in the presence of wortmannin (*A*) or LY294002 (*B*) as described under "Experimental Procedures." Data, expressed as the mean ± S.E. (n = 3), are reported as percent of control phagocytosis in the presence of vehicle alone.

suggest that the inhibitory effects of wortmannin and LY294002 on $Fc_\gamma R$-mediated phagocytosis were attributable to inhibition of one or more members of the PI 3-kinase family of enzymes.

Inhibition of Phagocytosis by Wortmannin Is Not via Inhibition of a PLA_2—A previous study demonstrated that wortmannin inhibits PLA_2 *in vivo* but not *in vitro* (31), suggesting that this class of phospholipases may lie downstream of a PI 3-kinase. In addition, $Fc_\gamma R$-mediated phagocytosis is accompanied by enhanced activation of one or more isoforms of PLA_2, and inhibition of PLA_2 activity leads to decreased phagocytosis (32). To determine whether the inhibition of phagocytosis by wortmannin is mediated via inhibition of PLA_2, we preincubated cells with either 100 nM wortmannin or 10 μM bromophenacyl bromide (an irreversible inhibitor of PLA_2) followed by the absence or presence of 2 μM exogenous arachidonate to attempt to bypass PLA_2 blockade (32). Both wortmannin and bromophenacyl bromide inhibited phagocytosis of EIgG. Addition of exogenous arachidonate restored phagocytosis by 38 ± 3% in cells incubated with bromophenacyl bromide but did not do so in cells treated with wortmannin. This indicates that the decrease in phagocytosis in cells treated with wortmannin was not attributable to inhibition of PLA_2 activity.

Fc_γ-mediated Phagocytosis Is Accompanied by Enhanced Production of PIP_3—To test whether ligation of Fc_γ receptors results in the activation of PI 3 kinase(s) *in vivo*, [^{32}P] orthophosphate-labeled macrophages were challenged with EIgG, phospholipids were extracted, and the phosphoinositide content was analyzed by thin layer chromatography. There was a rapid increase in the accumulation of PIP_3 after $Fc_\gamma R$ ligation, which peaked at 15 s and declined by 1–2 min (Fig. 2). Immunoprecipitation of the 85-kDa subunit of PI 3-kinase did not reveal enhanced phosphotyrosine content of this subunit (not shown). The increase in the accumulation of PIP_3 that occurred during phagocytosis is consistent with an increase in the activity of one or more isoforms of PI 3-kinase.

Actin Assembly in Response to Fc_γ Ligation Is Not Inhibited by Wortmannin or LY294002—PI 3 kinase activity has been previously shown to be necessary for actin assembly mediated by PDGF or insulin (12, 33). Like receptor tyrosine kinases, ligation of Fc_γ receptors results in activation of several tyrosine kinases, such as Syk, and tyrosine kinase activity is required for actin assembly mediated by this class of immunoreceptor tyrosine activation motif-bearing receptors (2). To determine

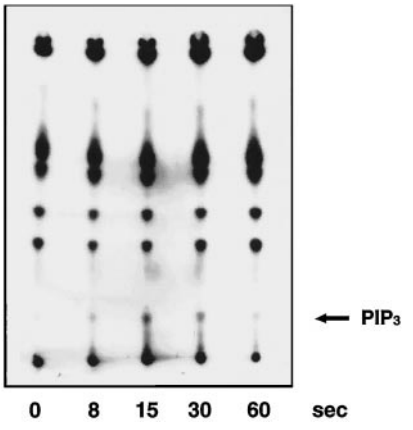

FIG. 2. **$Fc_\gamma R$-mediated phagocytosis is accompanied by enhanced production of PIP_3.** EIgG were added to adherent [^{32}P]orthophosphate-labeled RAW 264.7 cells for the indicated times, and lipids were extracted, separated by thin layer chromatography, and processed for autoradiography as described under "Experimental Procedures." The *arrow* denotes position of PIP_3 standard.

