Role of COPI in Phagosome Maturation*

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Phagosomes mature by sequentially fusing with endosomes and lysosomes. Vesicle budding is presumed to occur concomitantly, mediating the retrieval of plasmalemmal components and the regulation of phagosomal size. We analyzed whether fission of vesicles from phagosomes requires COPI, a multimeric complex known to be involved in budding from the Golgi and endosomes. The role of COPI was studied using IdIF cells, that harbor a temperature-sensitive mutation in e-COP, a subunit of the coatamer complex. These cells were made phagocytic toward IgG-opsonized particles by heterologous expression of human FcγRIIA receptors. Following incubation at the restrictive temperature, e-COP was degraded in these cells and their Golgi complex dispersed. Nevertheless, phagocytosis persisted for hours in cells devoid of e-COP. Retrieval of transferrin receptors from phagosomes became inefficient in the absence of e-COP, while clearance of the FcγRIIA receptors was unaffected. This indicates that fission of vesicles from the phagosomal membrane involves at least two mechanisms, one of which requires intact COPI. Traffic of fluid-phase markers and aggregated IgG-receptor complexes along the endocytic pathway was abnormal in e-COP-deficient cells. In contrast, phagosome fusion with endosomes and lysosomes was unimpaired. Moreover, the resulting phagolysosomes were highly acidic. Similar results were obtained in RAW264.7 macrophages treated with brefeldin A, which precludes COPI assembly by interfering with the activation of adenosine ribosylation factor. These data indicate that neither phagosome formation nor maturation are absolutely dependent on COPI. Our findings imply that phagosome maturation differs from endosomal progression, which appears to be more dependent on COPI-mediated formation of carrier vesicles.

Phagocytosis plays a key role in the host immune defense by sequestering invading microorganisms within vacuoles formed by invagination of the plasma membrane of neutrophils and macrophages (1–3). Such vacuoles, known as phagosomes, undergo sequential fusion with early and late endosomes and ultimately with lysosomes (4–8). Budding of vesicles from the phagosome is thought to occur in parallel with fusion (9–12), thereby maintaining the surface area of the phagosome approximately constant. Jointly, vesicular fusion and fission lead to remodeling of the phagosomal membrane and contents, a process known as phagosomal maturation (6). The final stage of this sequence is the phagolysosome, a highly acidic organelle, rich in hydrolases, where the internalized microorganisms are killed and degraded. The importance of phagosomal maturation is highlighted by the ability of some intracellular pathogens to arrest this process (13, 14). By interfering with normal maturation, several microorganisms like Mycobacterium species, are capable of surviving for extended periods within immature phagosomes of macrophages (15–18).

During the course of maturation, phagosomes progressively acquire a variety of proteins that are characteristic of endosomes and lysosomes (4, 9, 19–21). One of the protein complexes inserted into the phagosomal membrane through fusion with endomembranes, the vacuolar-type ATPase, mediates the acidification of the phagosomal lumen (22–24). Conversely, surface proteins that were internalized during the invagination of the plasma membrane are gradually removed from the phagosome as it matures (9, 25). At least some of these proteins appear to be recycled back to the plasma membrane (6, 10, 26, 27). In this regard, the phagosome has been shown to be a complete antigen processing compartment, so that MHC-II-antigen complexes formed therein appear on the plasma membrane (28).

Despite the importance of vesicular budding in the salvage of plasmalemmal components, antigen presentation and the maintenance of phagosomal size, little is known about the underlying mechanisms. In other organelles, such as the Golgi complex, several fission systems have been characterized, including the clathrin, COPI, and COPII complexes. Clathrin has been found to associate with the nascent phagosome, although its precise role in maturation remains unclear (9, 29). Amorphous coats, distinct from those generated by clathrin, have also been found on phagosomes (6). These may be related to the COP systems of other endomembranes. Of particular interest are recent findings indicating that normal delivery of cargo from early to late endosomes and lysosomes requires COPI. This was inferred from the observation that microinjection of antibodies to the β-COP subunit of COPI precluded early to late endosomal traffic (30). A similar conclusion was reached using the IdIF Chinese hamster ovary (CHO)1 cell line, which have a temperature-sensitive mutation in e-COP, a subunit of

1 The abbreviations used are: CHO, Chinese hamster ovary; ARF, adenosine ribosylation factor; DIC, differential interference contrast; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; LAMP, lysosome-associated membrane protein; RBC, red blood cells; Tf, transferrin; TR, transferrin receptor; V-ATPase, vacuolar-type H+-pumping ATPase; PAGE, polyacrylamide gel electrophoresis.

