Indirect Role for COPI in the Completion of Fcγ Receptor-mediated Phagocytosis*

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Phagocytosis of microbial pathogens by leukocytes is an essential component of the host defense against infection. Microorganisms become internalized into a membrane-bound vacuole called a phagosome, which subsequently matures upon fusion with endosomes and lysosomes into a powerful microbicidal organelle (1). The phagocytic capacity of neutrophils and macrophages is remarkable; individual cells can take up multiple and/or very large particles. As a result, the area of membrane internalized is significant and has in some cases been estimated to approach or exceed the total initial surface area of the phagocyte (2). Despite the internalization of a considerable membrane expanse, no net loss of surface has been detected, and, in fact, surface gains have been documented electrophysiologically (3) and by spectroscopic means (4). These observations therefore suggest that active exocytosis of endomembranes accompanies phagocytosis.

Pharmacological studies have in fact suggested that secretion of endomembranes is essential for optimal phagocytosis. First, inhibitors of phospholipase A2 precluded phagocytosis, with a concomitant accumulation of clear vesicles under the site of particle attachment (5). Second, Cox et al. (6) found that inhibition of phosphatidylinositol 3-kinase by wortmannin, which can inhibit exocytosis, prevented phagocytosis of large particles, with comparatively minor effects on the ingestion of smaller ones. Third, botulinum and tetanus toxins, which interfere with SNARE-mediated membrane fusion, were found to induce partial inhibition of phagocytosis (7). Finally, it was shown that exocytic vesicles enriched in VAMP3, a v-SNARE that mediates secretion of recycling vesicles (8), were locally secreted at sites of phagocytosis (9). Jointly, these studies suggest that extension of pseudopods during particle engulfment involves the focal exocytosis of endomembrane vesicles.

The mechanisms mediating the formation and traffic of these putative vesicles are not understood. However, existing information regarding endomembrane traffic may be applicable to the genesis of phagosomes. In particular, COPI has been convincingly shown to participate in the budding and traffic of vesicles between the Golgi complex and the endoplasmic reticulum (ER) (10–12) as well as vesicles involved in recycling and endosome maturation (13, 14). COPI exists as a protein complex in the cytosol and can assemble on docking sites of membranes, where it promotes the fission of vesicles (15, 16). We therefore considered the possibility that COPI may participate in the process of phagosome formation.

Recent evidence suggests that extension of pseudopods during phagocytosis requires localized insertion of endomembrane vesicles. The nature of these vesicles and the processes mediating their release and insertion are unknown. COPI plays an essential role in the budding and traffic of membrane vesicles in intracellular compartments. We therefore assessed whether COPI is also involved in phagosome formation. We used ldIF cells, a mutant line derived from Chinese hamster ovary cells that express a temperature-sensitive form of eCOP.

To confer phagocytic ability to ldIF cells, they were stably transfected with Fc receptors type IIA (FcγRIIA). In the presence of functional COPI, FcγRIIA-transfected ldIF cells effectively internalized opsonized particles. In contrast, phagocytosis was virtually eliminated after incubation at the restrictive temperature. Similar results were obtained impairing COPI function in macrophages using brefeldin A. Notably, loss of COPI function preceded complete inhibition of phagocytosis, suggesting that COPI is indirectly required for phagocytosis. Despite their inability to internalize particles, COPI-deficient cells nevertheless expressed normal levels of FcγRIIA, and signal transduction appeared unimpeded. The opsonized particles adhered normally to COPI-deficient cells and were often found on actin-rich pedestals, but they were not internalized due to the inability of the cells to extend pseudopods. The failure to extend pseudopods was attributed to the inability of COPI-deficient cells to mobilize endomembrane vesicles, including a VAMP3-containing compartment, in response to the phagocytic stimulus.