whether the inhibition of phagocytosis by wortmannin or LY294002 correlated with an inhibition of $Fc_\gamma R$-directed actin polymerization, we stained RAW 264.7 cells undergoing phagocytosis for F-actin using rhodamine-phalloidin. F-actin-rich phagocytic cups were visible in control cells treated with vehicle alone (Fig. 3, *A* and *B*), similar to those seen previously (27). In contrast to results obtained using growth factors, addition of wortmannin (Fig. 3, *C* and *D*) and LY294002 (not shown) had no obvious effect on the accumulation of F-actin beneath attached particles. However, careful observation of the cortical cytoplasm beneath attached cells revealed that, in nearly all cases, F-actin-rich pseudopods did not extend beyond ~50–70% of the circumferences of the particles (Fig. 3, *C* and *D*). This was apparent only for particles attached to the sides of the macrophages, because the degree of pseudopod extension around particles that were attached to the dorsal surfaces of macrophages was difficult to appreciate.

To determine whether the inhibition of PI 3 kinase in RAW 264.7 cells resulted in a failure to polymerize a sufficient quantity of actin necessary for complete pseudopod extension, we quantitated F-actin content in cells challenged with EIgG in the presence or absence of wortmannin or LY294002. In vehi-

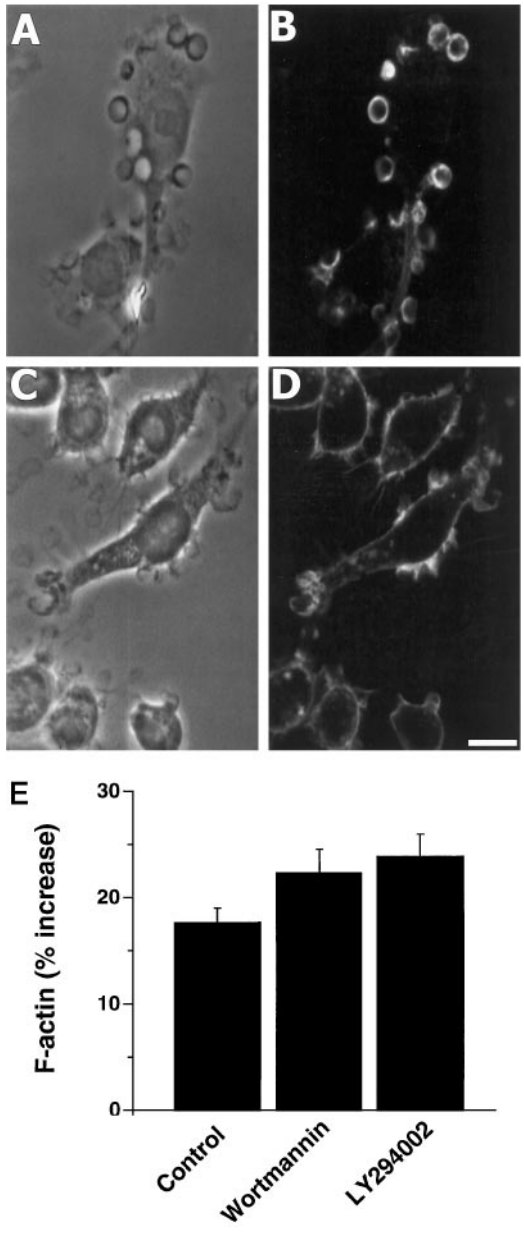

FIG. 3. **Phagocytic cup formation and actin assembly induced by EIgG in macrophages is not inhibited by wortmannin or LY294002.** RAW 264.7 cells were preincubated with vehicle (*A* and *B*) or 100 nM wortmannin (*C* and *D*) followed by a further incubation with EIgG for 5 min at 37 °C before fixation and staining for F-actin with rhodamine phalloidin as described under "Experimental Procedures." *A* and *C*, phase-contrast; *B* and *D*, rhodamine-phalloidin fluorescence. *Scale bar*, 10 μm. *E*, F-actin content of RAW 264.7 cells pretreated with vehicle, 100 nM wortmannin, or 100 μM LY294002 was determined after addition of EIgG for 5 min at 37 °C. Data, expressed as the mean ± S.E. (n = 7), are reported as percent increase compared with cells not challenged with EIgG.