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the COPI complex (31–35). Like wild-type CHO cells, IdIF cells are normally infected by vesicular stomatitis virus at the permissive temperature. At the restrictive temperature e-COP becomes unstable and is rapidly degraded. Under these conditions viral infection is completely arrested, indicating the failure of the virus to reach the acidic late endosomes (35). In addition, progression of fluid-phase markers like horseradish peroxidase into the late endocytic compartment was at least partially blocked (33, 35), as was the case for complexes formed between epidermal growth factor and its receptor (35). These results imply that COPI is essential for endosomal progression and are consistent with the reported effects of brefeldin A on endosomal function and morphology (36–38). Brefeldin A is a specific inhibitor of GTP exchange factors of the adenosine ribosylation factor (ARF) family of GTPases (39) which are essential for COPI function (40, 41).

The involvement of COPI in the endocytic pathway is consistent with both the “endosome maturation” and “vesicle shuttle” models, two competing hypotheses offered to explain how internalized molecules progress along the endocytic pathway. The vesicle shuttle model proposes that endosomes are stable, long-lived organelles that deliver cargo to the downstream compartment via vesicular intermediates (42–45). In contrast, the maturation model envisions the gradual modification of endocytic compartments by a series of fusion and fission events (19, 21, 42, 46–48). While endocytic progression is a subject of ongoing debate, there is little disagreement that phagosomes mature (6). Regardless of the uncertainties regarding endosomal progression, there is a striking resemblance between the endocytic and phagocytic pathways. In both cases, vesicles/vacuoles detach from the plasma membrane and acquire sequentially Rab5 and Rab7, followed by lysosome-associated membrane proteins (or LAMPS) and lysosomal enzymes such as cathepsin (4, 19, 21, 42, 46, 47, 49, 50). Moreover, both systems undergo a gradual acidification, suggestive of progressive insertion of V-ATPases (23, 24, 42, 51, 52). Lastly, as in the case of endosomes, both ARF and the β-COP subunit of COPI were reported to associate with phagosomal membranes in vitro (6, 30, 53).

The similarity between these processes, together with the established role of COPI in endosomal progression, prompted us to analyze the role of COPI in phagosomal maturation. Two approaches were used: (i) ARF exchange factors were inhibited in RAW264.7 macrophages using brefeldin A and (ii) the e-COP subunit of COPI was eliminated using the temperature-sensitive mutant IdIF cell line. The latter was derived from CHO RIIA receptor.

To label the early endocytic compartment, FCeRII CHO or FcR-ldl cells were incubated at 37 °C while IdIF and FCeRIIIdl were maintained at 4°C. The COPI complex (31–35). Like wild-type CHO cells, IdIF cells are normally infected by vesicular stomatitis virus at the permissive temperature. At the restrictive temperature e-COP becomes unstable and is rapidly degraded. Under these conditions viral infection is completely arrested, indicating the failure of the virus to reach the acidic late endosomes (35). In addition, progression of fluid-phase markers like horseradish peroxidase into the late endocytic compartment was at least partially blocked (33, 35), as was the case for complexes formed between epidermal growth factor and its receptor (35). These results imply that COPI is essential for endosomal progression and are consistent with the reported effects of brefeldin A on endosomal function and morphology (36–38). Brefeldin A is a specific inhibitor of GTP exchange factors of the adenosine ribosylation factor (ARF) family of GTPases (39) which are essential for COPI function (40, 41).

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was used instead when simultaneous FITC-dextran labeling and immunostaining for hamster LAMP1 was necessary. Murine LAMP1 was detected with rat anti-LAMP1 at 1:2 dilution after paraformaldehyde fixation and methanol permeabilization. FcγRIIA, the Golgi apparatus, and TIR were labeled with monoclonal antibodies to FcγRIIA (1:200), giantin (1:1000), or TIR (1:250), respectively, after fixation with 4% paraformaldehyde and permeabilization with 0.1% Triton. Fluorochrome-conjugated secondary anti-human, anti-mouse, anti-rat, and anti-rabbit were all used at 1:1000. Samples were analyzed with a Leica fluorescence microscope (model DMIRB) with a ×100 oil immersion objective and the appropriate filter set. Cells and phagosomes were clearly identifiable under DIC optics. Images were digitally acquired with a cooled charge-coupled device camera controlled by the Winview software (Princeton Instruments, Trenton, NJ). Confocal microscopy was performed with a Zeiss LSM 510 laser scanning confocal microscope.