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1 The abbreviations used are: ER, endoplasmic reticulum; BFA, brefeldin A; CHO, Chinese hamster ovary; EM, electron microscopy; FcγRIIA, Fc receptors type IIA; GFP, green fluorescent protein; Tfn, transferrin; ARF, ADP-ribosylation factor.
To this end, we used brefeldin A (BFA), which inactivates the GTP exchange factors of adenosine ribosylation factor, to inhibit COPI or the Chinese hamster ovary (CHO) mutant cell line called idlF, originally isolated by Hobbie et al. (10). These cells harbor a temperature-sensitive mutation in the gene encoding eCOP, a component of COPI (10, 11). When incubated at the permissive temperature (34 °C), such cells express functional eCOP, while incubation at the restrictive temperature (≥39 °C) results in destabilization and rapid degradation of the mutant eCOP, thereby inactivating COPI (10, 11). While extremely useful for the study of COPI function, idlF cells are not phagocytic. In order to analyze the role of COPI in phagocytosis, idlF cells were stably transfected with Fc receptors (FcγRIIA). Such heterologous transfection of opsonin receptors was shown earlier to confer phagocytic capacity to nonmyeloid cells, including CHO cells (17–19). We found that, like CHO cells, idlF cells transfected with FcγRIIA (called FcR-idl hereafter) effectively internalized IgG-opsonized particles when grown at the permissive temperature. However, following down-regulation of COPI, phagocytosis was progressively and drastically inhibited. Evidence is provided that COPI is indirectly required for phagocytosis by maintaining a VAMP3-containing endosome pool that appears to be required for pseudopod extension during FcγR-mediated phagocytosis.

**EXPERIMENTAL PROCEDURES**

**Materials, Constructs, and Antibodies—**Fura-2 acetoxymethyl ester, zymosan, FM1–43, rhodamine-123, and rhodamine-phalloidin were from Molecular Probes, Inc. (Eugene, OR). Hepes-buffered medium RPMI 1640, 0.8-μm dyed latex beads, BFA, and cycloheximide were obtained from Sigma. 125I-Diferric human transferrin (125I-Tfn) was from PerkinElmer Life Sciences, and [35S]methionine/cysteine was from Amersham Pharmacia Biotech. pEGFP, the plasmid encoding GFP, was from CLONTECH. The FAMP3-GFP chimera was previously described (9). Human IgG was from Baxter Healthcare Corp. (Glendale, CA). Rabbit anti-GFP and anti-catalase antibodies were from Molecular Probes, Inc. and Calbiochem (La Jolla, CA), respectively. The rabbit polyclonal antibodies to eCOP, α-mannosidase II, and calnexin were generous gifts from Drs. M. Krieger (Massachusetts Institute of Technology, Cambridge, MA), K. Moresen (Emory University, Atlanta, GA), and David Williams (University of Toronto), respectively. The phosphotyrosine antibody mixture, containing equivalent amounts of monoclonal antibodies PY-7E1, PY-1B2, and PY-20, was from Zymed laboratories Inc. (San Francisco, CA). Mouse anti-FcγRIIA monoclonal antibody IV.3 and the anti-β1-integrin antibodies to quantitate surface expression of FcγRIIA and β1-integrin, respectively. Fluorescence was analyzed using either a Leica model TCS4D or a Zeiss LSM 510 laser confocal microscope. Composites of confocal images were assembled and labeled using Photoshop (Adobe, Mountain View, CA) and Microsoft Powerpoint software. Quantification of immunofluorescence was performed using Scion Image (Scion Corporation, MA).

**Quantification of Surface FcγRIIA and β1-Integrin—**Surface β1-integrin was quantified using fixed, nonpermeabilized cells attached to glass coverslips by confocal microscopy. This was accomplished by acquiring confocal optical slices at a constant interval and integrating the total cellular fluorescence by stacking the collected slices. Fluorescence intensity of the reconstructed images was quantified using Scion Image. For flow cytometry, the cells were immunostained as above and then scraped off the coverslips in ice-cold divalent cation-free phosphate-buffered saline. After washing, the cells were analyzed as in Ref. 7.