cle-treated cells there was an increase in the total amount of F-actin present after the addition of EIgG (Fig. 3*E*), which was blocked by the presence of 2 μM cytochalasin D (data not shown). The presence of either wortmannin or LY294002 did not inhibit increases in F-actin content after addition of EIgG and, in fact, led to slightly enhanced accumulations of F-actin (Fig. 3*E*; p = 0.08 and 0.04, respectively). These data indicate that inhibition of PI 3-kinase did not impair actin assembly in response to $Fc_\gamma R$ ligation.

Efficacy of PI 3-Kinase Inhibition Is a Function of Particle Size, Not Number—The apparent block in maximal pseudopod extension in the presence of wortmannin (Fig. 3, *C* and *D*), with a concomitant preservation of $Fc_\gamma R$-directed actin polymerization (Fig. 3*E*), suggested that pathways leading to cytoskeletal assembly were not the target of PI 3-kinase inhibition. Because PI 3 kinase(s) have been implicated in the regulation of various membrane-trafficking events (for review, see Ref. 24), we considered the possibility that inhibition of this pathway produced a limitation in the membrane available for participation in pseudopod extension. In principle, the source of this membrane can be from the plasma membrane itself (*e.g.* from areas adjacent to those directly participating in particle ingestion) or from an intracellular membrane source (Fig. 4*A*). To distinguish these possibilities, we first assessed the effect of reducing the total number of particles added to the macrophages on sensitivity to PI 3-kinase inhibition. However, reduction in the number of particles did not lead to a restoration of phagocytosis in the presence of wortmannin (Fig. 4*B*), indicating that availability of plasma membrane was not a limiting factor for phagocytosis during PI 3-kinase inhibition.

To determine whether inadequate pseudopod extension in the presence of PI 3-kinase inhibitors was attributable to a failure of the recruitment of membrane derived from an intracellular source, we challenged macrophages with phagocytic particles of varying sizes. We determined whether the phagocytic blockade by PI 3-kinase inhibitors was relieved by reducing particle size and, hence, the magnitude of pseudopod extension required for complete particle engulfment. By screening a variety of latex particles derivatized by several proteins, we found that covalent modification of carboxylated polystyrene beads with sulfhydryl-modified BSA afforded the lowest extent of nonspecific binding to macrophages. We chose a subclone of RAW 264.7 cells to study, RAW LacR/FMLPR.2, because this clone among several others demonstrated the least tendency to bind latex particles nonspecifically. Finally, we chose to opsonize the BSA-derivatized particles with an equivalent surface density of IgG, because this would allow for changes in particle size without altering ligand density and, hence, efficiency of $Fc_\gamma R$ engagement. Quantitative immunoblotting was performed to determine IgG opsonin density, and batches of beads of varying sizes were prepared that varied by no more than 15% in opsonin density.

IgG-opsonized latex beads ranging from 1 to 6 μm in diameter were ingested by RAW LacR/FMLP.2 cells. Ingestion of these beads resembled phagocytosis of EIgG, because cytochalasin D inhibited phagocytosis by >90% regardless of the bead size. However, inhibition by cytochalasin D was slightly more effective in larger beads (*e.g.* 100 ± 0 *versus* 91 ± 6% for 6- and 1-μm beads, respectively). The greater sensitivity to cytochalasin for the larger beads may be attributable to a requirement for a greater magnitude of actin assembly needed to completely surround the 4.5- and 6-μm particles. Similar to results using EIgG (2), phagocytosis of all sizes of latex beads was completely blocked by genistein, a tyrosine kinase inhibitor (data not shown).