Ratiometric Fluorescence Microscopy—For measurement of intracellular pH, sorting and recycling endosomes were labeled with FITC-Tf as described above and were identified by their peripheral and juxtanuclear localization, respectively. To measure the lysosomal pH, cells were labeled with 1 mg/ml Oregon Green-dextran M, 10,000 for 1 h, chased for 4 h, and subsequently incubated at the indicated temperature overnight before imaging. Phagosomal pH was measured by covalently labeling zymosan with pH-sensitive probes, as detailed above.

Resting pH values were obtained in cells bathed in sodium-rich medium (140 mM NaCl, 5 mM glucose, 15 mM Hepes, pH 7.4) at 37 °C. Calibration of fluorescence versus pH was obtained by substituting the sodium-rich buffer with potassium-rich medium (140 mM KCl, 5 mM glucose, 15 mM Hepes, adjusted to the desired pH with KOH, followed by addition of the cation/H+ ionophores monensin (2 μM) and nigericin (5 μM). Internalized zymosan particles were identified by their insensitivity to abrupt changes in extracellular pH and by their responsiveness to the addition of 10 mM NH4Cl (see Ref. 60 for details). The microscope and software set up used for ratio imaging have been described in detail elsewhere (60, 61).

RESULTS

ldlF Cells Expressing the Human FcγRIIA Receptor Are Phagocytic—The interaction of β-COP and ARF with phagosomal membranes in vitro (6) and the role played by COPI in the progression of the endocytic pathway (30, 33, 35, 53) were suggestive of a role of COPI in vesicular budding during phagosome maturation. We used two different approaches to analyze the involvement of COPI in phagosomal maturation: (i) inhibition of ARF nucleotide exchange factors using brefeldin A in the professional phagocyte cell line RAW264.7 and (ii) elimination of β-COP in the temperature-sensitive mutant ldlF cells.

Because ldlF cells are not normally phagocytic, they were transfected with the human FcγRIIA receptor gene and stable clones were selected. The selected clones, termed FcR-ldl, expressed the FcγRIIA receptor on their plasma membrane (Fig. 1A) and were capable of binding IgG-opsonized RBC (Fig. 1B). More importantly, FcR-ldl cells were able to internalize opsonized RBC (Fig. 1C), as described earlier for FcγRIIA-transfected wild-type CHO cells (56). In fact, the phagocytic efficiency of the FcR-ldl clone selected for the subsequent experiments was greater than reported for FcγRIIA-transfected CHO cells (>50 versus <30%). Phagocytosis of RBC by ldlF cells was strictly dependent on opsonization of the particles with IgG and occurred only in cells transfected with FcγRIIA receptors (not illustrated).

FcR-ldl Cells Degrade β-COP at the Restrictive Temperature—We next examined whether FcR-ldl cells retained the temperature-sensitive mutation in β-COP. The effect of progressively longer incubations at the restrictive temperature (39 °C) on the β-COP content of FcR-ldl cells was analyzed by immunoblotting. To ensure comparable loading, the samples were also probed for tubulin, an abundant protein that should be unaffected by the ldlF mutation. For comparison, wild-type CHO cells transfected with FcγRIIA (named hereafter FcR-CHO) were also incubated at 39 °C for identical periods. When maintained at the permissive temperature (34 °C) the β-COP content of FcR-ldl cells is about 3–4-fold lower than that of FcR-CHO cells (not shown). In addition, as shown in Fig. 2,
e-COP rapidly diminishes when the mutant cells are warmed to 39 °C, while the level of tubulin remains unaffected. By contrast, e-COP in FcR-CHO cells did not diminish after 6 h at the restrictive temperature (leftmost lane in Fig. 2), as reported for wild-type CHO cells (32). After 6 h at 39 °C, the e-COP content of FcR-ldl cells is less than 5% of the content in FcR-ldl cells maintained at the permissive temperature and less than 2% of the content of FcR-CHO cells. These findings confirm that the temperature-sensitive mutation of the parental ldlF cell line was preserved in our FcR-ldl clonal line. All subsequent experiments were performed in cells incubated at 39 °C for 8 h.

