Particle internalization in macrophages is followed by a complex maturation process. We have previously observed that proteins bound to phagocytosed particles are sorted from phagosomes into a heterogeneous population of vesicles that fuse with endosomes. However, the mechanism and the protein machinery involved in the formation of these phagosome-derived vesicles are largely unknown. It has been shown that vesicles coated with coat protein complex type I (COPI) have a role in both secretion and endocytosis. To address the possibility that COPI proteins might participate in the formation of phagosome-derived vesicles we studied the recruitment of β-COP to highly purified phagosomes. The binding of β-COP to phagosome membranes was regulated by nucleotides and inhibited by brefeldin A (BFA).

An ADP-ribosylation factor 1 (ARF1) mutant defective in GTP hydrolysis supported the binding of β-COP to phagosomes independently of added nucleotide. AlF₃ and Gβγ subunits, agents known to modulate heterotrimeric G-protein activity, were tested in the β-COP binding assay. AlF₃ increased β-COP association, whereas binding was inhibited by the addition of Gβγ subunits. Our results suggest that COP proteins are recruited to phagosomal membranes by a mechanism that involves heterotrimeric GTP-binding proteins and a BFA-sensitive ARF. In addition, our findings indicate that COPI proteins are involved in the recycling of components from phagosomes to the cell surface.

Key words: phagocytosis, phagosomes, recycling, vesicular transport.

INTRODUCTION

Phagocytosis is responsible for the internalization of microorganisms, damaged cells or inert particles [1,2]. During this process, phagosomes actively exchange material with other intracellular compartments, mainly from the endocytic pathway, finally acquiring hydrolytic enzymes that eventually degrade the internalized particle. The mechanisms responsible for recycling from the phagosomal compartment are still largely unknown. However, they seem to involve the formation of tubules and transport vesicles that selectively include molecules to be recycled back to the plasma membrane [3].

Transport vesicles forming from a given compartment are known to be coated by specific sets of peripheral membrane-associated proteins such as clathrin, adaptor proteins (reviewed in [4]) and coat protein complex type I (COPI) and COPII [5,6]. Both COPI and COPII are involved in transport through the biosynthetic pathway. These coats participate in anterograde and retrograde transport between the endoplasmic reticulum and the Golgi apparatus [7,8]. COPI is a hetero-oligomeric complex composed of seven subunits (α, β, β', γ, δ, ε and ζ) that is recruited to the membrane after the activation of the small GTPase ADP-ribosylation factor 1 (ARF1) (reviewed in [9]). Binding of ARF to the membranes depends on GDP–GTP exchange on the protein. The drug brefeldin A (BFA) inhibits the ARF exchange factor [10–14], blocking the assembly of COPI and altering normal Golgi transport [15].

Biochemical and immunocytochemical evidence has implicated both clathrin and coatamer coats in endocytic sorting and vesicle budding [16]. It has been shown that one of the subunits of the COPI complex, β-COP, is present on endosomes and is required for the formation of vesicles that mediate transport to late endosomes [17,18]. Microinjection of antibodies against β-COP blocks infection by vesicular stomatitis virus, a virus internalized via the endocytic pathway [17]. In addition, a mutant Chinese hamster ovary (CHO) cell line with a temperature-sensitive defect in α-COP has a defect in the sorting and recycling functions of endosomes [19,20]. There is also evidence that ARF is involved in the endocytic pathway. Studies in vitro have indicated that an ARF protein regulates endosome fusion [21,22]. Additionally, overexpression of an ARF6 mutant defective in GTP binding altered transferrin recycling and causes the accumulation of non-clathrin-coated structures on peripheral tubes [23,24]. Moreover, BFA, a drug that inhibits the Golgi-associated ARF1 exchange factor [10–12], changes the morphology of endosomes [15]. Indeed, it was shown recently that ARF1 regulates the binding of COP proteins to endosomes [25].