The efficacy of PI 3-kinase blockade on phagocytosis varied with bead size. Wortmannin or LY294002 potently inhibited the ingestion of beads of 4.5 and 6 μm in diameter but had progressively less efficacy of inhibiting ingestion of smaller beads (Fig. 4*C*). Because the magnitude of pseudopod extension required for complete internalization varies with particle diameter, these results suggest that intact signaling through PI 3-kinase(s) is critical when pseudopods are required to extend from the margins of the cells by more than the circumference of 2–3-μm beads. These results are consistent with a requirement for surface recruitment of a latent intracellular pool of membrane for optimal pseudopod extension.

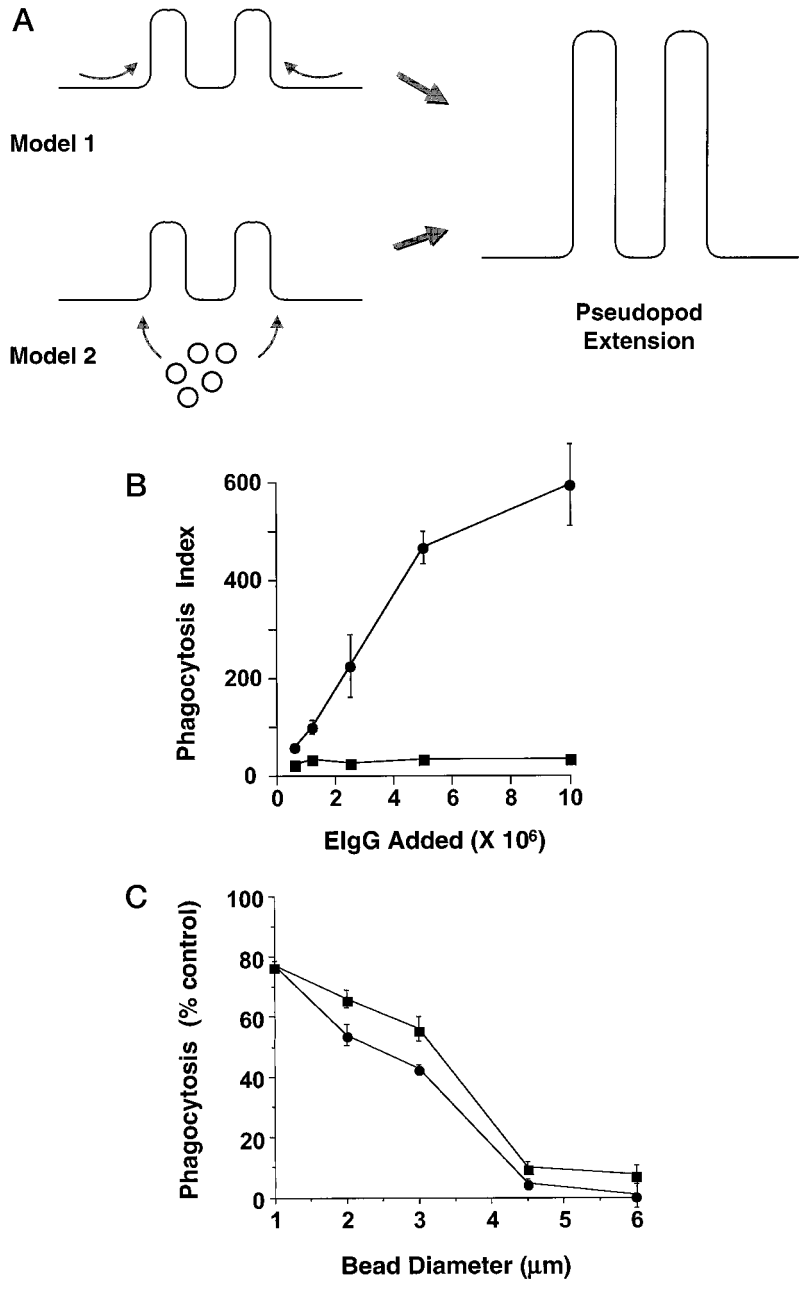

FIG. 4. **Sensitivity of phagocytosis to PI 3-kinase inhibition is independent of the number of particles added but is dependent on particle size in RAW LacR/FMLPR.2 cells.** *A*, model for potential sources of recruited membrane necessary for pseudopod extension and successful completion of phagocytosis. In *Model 1*, the source of membrane is directly from the plasma membrane itself (*i.e.* from areas adjacent to those directly participating in particle ingestion). In *Model 2*, the source of membrane is from a latent intracellular vesicular pool. *B*, phagocytosis of EIgG as a function of increasing total number of EIgG added in the absence (●) or presence (■) of 100 nM wortmannin. Data are expressed as the mean ± S.E. (n = 3). *C*, phagocytosis of IgG-coated beads of the indicated diameters in the presence of 100 nM wortmannin (●) or 100 μM LY294002 (■). Data, expressed as the mean ± S.E. (n = 3), are reported as the percent ingestion of beads in the presence of vehicle.