In ldlF cells, the loss of e-COP alters the function of the COPI

Fig. 3. Golgi and endosome functions are disrupted in COPI-deficient FcR-ldl cells. A-B, FcR-ldl cells treated at 34 °C (A) or 39 °C (B) were immunostained with a monoclonal anti-giantin antibody. In COPI-containing cells (A) the Golgi was seen as a dense juxtanuclear cluster, likely a stack of cisternae. In COPI-deficient cells (B), the Golgi was dispersed and vesiculated. C and D, endosome function in FcR-ldl cells maintained at 34 °C. The cells were labeled with 250 μg/ml of lysine-fixable FITC-dextran for 30 min and chased for 4 h. Cells were then fixed, permeabilized with methanol, and immunostained for LAMP1. C, distribution of LAMP1. D, distribution of FITC-dextran. E and F, endosome function in cells lacking e-COP. FcR-ldl cells were shifted to 39 °C for 8 h and treated as described for C and D. E, LAMP1; F, FITC-dextran. Insets show images of cells acquired immediately after the dextran pulse, demonstrating that pinocytosis of dextran persisted in the absence of COPI. Scale bar: 10 μm. Representative of three similar experiments.

Fig. 4. e-COP deficiency alters the subcellular distribution of aggregated IgG. FcR-ldl cells were preincubated at 34 °C (A and B) or at 39 °C (C and D) and then allowed to internalize aggregated human IgG for 30 min, followed by a 2-h chase. After fixation, cells were immunostained for LAMP1 (A and C) and treated with FITC-conjugated anti-human IgG antibodies to reveal the location of the aggregates (B and D). Representative of two experiments.
complex, as judged by alteration of the Golgi morphology and by disruption of endosome function (31, 33, 35). We tested whether depletion of $\varepsilon$-COP had similar functional consequences in FcR-ldl cells. Immunofluorescence was used to analyze the distribution of giantin, a Golgi resident protein. FcR-ldl cells maintained at 34 °C displayed a typical Golgi morphology, consisting of tightly packed juxtanuclear cisternae (Fig. 3A). Following incubation for 8 h at the restrictive temperature, the Golgi marker became dispersed and vesiculated (Fig. 3B), consistent with inactivation of COPI upon loss of $\varepsilon$-COP (31).

Normal progression of endocytic markers from sorting to late endosomes has also been shown to depend on COPI. Thus, infection of cells with vesicular stomatitis virus, which requires entry into acidic late endosomes, was inhibited by microinjection of inhibitory antibodies to $\beta$-COP (30) and was also impaired in ldlF cells incubated at restrictive temperatures (35). We tested the traffic along the endocytic pathway of FcR-ldl cells by monitoring the fate of fixable FITC-dextran internalized by fluid phase uptake. FcR-ldl cells maintained at 34 °C or preincubated for 8 h at 39 °C were allowed to take up the dextran for 30 min and then chased for 4 h. Cells were then fixed and immunostained for LAMP1. The distribution of LAMP1 (C and E) and FITC-dextran (D and F) was visualized by confocal microscopy. C and D, control cells. E and F, brefeldin-treated cells. Representative of three experiments.

Traffic of Immune Complexes along the Endocytic Pathway Is Abnormal in the Absence of $\varepsilon$-COP—Fc$\gamma$ receptors bound to IgG immune complexes were shown to be internalized and targeted for degradation in late endosomes and lysosomes (62, 63). This is an essential step for antigen processing and presentation. However, the mechanism by which immune complexes travel along the endocytic pathway is not known. We examined whether COPI is involved in this process using FcR-ldl cells. FcR-ldl cells treated at the permissive or restrictive temperatures were allowed to take up aggregated IgG and were subsequently chased and stained for LAMP1. As expected, aggregated IgG overlapped extensively with LAMP1-positive organelles in cells expressing functional COPI (Fig. 4, A and B). These structures were usually small, punctate, and numerous. In the absence of $\varepsilon$-COP, IgG complexes were also internalized and found to accumulate in LAMP1-positive organelles (Fig. 4, C and D). However, significant differences were noted. First, the number of IgG-positive organelles was considerably lower in COPI-deficient cells. Second, these structures often appeared to be larger and mainly perinuclear (cf. Fig. 4, D and B). As a result, only a subset of LAMP1-containing organelles is accessible to the Fc receptor-ligand complex in cells lacking $\varepsilon$-COP. We
conclude that COPI plays a role in the normal traffic of immune complexes along the endocytic pathway.