**Measurement of Free Cytosolic Calcium (Ca²⁺) —**Cells grown on glass coverslips were incubated overnight at either 34 or 39 °C and then loaded with fura-2 by incubation with 10 μM of the parental acetoxyethyl ester for 30 min. Coverslips were then mounted in a thermostat-stabilized Leiden holder on the stage of a Zeiss IM-35 microscope, equipped with a ×63 oil immersion objective. The microscope set-up has been previously described in detail (18). Calibration of fluorescence ratio versus [Ca²⁺], was performed as described (20). All measurements were at 37 °C.

**Scanning and Transmission Electron Microscopy—**For scanning electron microscopy, cells were fixed in 2% glutaraldehyde and post-fixed with 1% OsO₄. Following washing, the cells were dehydrated in a graded series of ethanol. The samples were then critical point-dried in a Pelo CPD-2 critical point drying device and mounted on EM stubs with colloidal silver glue. They were then coated with evaporated gold/palladium with a Pelco sputter coater model 3 for 50 s at 18 mA. The samples were then examined with a Philips XL 30 scanning electron microscope modified from the manufacturer.

For transmission EM, cells were fixed as for scanning EM. The cells were washed extensively and then en bloc stained with 1% aqueous uranyl acetate for 30 min. Following washing, the samples were dehydrated through a graded series of ethanol and then embedded in Epon as we have described previously (21). Thin sections were cut and stained with lead citrate and uranyl acetate and observed with a Philips CM-12 electron microscope.

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—**Samples were solubilized in Laemmli’s sample buffer (22), resolved by SDS-polyacrylamide gel electrophoresis using the Protean II minigel system (Bio-Rad), and transferred onto polyvinylidene difluoride membranes. Membranes were then immersed in blocking buffer (5% milk and 0.05% Tween 20) overnight at 4 °C. Blots were incubated with anti-eCOP antibody (1:8000) and anti-tubulin antibody (1:500) for 1 h at room temperature. The blots were then washed three times for 10 min each in antibody buffer (50 mM Tris/HCl, 150 mM NaCl, 0.05% Tween 20, 0.04% Nonidet P-40, pH 7.5) and next incubated with peroxidase-conjugated anti-rabbit IgG (1:5000) for 1 h. Membranes were washed and developed using enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Receptor-mediated Endocytosis and Recycling of 125I-Tranferrin—**FcR-idl cells were either maintained at 34 °C or incubated at 39 °C for ≥12 h and then serum-starved for 1 h. The rate of receptor-mediated endocytosis of 125I-Tranferrin was then measured by incubating cells with 0.4 μCi/ml of 125I-Tranferrin for either 10 or 30 min. Extracellular 125I-Tranferrin was then washed and stripped with phosphate-buffered saline solution at
pH 3.0. To measure the rate of $^{125}$I-Tfn recycling, cells were incubated for 15 or 30 min in the absence of $^{125}$I-Tfn after a pulse of 30 min. All steps were performed at 37 °C. The cells were then lysed, and the amount of internalized $^{125}$I-Tfn was quantified with a $\gamma$ counter.

**RESULTS**

*Modulation of eCOP Expression in FcR-ldl Cells*—To study the role of COP in phagocytosis, ldIF cells were stably transfected with FcRRIIA, yielding FcR-ldl cells. To verify that the mutation characteristic of ldIF cells persisted in FcR-ldl cells, the eCOP content of both cell types was assessed by immunoblotting following incubation at the permissive (34 °C) or restrictive (39 °C) temperature. For comparison, wild-type CHO stably transfected with FcRRIIA (FcR-CHO cells) were also analyzed. Tubulin, which is constitutively expressed in these cells in a temperature-independent manner, was used as a reference. When grown at 34 °C, both ldIF and FcR-ldl express eCOP, although at levels that are 2–4 times lower than that found in FcR-CHO cells (Fig. 1A). A similar differential expression of eCOP at 34 °C was reported earlier between ldIF and wild-type CHO cells (12). Incubation at the restrictive temperature for $\geq$18 h resulted in the complete disappearance of eCOP in ldIF and FcR-ldl cells but had no detectable effect on the expression of this protein in FcR-CHO cells (Fig. 1A), as found earlier for wild-type CHO cells (12).