Spreading of Thio-macrophages on Human IgG and Concomitant Exocytic Insertion of Membrane Are Inhibited by Wortmannin or LY294002—The above results using beads of 1–2 μm in diameter suggested that the PI 3-kinase blockade impaired pseudopod extension rather than phagosomal closure (21). To confirm this, we used an assay of $Fc_{\gamma}R$-based motility that does not require phagosomal closure. We coated coverslips with human IgG and observed the extent of spreading as a function of time and PI 3-kinase activity. We chose thio-macrophages because RAW 264.7 cells and similarly derived lines do not spread readily on this substrate (data not shown). Thio-macrophages, like RAW LacR/FMLP.2 cells, ingested small beads in the presence of wortmannin of LY294002 (data not shown).

Thio-macrophages spread rapidly on IgG-coated substrates, demonstrating an ~4-fold increase in their apparent adherent surface areas. Spreading of macrophages on IgG was inhibited by either wortmannin or LY294002, whereas the peripheral appearance of F-actin was not, consistent with a lack of inhibition of cytoskeletal assembly by PI 3-kinase inhibitors (Fig. 5). The extent of spreading in controls was appreciable and suggested that newly recruited membrane might be required for efficient spreading. We measured the recruitment of membrane to the cell surfaces using FM1-43, a styryl dye with fluorescence that increases after binding the outer leaflet of plasma membranes (34, 35). As new membrane is recruited to the surface during exocytosis, fluorescence increases in cells continuously maintained in the presence of the dye. Thus, the dye reports cumulative exocytic membrane insertion. We measured FM1-43 fluorescence using saturating concentrations of dye (Fig. 6*A*) and found that macrophages displayed a time-dependent increase in FM1-43 fluorescence during spreading on human IgG. Similar to results of cell spreading (Fig. 5), increases in FM1-43 fluorescence were inhibited by wortmannin and LY294002 (Fig. 6*B*). Collectively, these data indicate that spreading of macrophages on human IgG is accompanied by an apparent increase in the plasma membrane surface area concomitant with an increase in exocytic insertion at the plasma