**Brefeldin A Disrupts the Golgi and Impairs Endosome Function in RAW264.7 Macrophages**—To study the role of COPI in phagosome maturation in cells of myeloid origin that are intrinsically phagocytic, we used brefeldin A. Brefeldin A is a fungal metabolite widely used to disrupt COPI function. It associates with and blocks the activity of nucleotide exchange factors that regulate ARF (39, 40). Because brefeldin A is not universally effective in all cell types (36, 64) we tested its effectiveness in RAW264.7 cells.

As above, the morphology of the Golgi complex in untreated RAW264.7 cells was compact and juxtanuclear (Fig. 5A). Following treatment with brefeldin, however, the Golgi marker giantin became dispersed and punctate (Fig. 5B). Next, endosome function was examined in RAW264.7 macrophages treated with brefeldin A. As observed for FcR-ldl cells, FITC-dextran efficiently accumulated in LAMP1-positive organelles (Fig. 5, C and D). However, treatment with brefeldin significantly reduced, but did not entirely preclude, the accumulation of FITC-dextran in these organelles (Fig. 5, E). Analysis of the cells immediately after the pulse revealed that pinocytic uptake of FITC-dextran was not greatly affected, as described above for FcR-ldl cells. These data are consistent with the inhibition of HRP progression to late endosomes/lysosomes in brefeldin A-treated NRK and rat yolk epithelial cells (37, 51), although discrepant results have been reported in other cell types (38). Our results imply that brefeldin is an effective tool to study the role of COPI in macrophages.

**Phagocytosis in COPI-deficient Cells**—Analysis of the role of COPI in phagosomal maturation requires that particle internalization be preserved in the absence of functional COPI. Zhang et al. (65) described earlier that phagocytosis persisted in RAW264.7 cells treated acutely with brefeldin A (65). We
were able to confirm these observations in RAW264.7 cells incubated with 100 μM brefeldin for 45 min, conditions shown above to disrupt the Golgi and to interfere with endosomal traffic. In addition, we found that FcR-ldl cells that had no detectable e-COP following incubation at 39 °C for 8 h were nevertheless able to internalize IgG-opsonized RBC, although the phagocytic efficiency was only ~50% relative to cells maintained at the permissive temperature. This partial inhibition of phagocytosis was specific to the disappearance of e-COP and was not a deleterious effect of heat shock, since FcR-CHO cells incubated at 34 and 39 °C had similar phagocytic efficiencies. The mechanism underlying the inhibition of phagocytosis in e-COP-deficient cells will be described in detail elsewhere. Nevertheless, the persistence of phagocytosis in the absence of e-COP enabled us to study the role of COPI in phagosomal maturation.

Role of COPI in Removal of TfR from the Phagosomal Membrane—Transferrin receptors are incorporated into the phagosomal membrane during particle ingestion and are also inserted by fusion with early endosomes (15, 56, 66). However, the residence time of TfR in the phagosome is limited, as they are removed by vesicular budding within 10–20 min of phagosome closure (6, 25, 56, 67). The possible role of COPI in such budding was analyzed by immunofluorescence.

Phagocytosis was initiated by exposing e-COP-containing or depleted FcR-ldl cells to IgG-opsonized RBC for 15 min at 34 °C. After this phagocytic pulse, cells were either fixed immediately or chased (i.e., incubated without RBC) for 15 or 30 min at 34 °C and the distribution of TfR determined by immunostaining. As anticipated, TfR were detected on some phagosomes immediately after the phagocytic pulse (Fig. 6, A and B; note that at this early time approximately 60% of the phagosomes had lost or never acquired TfR). However, after a 30-min chase, practically all phagosomes had no detectable TfR (Fig. 6, C and D). A comparison of the rates of disappearance of TfR from the phagosomes is presented in Fig. 6E, which summarizes the results of three experiments, where a total of 450 phagosomes were counted. At all times studied, COPI-expressing FcR-ldl cells had more effectively removed TfR receptors from their phagosomes than their COPI-deficient counterparts. The differences in the number of TfR-positive phagosomes were found to be statistically significant 15, 30, and 45 min after initiation of phagocytosis (i.e., 0, 15, or 30 min of chase, respectively, in Fig. 6). These findings suggest that COPI plays a role in the early stages of phagosomal maturation.