Given the similarities between the phagocytic and endocytic pathways, it is very likely that related COP-coated vesicles also participate in transport from the phagosomal compartment. To address the possibility that COPI-proteins might participate in the formation of phagosome-derived vesicles, we studied the recruitment of β-COP and β'-COP to highly purified phagosomes. The binding of β-COP to phagosomes was regulated by monomeric and heterotrimeric GTPases. The binding was sensitive to BFA.

With the use of a recycling assay to study the efflux of phagosomal components into the extracellular medium, we observed that recycling from phagosomes was partly inhibited in cells treated with BFA. Our findings indicate that COPI is involved in the recycling of components from the phagocytosis compartment.
EXPERIMENTAL

Biological materials

Clone J774-E, a mannose-receptor-positive macrophage cell line, was grown at 37 °C in minimal essential medium containing Earle's salts and supplemented with 10% (v/v) fetal bovine serum. Macrophage cytosol aliquots were gel-filtered through a 1 ml Sephadex G-25 spin column just before use in the binding assay.

Proteins were measured with BSA as a standard. Purified β/γ subunits of G-proteins were kindly provided by P. Casey (Duke University, Durham, NC, U.S.A.) and R.J. Lefkowitz (Duke University). M3A5, a mouse monoclonal antibody against β-COP, was kindly provided by T. Kreis (Universite of Geneva, Geneva, Switzerland). An affinity-purified polyclonal antibody directed against β-COP was a gift from Felix Wieland (Universität Heidelberg, Heidelberg, Germany). BFA analogues were kindly provided by J. Donaldson (Laboratory of Cell Biology, NHLBI, National Institutes of Health, Bethesda, MD, U.S.A.). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Phagocytic probes

Formaldehyde-fixed Staphylococcus aureus (IgGsorb; The Enzyme Center) were washed with HBSA [Hanks balanced salt solution buffer with 10 mM HEPES and 10 mM Tris, pH 7.4, and supplemented with 1% (w/v) BSA]. After incubation of 200 μl of S. aureus suspension (10% cell suspension, approx. 4 × 10^10 particles/ml, 2 mg/ml IgG binding capacity) with 200 μg of rabbit anti-mouse IgG polyclonal antibody (IgG fraction; Organon Teknika Corporation) for 1 h at 20 °C, the particles were washed three times with HBSS and incubated with 25 μg of IgG raised against 2,4-dinitrophenol (Nph) for 1 h at 20 °C. Coated S. aureus was washed three times and resuspended in HBSS.

Preparation and isolation of phagosomes

J774-E clone macrophages (10^6 cells) were incubated with antibody-coated S. aureus (200 μl) for 1 h at 4 °C. Uptake was initiated by the addition of prewarmed HBSS. After 5 min at 37 °C, the incubation was stopped by the addition of ice-cold HBSS. Cells were subsequently washed with HBSS, HBSS supplemented with 5 mM EDTA and finally HBE buffer [250 mM sucrose/0.5 mM EGTA/20 mM Hepes/KOH (pH 7.2)] by centrifugation (300 g for 3 min). Cells were resuspended in HBE buffer to a final concentration of 2 × 10^2 cells/ml and homogenized in a homogenizer using ball bearings. Homogenates were centrifuged at 300 g for 3 min to eliminate nuclei and intact cells. The supernatants were combined and centrifuged again. The resulting supernatant (Triton-extracted proteins).

Labelling of plasma membrane

J774-E clone macrophages (10^6 cells) were incubated with 10μg/ml anti-Nph IgG and 10 μg/ml Nph-β-glucuronidase for 1 h at 4 °C. Cells were subsequently washed with HBSS, HBSS supplemented with 5 mM EDTA and finally HBE buffer by centrifugation (300 g for 3 min at 4 °C). Cells were resuspended in HBE buffer to 2 × 10^6 cells/ml and homogenized in a homogenizer using ball bearings. Homogenates were centrifuged at 300 g for 3 min to eliminate nuclei and intact cells. The PNSs were quickly frozen in liquid nitrogen and stored at −80 °C.