The disappearance of eCOP was also apparent by analyzing the functional consequences of incubation at 39 °C. As illustrated in Fig. 1B, i, the Golgi complex normally displays a tight juxtanuclear structure composed of cisternae and vesicles. Maintenance of this structure is known to depend on the continued function of COPI, which in turn requires the presence of COP (13, 14). Degradation of eCOP in FcR-ldl cells was associated with dispersal of the juxtanuclear complex, resulting in a diffuse punctate staining of a–mannosidase II-reactive vesicles (Fig. 1B, iv). Dispersal of the Golgi complex was due to disappearance of eCOP and not to the temperature shift itself, since FcR-CHO cells were found to preserve their juxtanuclear Golgi cisternae after overnight incubation at 39 °C (Fig. 1B, iii).

In contrast to the Golgi complex, incubating cells at 39 °C did not appear to cause detectable changes to peroxisomes, mitochondria, and the ER. The punctate distribution of catalase, a marker of peroxisomes, was indistinguishable in cells incubated at 34 and 39 °C (not shown), implying that peroxisomes are able to import and retain this soluble protein in their lumen. Furthermore, rhodamine-123 accumulated normally in the mitochondria of cells incubated at 39 °C (Fig. 1C), indicating that the mitochondrial membrane potential was unaffected, which implies that the activity of the respiratory chain is normal. In addition, the reticulate morphology of the ER, revealed by immunostaining of calnexin, was similarly unaltered by prolonged incubation at the restrictive temperature (not illustrated). These results were consistent with ultrastructural analysis of thin sections by transmission EM, which showed no alterations in the structure or distribution of mitochondria or ER in cells treated at 39 °C (100 sections from three different experiments; not illustrated). Together, these observations imply that treatment of the cells at the restrictive temperature for $\geq$15 h does not induce wholesale, nonspecific disorganization of the cellular ultrastructure and that only organelles dependent on COPI for their homeostasis, such as the Golgi apparatus, undergo visible alterations, as shown previously (11, 13, 14).

**Effect of eCOP Depletion on Phagocytosis**—Previously, it had been shown that ldIF cells stably transfected with FcRRIIA were able to internalize IgG-opsonized particles (17). Therefore, we next investigated whether COPI is required for phagocytosis. FcR-ldl cells were maintained at either 34 or 39 °C and then exposed to IgG-opsonized SRBC to initiate phagocytosis. As illustrated in Fig. 2A and quantified in Fig. 2C, FcR-ldl cells maintained at the permissive temperature internalize SRBC effectively; phagocytosis occurred in upwards of 50% of the cells. This compares favorably with the phagocytosis efficiency of FcR-CHO cells (~30%; Fig. 2C). The greater phagocytic ability of FcR-ldl cells is most likely attributable to clonal differences. Depletion of eCOP by overnight incubation at 39 °C profoundly reduced the ability of FcR-ldl cells to perform phagocytosis. SRBC were observed in only 4 ± 3% of the eCOP-depleted cells. By contrast, FcR-CHO cells internalized SRBC slightly more effectively following incubation at 39 °C than when maintained at 34 °C. These observations imply that the effect noted in FcR-ldl cells is not due to the temperature per se but instead that the presence of COPI is essential for optimal phagocytosis.

Consistent with the findings of Daro et al. (13) in ldIF cells, the
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cells stably transfected with Fc receptors (FcR-ldl) were found to take up and recycle Tfn, albeit at reduced rates. As shown in Fig. 2D, the initial rate of uptake was >2-fold lower in cOP-depleted cells, although the extent of uptake after 30 min was only ~30% lower. Also in agreement with Daro et al. (13), we found that the slower rate of Tfn uptake was compensated by a reduced rate of recycling (Fig. 2D, lanes 5–8), accounting for the nearly normal Tfn content at steady state. The reduced recycling of transferrin is consistent with the proposed role of COPI in traffic along the endocytic pathway.