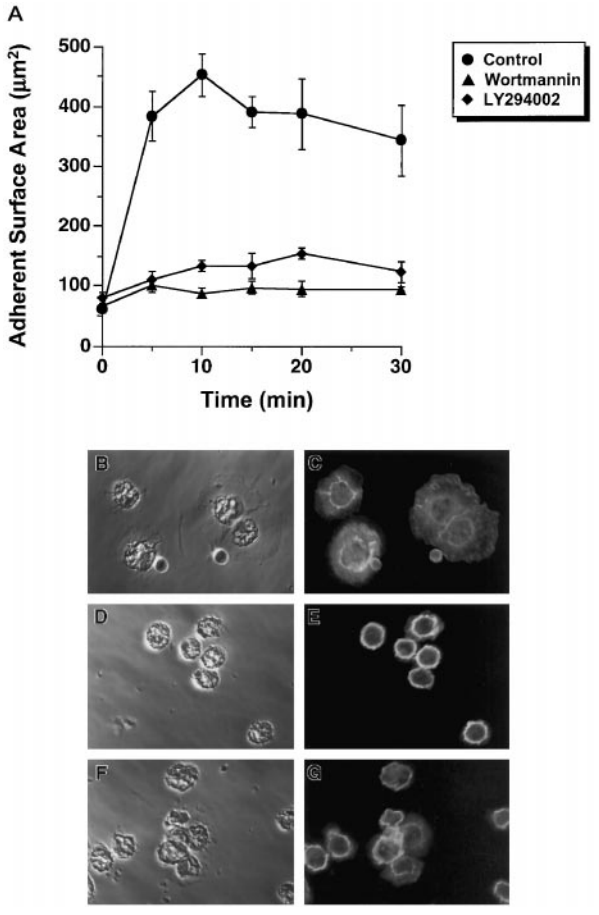

FIG. 5. **Spreading but not F-actin accumulation of thio-macrophages on human IgG is inhibited by wortmannin or LY294002.** *A*, cells, preincubated with vehicle, 100 nM wortmannin, or 100 μM LY294002, were allowed to spread on IgG-coated coverslips at 37 °C for the indicated times before fixation. Data, expressed as the mean ± S.E. (n = 3), are reported as mean adherent surface areas. *B–G*, thio-macrophages spreading on IgG-coated coverslips in the presence of vehicle (*B* and *C*), 100 nM wortmannin (*D* and *E*), or 100 μM LY294002 (*F* and *G*) were fixed and stained for F-actin with rhodamine-phalloidin. Phase-contrast (*B*, *D*, and *F*) and fluorescence micrographs (*C*, *E*, and *G*) depict presence of F-actin-rich areas at the peripheries of the cells despite the presence of wortmannin or LY294002.

membrane from an intracellular source. Both processes are regulated in a PI 3-kinase-dependent fashion.

DISCUSSION

The data presented here demonstrate a requirement for one or more isoforms of PI 3-kinase in $Fc_\gamma R$-mediated phagocytosis. Although these results are not surprising in light of earlier reports of the requirement for this family of enzymes in phagocytosis, the role for PI 3-kinase in phagocytosis that we are proposing is somewhat unexpected. Studies of the PDGF receptor signaling cascade indicate that PI 3-kinase activity is required for membrane ruffling and actin polymerization (8, 33), both of which occur in a Rac1-dependent manner. Similar to growth factor receptor signaling, $Fc_\gamma R$-dependent signaling pathways that culminate in actin assembly are tyrosine kinase- and Rac1-dependent (2, 3). However, our results clearly indicate that PI 3-kinase activity is not required for $Fc_\gamma R$-mediated actin assembly. Rather, blockade of PI 3-kinase(s) appears to result in the functional dissociation between cytoskeletal assembly and pseudopod extension, events that are normally coupled. These data are somewhat different than those of Araki *et al.* (21), who described a block in phagosomal closure and macropinocytosis in the presence of PI 3-kinase inhibitors. Our results support of role for PI 3-kinase in an earlier step in phagocytosis. Because the cellular components required for the terminal fusion of vesicles, an event akin to phagosomal closure, are likely to be different than those governing process extension, this distinction has mechanistic consequences. Indeed, given the results described in Fig. 6 and studies that show a requirement for PI 3-kinase in membrane trafficking (36–40), we propose that up-regulation of one or more intracellular membrane compartments is required for optimal pseudopod extension, and that this event is dependent on one or more isoforms of PI 3-kinase.

The identity of PI 3-kinase-sensitive membrane compartments is under extensive study by many groups (for review, see Ref. 24). The bulk of evidence shows a requirement for PI 3-kinase in trafficking from one or more recycling compartments that contain transferrin receptors and/or glucose transporters to the plasma membrane (10, 36, 41–44). In addition, several studies demonstrate a requirement for PI 3-kinase in the secretory pathway (45–47). Conceivably, one or more of these pathways are up-regulated during, and are required for, pseudopod extension. Interestingly, a recent ultrastructural study of phagocytosis in monocytes demonstrated the accumulation of plasma membrane-derived, electron-lucent vesicles beneath nascent phagosomes, particularly in the presence of PLA_2 inhibitors (48). However, the lack of restoration of phagocytosis by arachidonate in the presence of PI 3-kinase blockade and the results of Fig. 4*B* indicate that this membrane compartment is not likely to be directly regulated by PI 3-kinase.