Similar conclusions were drawn when comparing the fate of TfR in control and brefeldin A-treated RAW264.7 macrophages. As illustrated in Fig. 7, A and B, TfR was enriched in phagosomes immediately after phagocytosis was completed. As reported earlier (15, 56, 66), the receptors were rapidly cleared from the phagosomes of otherwise untreated cells (Fig. 7, C and D). As in the case of COPI mutants, the rate of disappearance of TfR receptors from the phagosome was noticeably slower when brefeldin was present (Fig. 7E). Even at the earliest time measured (0 min chase after the 15-min phagocytic pulse) the fraction of TfR-positive phagosomes was significantly greater in brefeldin-treated samples than in controls (p < 0.05). Because only partial inhibition was observed when COPI was genetically ablated or inhibited pharmacologically, we conclude that TfR removal from phagosomes is mediated by at least two distinct mechanisms: one that is COPI-dependent and one or more COPI-independent processes.

FIG. 8. FcγRIIA removal from phagosomes in FcR-ldl cells. FcR-ldl cells were allowed to internalize IgG-opsonized RBC for 15 min. Cells were then fixed immediately, or following a 15- or 30-min chase, and immunostained with a monoclonal anti-human FcγRIIA antibody. A, FcγRIIA staining in cells fixed immediately after the phagocytic pulse. B, corresponding bright field image. C, FcγRIIA staining in cells fixed 60 min after the phagocytic pulse. D, corresponding bright field image. Arrowheads point to RBC. Bar represents 10 μm. E, course of disappearance of FcγRIIA from phagosomes. FcR-ldl cells maintained at 34 °C (solid bars) or 39 °C (open bars) were treated as described above. The cells were analyzed microscopically and phagosomes lined by observable FcγRIIA were defined as FcγRIIA-positive. Results were collected from three different experiments with 150 phagosomes per condition. Data are mean ± S.E. No statistically significant difference between e-COP containing and deficient cells was found at any time (p > 0.05, Student’s t test).

Role of COPI in Removal of Fcγ Receptors from the Phagosomal Membrane—After signaling particle internalization, Fcγ receptors (FcγR) are no longer required on the phagosomal membrane and are eventually cleared (9, 63). Because little is known about the mechanism(s) underlying FcγR removal from phagosomes, we investigated the possible role of COPI in this process. The occurrence of this phenomenon, which has been described in professional phagocytes, was initially verified in FcR-ldl cells grown under permissive conditions. Fig. 8, A-D, shows that, while FcγRIIA receptors are highly concentrated in nascent and early phagosomes, they gradually disappear over time. After a 60-min chase, Fcγ receptors were detectable in only ~40% of the phagosomes. In contrast to the results obtained with TfR, the rate of clearance of Fcγ receptors was unaffected when e-COP was eliminated by preincubating the cells at the restrictive temperature (Fig. 8E). Therefore, FcγRIIA receptors are removed from phagosomes in a COPI-independent manner.

2 D. J. Hackam Botelho, R. J., C. Sjolin, D. D. Rotstein, J. M. Robinson, A. D. Schreiber, and S. Grinstein, manuscript in preparation.
Fusion of Early Endosomes with Phagosomes in the Absence of COPI—Phagosomes acquire proteins which are essential for their microbicidal function, such as cathepsins and V-ATPases, by fusing with endosomes and lysosomes. The sequential fusion steps undergone by phagosomes closely resemble the process of endosomal maturation (42, 46, 50). Because the latter was shown to be arrested by deletion of \( \alpha \)-COP, we tested whether phagosome-early endosome fusion proceeded in the absence of COPI.

We initially used FcR-I\(d\)l cells to assess the role of \( \alpha \)-COP. To monitor the fusion of early endosomes with the phagosome, cells were pre-loaded with rhodamine-Tf for 1 h, followed by a chase period intended to clear any Tf bound to the plasma membrane. The cells were next allowed to internalize opsonized RBC and, after arresting the reaction, the subcellular distribution of Tf was analyzed. As reported for FcR-CHO (56), FcR-I\(d\)l maintained at 34 °C undergo rapid and efficient fusion with Tf-containing endosomes (Fig. 9, A and B). The distribution of rhodamine-Tf on the phagosomal membrane was often uneven, perhaps reflecting asynchrony in the onset of phagocytosis. When FcR-I\(d\)l cells were pretreated at 39 °C to eliminate \( \alpha \)-COP, phagosomes were nevertheless able to acquire Tf, indicating that phagosomal maturation was similarly dependent on COPI.