Effect of cOP Depletion on Cell Morphology—During the course of the phagocytosis experiments described above, a striking morphological difference between cells incubated in different conditions became apparent. When incubated at the restrictive temperature, FcR-ldl cells were found to become more rounded (Fig. 2, compare A and B). As before, the effect was attributable to the depletion of COPI and not to the incubation at 39 °C, since FcR-CHO cells maintained their spread morphology at this temperature (not shown).

Further evidence that this effect was specifically related to loss of COPI was obtained by treating FcR-CHO and FcR-ldl cells (maintained at 34 °C) with the fungal metabolite BFA. BFA associates with and blocks the activity of nucleotide exchange factors that regulate ARF, thereby preventing the association of coatamer subunits with membranes (23, 24). Treatment with 100 μM BFA for 30–60 min induced rounding of both cell lines (not shown).

The cell rounding observed upon inactivation of COPI was associated with an apparent decrease in net surface area. This was determined by confocal microscopy, quantifying the amount of cell-associated FM1–43 (not shown), a dye that becomes fluorescent when it intercalates into the plasmalemma, thereby providing a measure of cell surface area (6, 25). The decrease in cOP-depleted cells was reproducible and statistically significant, but since fluorescence is not a simple function of the area, the change in plasmalemmal surface was not quantified precisely.

Several separate lines of evidence argue that rounding of COPI-deficient cells is not an indication of detrimental effects on cell function or viability. First, the cells remained impermeant to vital dyes and to the fluorescent dye FM1–43. Second, the cytosolic free calcium concentration, a sensitive measure of cell integrity and well being, was unaltered by depletion of cOP (see below). Third, and as reported earlier for ldlF cells (13, 14), FcR-ldl cells treated overnight at 39 °C displayed endocytosis and recycling of transferrin (Fig. 2D). Fourth, the rate of incorporation of [35S]methionine/cysteine into newly synthesized proteins was not significantly changed (not shown). Fifth, peroxisomes, the ER, and mitochondria in cOP-depleted cells resembled those of control FcR-ldl cells, and the mitochondrial potential appeared unaffected. Sixth, cells responded to the addition of stimuli with increased tyrosine phosphorylation and actin assembly (see below). Finally, the effects of incubation at 39 °C were reversible. These findings imply that within the time period examined, suppression of COPI function does not cause generalized detrimental effects on the cell.

Effect of COPI Depletion on Receptor Expression and Function—In view of the apparent loss of surface area, the effects of COPI depletion on phagocytosis could result from alterations in Fc receptor expression, distribution, or function. This possibility was considered in the experiments illustrated in Fig. 3. At the permissive temperature, FcγRIIA receptors were clearly observable by immunostaining on the plasmalemma of FcR-ldl cells (Fig. 3A). Neither the distribution (Fig. 3B) nor the amount of receptors appeared to be altered following incubation at the restrictive temperature. Quantitation of receptors by flow cytometry indicated that similar levels of FcγRIIA were exposed after incubation at 34 or 39 °C for 18 h in both FcR-CHO and FcR-ldl cells (Fig. 3E).

The ability of the receptors to engage their ligand was also assessed, quantifying the number of opsonized SRBC bound to the surface of the FcγRIIA-transfected cells. As illustrated by the scanning electron micrographs in Fig. 3, C and D, and summarized in Fig. 3E, SRBC bound readily to FcR-ldl cells both before and after depletion of cOP by overnight incubation at 39 °C. Comparable results were obtained with FcR-CHO cells (Fig. 3E).

The Effect of Brefeldin A on Phagocytosis by FcR-ldl and
Macrophage Cells—Our results indicated that functional COPI was required for phagocytosis in FcR-ldl cells. These findings appear to be in conflict with earlier findings of Zhang et al. (26), who reported that in RAW 264.7 macrophages phagocytosis was insensitive to BFA. BFA is a specific inhibitor of GTP exchange factors of the ARF family of GTPases, which are essential for COPI function (23, 24, 27). We therefore tested the effect of BFA on phagocytosis in FcR-ldl cells and macrophages. The inhibitor induced a rapid dispersal of α-mannosidase II from a perinuclear distribution (Fig. 4A) to a diffuse intracellular appearance (Fig. 4B), resembling that found in eCOP-depleted cells. Despite the demonstrated effect of BFA on COPI function, phagocytosis was virtually unaffected when measured 2 h after the addition of the drug (Fig. 4C), consistent with earlier observations (26).