This study did not address the specific isoforms of PI 3-kinase involved in phagocytosis and pseudopod extension. The list of PI 3-kinase family members is long (for review, see Ref. 49), and many have been only partially characterized. The isoform most often implicated in cellular signaling, p85/p110, is activated by many growth factor receptors; during receptor clustering, the p85 regulatory subunit is recruited to the receptor itself (50–52) or interacts with adaptor proteins such as Grb2 (53), c-Cbl (54, 55), and cytosolic tyrosine kinases (56, 57). p85/p110 has been shown to associate with Fc_γ receptors and with Syk, although it is not clear whether such an association is direct (58, 59). We could not definitively determine whether this isoform was required for phagocytosis. Although transfection of RAW LacR/FMLPR.2 cells with Δp85, a p110 binding-defective allele of p85, did not result in impaired phagocytosis despite apparent expression (data not shown), it also produced no detectable phenotypic changes in the cells; therefore, we could not verify that it functioned in a dominant-negative fashion. Other members of the PI 3-kinase family, in addition to p85 binding isoforms, may be activated after Fc_γ receptor activation (60).

Several molecular targets of the lipid products of PI 3-kinase have been identified. One of these, ARNO (cytohesin-2), contains a PH domain that is capable of interacting with acidic phospholipids *in vitro* (61). Recent studies indicate that cytohesin-2 has guanine nucleotide exchange activity for ARF6 (62), and intact ARF6 has been shown to be required in phagocytosis (63). Other likely targets include proteins that interact with or stimulate guanine nucleotide exchange activity of members of the Rab family of GTPases. Wortmannin inhibits Rab5-mediated stimulation of endocytosis (64) and blocks insulin-stimulated binding of ^{35}S-GTPγS to Rab4 (65). It is possible that lipid products of PI 3-kinase bind to PH domains on guanine nucleotide exchange factors for ARF and Rab family members, thereby increasing their exchange activity. For example, a role for phosphoinositide-stimulated guanine nucleotide exchange factor activity has been described for Vav (66).