![Figure 9: Fusion of phagosomes with early endosomes in FcR-I\(d\)l cells.](image)

**A** and **B** represent the Tf distribution in FcR-I\(d\)l cells maintained at 34 °C and 39 °C, respectively. **C** and **D** are corresponding DIC images. Bar represents 10 μm. Representative of three experiments.

We concluded that COPI was not essential for fusion between phagosomes and endosomes/lysosomes was confirmed in RAW264.7 cells using brefeldin. Pretreatment of the cells with this inhibitor had no effect on the extent of LAMP1 fusion with phagosomes (not illustrated). We conclude that COPI is not required for the intermediate stages of phagosomal maturation.

Acidification of COPI-deficient Phagosomes and Endosomes—Acquisition of LAMP1 is not a definitive indicator of the completion of phagosomal maturation. Thus, phagosomes containing live mycobacteria do not fuse with lysosomes nor do they acidify fully, yet acquire LAMP1 (15, 16, 61). To ensure that COPI-deficient phagosomes are formed in the absence of COPI, we measured phagosomal pH in FcR-I\(d\)l cells. Following incubation at either the permissive or restrictive temperature, FcR-I\(d\)l cells were allowed to internalize zymosan particles covalently labeled with pH-sensitive fluorescent probes and the phagosomal pH was measured by ratio microfluorimetry. As described earlier, internalized zymosan particles were identified by their responsiveness to addition of NH\(_4\)Cl and by their insensitivity to abrupt changes in extracellular pH (not shown). In cells with functional COPI, the phagosomal pH averaged 4.2 ± 0.2 (mean ± S.E. of 23 determinations; Table I). Follow-
TABLE I
Luminal pH of peripheral and juxtanuclear endosomes, lysosomes, and phagosomes

|       | PE  | JE  | Ly  | Ph  |
|-------|-----|-----|-----|-----|
| 34°C  | 6.2±0.2 | 6.6±0.2 | 4.9±0.4 | 4.2±0.2 |
| 39°C  | 6.4±0.2 | 6.7±0.1 | 4.7±0.2 | 4.5±0.2 |

The following abbreviations are used in the table: PE and JE, peripheral and juxtanuclear endosomes; Ly, lysosomes; Ph, phagosomes. The data presented are the mean ± S.E. of n ≥ 12 experiments. For each experiment, the pH of multiple organelles in one or more cells was measured and averaged.

To our knowledge, only e-COP is directly affected by the shift to the restrictive temperature in these cells. However, the comparatively long time required for the total disappearance of e-COP (>6 h) may have secondary consequences. Indeed, the efficiency of phagocytosis was significantly decreased after 6–8 h at 39°C in FcR-ldl, but not in wild-type FcR-CHO cells (not illustrated). However, the remaining phagocytosis (∼50% of the control) sufficed for analysis of maturation. Last, although phagosomal formation and maturation in the FcR-transfected ldIF cells seem to parallel the events reported in professional phagocytes, we cannot a priori disregard the possibility that different budding mechanisms were involved in professional and engineered phagocytes.

Despite the individual limitations of the two experimental models, the results obtained regarding the role of COPI in phagosomal maturation are internally consistent. First, we confirmed earlier findings that, at the time points studied,

FIG. 10. Fusion of phagosomes with late endosomes and/or lysosomes in FcR-ldl cells. FcR-ldl cells were allowed to internalize IgG-opsonized RBC for 1 h, treated hypotonically to remove external RBC and fixed. The cells were then immunostained with monoclonal anti-hamster LAMP1 antibody. A, LAMP1 staining in cells maintained at 34°C. B, corresponding DIC image. C, LAMP1 staining in cells treated at 39°C degrade e-COP. D, corresponding DIC image. Bar = 10 μm. E, course of LAMP1 acquisition by phagosomes. FcR-ldl cells maintained at 34°C (solid bars) or 39°C (open bars) were allowed to internalize RBC for 15 min and either fixed immediately or chased for the indicated times. The cells were analyzed microscopically and phagosomes lined by observable LAMP1 were defined as LAMP1-positive. Results were collected from three different experiments with 100 phagosomes per condition. Data are mean ± S.E. No statistically significant difference between e-COP containing and deficient cells was found at any time (p > 0.05, Student’s t test).
COPI and Phagosome Maturation