The primary difference between the two protocols used to inactivate COPI is the time involved in the development of the inhibitory effect. To resolve the apparent inconsistency, we compared the rate of disappearance of eCOP with the onset of inhibition of phagocytosis. The results are summarized in Fig. 4D. Significantly, the course of disappearance of eCOP preceded the complete ablation of phagocytosis. After 6 h at 39 °C, when eCOP was no longer detectable, phagocytosis was diminished but still clearly observable (approximately half-maximal; see Fig. 4D). Similarly, while no significant effects were noted at the early stages of BFA incubation, phagocytosis was inhibited by nearly 35% after 3 h and by 100% after 20 h (Fig. 4D).

BFA also inhibited phagocytosis in professional phagocytes; particle ingestion was inhibited by 50% and by >90% in J774.1 and RAW cells incubated with the ARF antagonist for 6 and 12 h, respectively (not shown). Importantly, these data argue that a significant attenuation of phagocytosis can be observed at early time periods (4–8 h) after COPI deficiency and further suggest that our observations are not due to generalized, non-specific detrimental effects on cell function due to prolonged absence of COPI.

However, these findings imply that COPI is not directly involved as an essential component of the phagocytic process. Instead, loss of COPI function abolishes phagocytosis indirectly, perhaps by gradual depletion of an essential component required for the late stages of signal transduction, actin polymerization, and/or pseudopod extension. There is precedent for depletion of membrane components in IdlF cells, which were initially isolated on the basis of reduced Idl receptor expression.
at the restrictive temperature (10). Reduction of these essential components may conceivably occur by inhibition of the biosynthetic pathway when COP-I is inactivated (11). Nonetheless, treatment of cells with cycloheximide for 24 h did not preclude phagocytosis, although protein synthesis was demonstrably arrested (not shown).

We also investigated if the prolonged absence of functional COP-I was equally effective at blocking phagocytosis of smaller particles. RAW cells were treated with BFA for 8–10 h and then allowed to internalize either SRBC, which have a diameter of ~4 μm, or 0.8-μm latex beads coated with IgG, the latter approaching the size of bacterial pathogens. At this time, phagocytosis of SRBC was blocked by ~65% ± 10% relative to control cells. The uptake of 0.8-μm beads was also clearly inhibited by depletion of eCOP, although the inhibition was significantly smaller (40 ± 9%; Fig. 4E).

**Effect of eCOP Depletion on Receptor Signaling**—The preceding results indicate that the inhibition of phagocytosis induced by depletion of COP-I cannot be attributed to a decrease in the number of FcγRIIA receptors or in their ability to ligate SRBC. Instead, the effect of COP-I is probably exerted at a later stage, affecting signal transduction or downstream effectors. These possibilities were analyzed next. Clustering of Fcγ receptors upon interaction with IgG-opsonized particles leads to the activation of tyrosine kinases and to accumulation of F-actin around nascent phagosomes (18, 28, 29). The occurrence of these steps was tested in COP-I-deficient cells. As shown in Fig. 5, A and B, accumulation of tyrosine-phosphorylated proteins was detected at sites of particle attachment to Fcγ-ldl cells pretreated at 34 °C (not shown) and 39 °C. Moreover, activation of downstream effectors was also readily observable. Accumulation of F-actin in the phagosomal cup, which is evident in the phagocytosis-competent Fcγ-ldl cells kept at 34 °C (Fig. 5D), was in fact more pronounced in cells shifted to 39 °C (Fig. 5E). In fact, scanning EM revealed that SRBC were often found tightly attached to “pedestals” that protruded from the eCOP-depleted Fcγ-ldl cells (Fig. 5C). F-actin was concentrated at the junction between the opsonized particles and these pedestals (Fig. 5E).