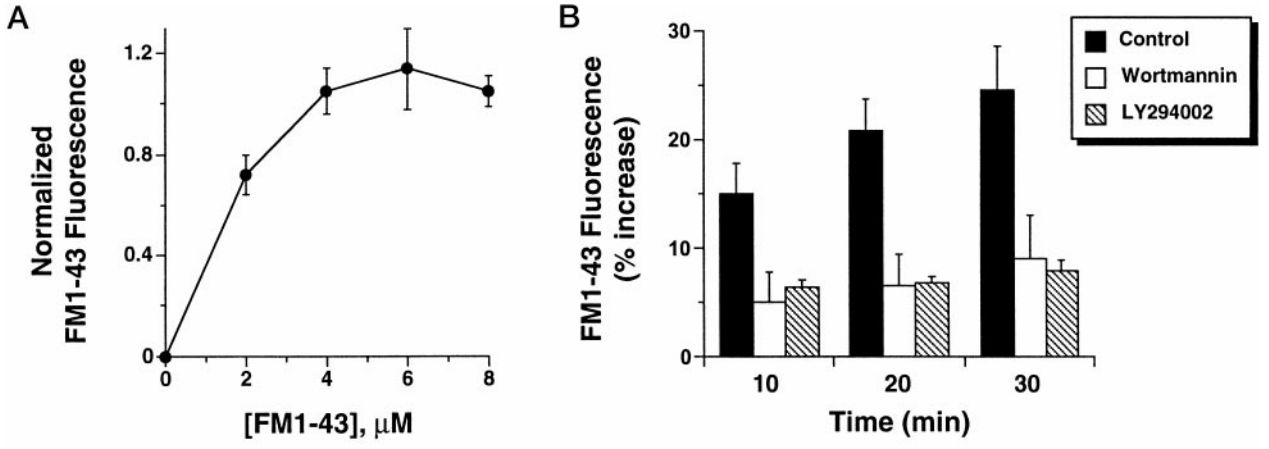

FIG. 6. **Exocytic insertion of plasma membrane is inhibited by wortmannin or LY294002.** *A*, FM1-43 fluorescence saturates with increasing dye concentration. 5×10^4 cells were incubated with the indicated concentrations of FM1-43. Data are expressed as the mean ± S.E. of FM1-43 fluorescence normalized to cell number ($n = 3$). *B*, fluorescence of cells spreading on adherent human IgG in the continuous presence of 8 μM FM1-43 and vehicle, 100 nM wortmannin, or 100 μM LY294002. Data, expressed as the mean ± S.E. ($n = 5$), are reported as percent increase in baseline fluorescence ($t = 0$). The difference between control- and wortmannin- or LY294002-treated cells is statistically significant ($p < 0.01$).

Another product of PI 3-kinase, phosphatidylinositol-3-phosphate, may be required for Rab-dependent membrane fusion. Phosphatidylinositol-3-phosphate binds the FYVE finger domain of EEA1, a protein that is required for Rab5-dependent endosomal fusion *in vitro*. This phospholipid is required for membrane localization of EEA1 (for review, see Ref. 67). Thus, multiple lipid products of PI 3-kinase may be required for promoting membrane fusion events *in vivo*, including those that accompany phagocytosis.

Although it could be argued that ingestion of beads of 1–2 μm in diameter differ mechanistically from ingestion of larger beads, our results suggest that they share certain common elements, including cytochalasin sensitivity and a requirement for one or more tyrosine kinases. The slightly greater sensitivity of large bead phagocytosis to cytochalasin may reflect a requirement for sustained actin polymerization necessary to support the formation of large pseudopods. Phagocytosis of small (*i.e.* 1–2 μm) beads would be expected to require less sustained actin assembly to achieve complete particle engulfment. Thus, the typically incomplete inhibition of barbed end actin filament growth by cytochalasin may result in complete failure in the engulfment of large beads and only partial inhibition in the engulfment of small beads. These data are similar to recent findings by Koval *et al.* (68), in which complete inhibition of the ingestion of latex particles by macrophages required high (2.5 μM) concentrations of cytochalasin D. Although we did not test the phagocytosis of beads smaller than 1 μm, it is anticipated that the cellular machinery involved in the ingestion of progressively smaller test particles may not require the active participation of the actin-based cytoskeleton, as suggested by Koval *et al.* (68), or the membrane recruitment of tyrosine kinases. It is difficult to draw general conclusions regarding the requirement of PI 3-kinase in the phagocytosis of other phagocytic targets, such as bacteria, because they are geometrically distinctive and may not necessarily be opsonized uniformly, as is the case here. However, a demonstration of a requirement for PI 3-kinase was reported for the ingestion of *Listeria monocytogenes* by epithelial cells (69); it is possible that the role of this enzyme in phagocytosis of *Listeria* is similar to that described in the current study.

Several studies that attempt to elucidate how a cell extends surface protrusions focus on the role of actin as a protrusive force driving pseudopod extension and view the membrane as a passive component in this process (for review, see Ref. 70). Other models for membrane protrusion have proposed a role for a polarized endocytic cycle that involves exocytosis of vesicles at the leading edge (71). In support of this model, recycled transferrin receptors are concentrated at the leading lamella in migrating fibroblasts (72), and protrusion of the cell surface in plasmodia has been linked with exocytic events (73). Studies of macrophage phagosomes indicate the presence of multiple syntaxins, and addition of tetanus toxin light chain, a v-SNARE protease, inhibited Fc_γR-mediated phagocytosis, underscoring the close association of the endocytic and exocytic compartments (74, 75). Our data support a model of pseudopod extension that requires active participation of tyrosine kinases, actin polymerization, and PI 3-kinase. All are necessary for the coordinated extension of pseudopods that culminate in the formation and eventual closure of the phagosome.

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