COPI is not essential for particle internalization (65). Second, unlike endosomal progression, we observed that phagosomal maturation proceeded to completion in the absence of e-COP and in the presence of brefeldin. It is noteworthy that endosomal maturation is not equally sensitive to brefeldin in all systems tested (cf. Refs. 37 and 38) and that progression of viruses or molecules internalized via fluid-phase or receptor-mediated endocytosis is not equally affected by deletion of e-COP (35). The alternate pathways that underlie these differences may play a dominant role in phagosome maturation.

While phagosome maturation proceeds to completion in the absence of functional COPI, as judged by the acquisition of LAMP1 and by the attainment of a very acidic pH, the traffic of some components is altered. Specifically, both experimental systems indicated that budding of TIR from the phagosome is affected by impairment of COPI function. It is important to note, however, that only partial inhibition of TIR recycling was observed in e-COP-deficient or brefeldin-treated cells, implying that at least one other COPI-independent pathway can be used for membrane budding off phagosomes. The possible role of clathrin-dependent vesicle formation in phagosome maturation is currently under investigation. Alternatively, it is conceivable that protein transfer between phagosomes and the endocytic compartment may occur by "kiss-and-run" (4). In this model, phagosomes and endocytic vacuoles do not coalesce into one organelle. Rather, membranes and luminal content are exchanged between the two compartments by a momentary fusion followed by fission.

Not all receptors are cleared from the phagosome via identical pathways. FcyRIIA remains on the phagosomal membranes long after TIR have pinched off and, more importantly, the gradual disappearance of Fc gamma RIIC is unaffected by inactivation of COPI. The fate of phagosomal Fc receptors remains unclear: they may be recycled to the plasma membrane or transferred to lysosomes, as found for Fc receptors cross-linked with specific antibodies (62, 63), or they may be degraded into functionally intact proteins (64).

References

1. Kwiatkowska, K., and Sobota, A. (1999) Biosci. Rep. 19, 221–231
2. Silverstein, S. C., Greenberg, S., Di Virgilio, F., and Steinberg, T. H. (1989) in Fundamental Immunology (Paul, W. E., ed) pp. 703–719, Raven Press, Ltd., New York
Grinstein, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11691–11696
60. Hackam, D. J., Rotstein, O. D., Zhang, W., Gruenheid, S., Gros, P., and Grinstein, S. (1998) J. Exp. Med. 188, 351–364
61. Hackam, D. J., Rotstein, O. D., Zhang, W. J., Demaurex, N., Woodside, M., Tsai, O., and Grinstein, S. (1997) J. Biol. Chem. 272, 29810–29820
62. Mellman, I., and Plutner, H. (1984) J. Cell Biol. 96, 1170–1177
63. Mellman, I. S., Plutner, H., Steinman, R. M., Unkeless, J. C., and Cohn, Z. A. (1983) J. Cell Biol. 96, 887–895
64. Kitakas, N. T., Roth, M. G., and Bloom, G. S. (1991) J. Cell Biol. 113, 1009–1023
65. Zhang, Q., Cox, D., Tseng, C. C., Donaldson, J. G., and Greenberg, S. (1998) J. Biol. Chem. 273, 19977–19981
66. Alvarez-Dominguez, C., Roberts, R., and Stahl, P. D. (1997) J. Cell Sci. 110, 731–743
67. Muller, W. A., Steinman, R. M., and Cohn, Z. A. (1980) J. Cell Biol. 86, 292–303
68. Moss, J., and Vaughan, M. (1998) J. Biol. Chem. 273, 21431–21434
69. Ooi, C. E., Dell’Angelica, E. C., and Bonifacino, J. S. (1998) J. Cell Biol. 142, 391–402
70. Dell’Angelica, E. C., Mullins, C., and Bonifacino, J. S. (1999) J. Biol. Chem. 274, 7276–7285
71. Hirst, J., Bright, N. A., Rous, B., and Robinson, M. S. (1999) Mol. Biol. Cell 10, 2787–2802