We also investigated whether increase in cytoplasmic free Ca2+ concentration ([Ca2+]i) was normal in COP-I-deficient cells. An increase in [Ca2+]i, mediated by activation of phospholipase C and release of Ca2+ from intracellular stores is one of the earliest consequences of Fc receptor ligation (30, 31). Although not essential for phagocytosis, this [Ca2+]i transient is invariably associated with receptor clustering and activation in professional phagocytes and nonprofessional engineered phagocytes (32, 33). In Fcγ-ldl cells maintained at 34 °C, [Ca2+]i spikes were similarly triggered by binding of IgG-opsonized particles (Fig. 5F, circles). Such spikes were absent in Iδ cells lacking Fc receptors (Fig. 5F, squares). Importantly, [Ca2+]i, transients were elicited by particles in Fcγ-ldl cells pretreated at 39 °C (Fig. 5G). Similar observations were obtained with Fcγ-CHO cells preincubated at both temperatures (not illustrated). In contrast, [Ca2+]i transients were not observed upon the addition of opsonized particles to the parental wild-type CHO (not shown) and IδIF cells (Fig. 5F, circles), which do not express FcγRIIA. These findings imply that COP-I is not essential for this early response and confirm the notion that incubation at 39 °C does not have generalized detrimental effects on cells.

**Effect of eCOP Depletion on Integrin Expression**—Integrins are important in cell spreading (34) and have been reported to act synergistically with Fc receptors to promote phagocytosis (35–37). Since COP-I-deficient cells were morphologically altered (i.e. rounded), we hypothesized that integrin function was compromised in these cells. We therefore assessed the presence and distribution of β1-integrins. We found that focal contacts were formed in Fcγ-ldl cells incubated at both 34 and 39 °C (Fig. 6, A and C). Actin stress fibers were often seen to abut these focal adhesions under both conditions, although the length of the stress fibers was reduced in eCOP-deficient cells (Fig. 6, B and D). The total content of β1-integrins in unstimulated Fcγ-ldl cells was compared at 34 or 39 °C using flow cytometry of suspended cells and also by quantitative fluorescence microscopy of adherent cells. No significant difference was found using the former method, and only a small decline was observed microscopically (Fig. 6E). Similarly, the F-actin content was unaffected by incubation at 39 °C.

**Ultrastructural Analysis of Phagocytosis in COP-I-containing or -depleted Cells**—We next used transmission EM to compare the ultrastructure of normal and COP-I-deficient cells during the course of phagocytosis. Fcγ-ldl cells preincubated at either 34 or 39 °C were exposed briefly to opsonized SRBC, fixed, and processed for EM. As described earlier for both professional (38) and engineered phagocytes (18), the early stages of phagocytosis in cells with functional COP-I are characterized by extension of elaborate pseudopods that surround and ultimately engulf the particles (Fig. 7, A and B). In these cells, SRBC located well within the cytosol (often near the nucleus; Fig. 7B) were seen frequently, probably representing complete

![Fig. 5. Effect of eCOP depletion on early signaling events.](image-url)
orometry. Data are means ± S.E. of at least three experiments each.

34°C

39°C

Fig. 6. Effect of COPI depletion on integrins. FcR-Idl cells maintained at 34 °C (A and B) or at 39 °C for 18 h (C and D) were stained for β1-integrin (A and C) and for actin (B and D). Focal adhesion complexes that co-localize with actin fibers are indicated with arrows. E, β1-integrins (black and striped bars) and F-actin (white bars) were quantified in FcR-Idl cells maintained at the permissive (34 °C) and restrictive (39 °C) temperatures. β1-Integrin content was measured by fluorescence-activated cell sorting analysis of suspended cells (black bars) or by quantitative confocal microscopy of adherent cells (striped bars) and F-actin stained with rhodamine-phalloidin by plate microfluorometry. Data are means ± S.E. of at least three experiments each.

VAMP3, which dictates its intracellular targeting, while the GFP module is in the lumen of endosomes and appears on the exofacial side of the plasma membrane upon exocytosis. As a result, the occurrence of exocytosis of VAMP3-containing vesicles can be detected by staining intact (nonpermeabilized) cells with anti-GFP antibodies as described (9).

VAMP3-GFP was found both in endomembrane vesicles and on the surface of RAW cells performing phagocytosis (Fig. 8, B–D). Importantly, as reported in FcR-CHO cells (8), VAMP3-GFP accumulated in the membrane of nascent phagosomes (Fig. 8C). The accumulated VAMP3-GFP was inserted on the plasma membrane and not simply concentrated in vesicular form below the surface. This was confirmed by the observation that the phagosomal VAMP3-GFP was accessible to anti-GFP antibodies in intact cells (Fig. 8D). In contrast, VAMP3-GFP was often intracellular in cells treated with BFA, distributed in tubules or cisternae (Fig. 8E). Importantly, little accumulation was generally observed at the sites of aborted phagocytosis where particles attached (Fig. 8, F and G). These results suggest that COPI deficiency precludes the mobilization of a VAMP3-expressing endomembrane pool necessary for pseudopod extension during phagocytosis.

DISCUSSION

The central observation described in this paper is that phagocytosis was impaired when COPI function was inhibited. The requirement for COPI was documented by two independent approaches: the use of a mutant cell line expressing a thermo-labile form of COPI and the inhibition of ARF with BFA. It is noteworthy that within the time frame studied, COPI inactivation did not produce nonspecific deleterious effects on cell viability or function. Thus, COPI-deficient cells accumulated Tfn, had seemingly normal mitochondrial potential, and synthesis of proteins at nearly normal rates, and the morphology of the mitochondria, ER, and peroxisomes was unaltered. More importantly, the cells retained the ability to bind SRBC, triggered cytosolic calcium changes, and induced the focal assembly of F-actin at sites of frustrated phagocytosis. We believe,
ARF function rapidly, and almost 50% of the phagocytic rate factors. Near normal phagocytosis was seen for up to 2 h after visible at normal laser exposure (and zymosan particles, which have modest background fluorescence not intensified image of the main panel

The inhibition of phagocytosis lagged behind the disappearance of eCOP or the inactivation of ARF nucleotide exchange factors. Near normal phagocytosis was seen for up to 2 h after the addition of BFA, which enters the cells and interferes with ARF function rapidly, and almost 50% of the phagocytic rate remained immediately after eCOP was virtually eliminated. For these reasons, we felt that COPI was necessary for the maintenance and/or localization of a critical pool of elements of the phagocytic machinery, instead of playing a direct role in pseudopod extension. Last, as in the case of wortmannin depletion, and focal adhesions were clearly visible in adherent cells grown at 39 °C (Fig. 6, A and C). Actin stress fibers were seen to abut these focal adhesions, and the total content of F-actin of unstimulated FcR-ldl cells was essentially identical when phosphatidylinositol-3'-kinase was inhibited. In the latter, impairment of phospholipase A2 also limited the extension of pseudopods and in addition promoted the accumulation of small electron lucent vesicles underneath the sites of particle attachment. In both cases, it was suggested that failure to perform exocytosis was responsible for the inability of the cells to extend pseudopods. In accordance with this interpretation, there is mounting evidence that secretion of endomembranes is involved in pseudopod extension (see Introduction). It is therefore conceivable that delivery of vesicles required for pseudopod elongation is impaired by neutralization of COPI and ARFs.

In summary, our findings and those of others suggest that COPI and ARF are important for the homeostasis of the endocytic recycling compartment. Recycling endosomes, in turn, seem to provide at least part of the membrane required for pseudopod extension. Last, as in the case of wortmannin-treated cells, our observations dissociate the polymerization of actin from the extension of pseudopods during phagocytosis, suggesting that multiple events are required for successful pseudopodial elongation.

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