The PNSs containing the plasma membrane markers were processed after the same steps used in the phagosome isolation protocol. To measure the plasma membrane markers, samples were mixed with solubilization buffer and the immune complex Nph-β-glucuronidase-anti-Nph IgG was immunoprecipitated in multwell plates coated with rabbit anti-mouse IgG. After 30–45 min of incubation at room temperature, the wells were washed three times with solubilization buffer and the β-glucuronidase activity was measured with 4-methylumbelliferyl β-D-glucuronide as a substrate.

Preparation of Golgi membranes

A Golgi-enriched membrane fraction was obtained from J774-E clone macrophages by sucrose-gradient centrifugation as described previously [26]. Membranes were collected at the 0.8/1.2 M sucrose interface, frozen in liquid nitrogen and stored at −80 °C until use. Galactosyltransferase activity, a Golgi marker, was measured with an assay modified from Brew et al. [27].

β-COP binding assay

Isolated phagosomes (5–10 μg of membrane proteins) were resuspended in a buffer containing 250 mM sucrose, 0.5 mM EGTA, 20 mM Hepes/KOH, pH 7.0, 1 mM dithiothreitol, 1.5 mM MgCl2, 50 mM KCl, 1 mM ATP, 8 mM creatine phosphate and 31 units/ml creatine phosphokinase, supplemented with gel-filtered cytosol (1.2 mg/ml) in a final volume of 50 μl, and incubated at 37 °C for 15 min. The reaction was stopped by chilling on ice and the addition of cold HBE buffer. The phagosomes were recovered by centrifugation (12000 g for 1.5 min at 4 °C) and resuspended in a buffer containing 1% (v/v) Triton X-100, 0.2% methylenbenzthonium chloride, 1 mM EDTA, 150 mM NaCl and 10 mM Tris/HCl, pH 7.4, incubated for 15 min on ice and centrifuged again. The resulting supernatant was mixed with reducing sample buffer, boiled for 5 min and analysed by SDS/PAGE.

Western blotting

After resolution by SDS/PAGE, the proteins were transferred to nitrocellulose membranes with standard procedures. Membranes were blocked overnight at 4 °C in Blotto (5% (w/v) low-fat dried milk/80 mM Na2HPO4/20 mM NaH2PO4/100 mM NaCl (pH 7.5)) and then incubated for 1 h at 37 °C with a monoclonal antibody against β-COP (M3A5). Blots were incubated sequentially with rabbit anti-mouse and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins. β-COP was detected with a specific affinity-purified antibody. Detection was per-
formed with the enhanced chemiluminescence Western blotting system (Amersham Corp.) in accordance with the supplier’s recommendations.

**Phagosome recycling assay**

The recycling pathway between phagosomes and the cell surface was assayed as described by Pitt et al. [3]. In brief, J774-E clone macrophages were incubated for 30 min at 4 °C with formaldehyde-fixed *S. aureus* coated sequentially with rabbit anti-mouse antibody and then with mouse 125I-labelled anti-Nph IgG. To optimize contact between cells and the phagocytic probe, the samples were centrifuged for 10 min at 1800 rev./min (500 g) in a benchtop centrifuge. Uptake was initiated by incubation at 37 °C for 5 min in a water bath and stopped by addition of cold PBS. After extensive washing at 4 °C to remove free particles, cells were incubated at 37 °C for different periods in HBSA. At the appropriate times, the culture medium was removed and assayed for trichloroacetic-acid-precipitable radioactivity (recycled material). The cell-associated radioactivity was assayed after cell solubilization with 0.5% (v/v) Triton X-100 in HBSA. This fraction was centrifuged for 5 min at 12000 g; *S. aureus*-associated radioactivity was measured in the pellet.

**Electron microscopy**

Purified phagosomes resuspended in HBE buffer were fixed with 1% (w/v) glutaraldehyde (prepared in HBE buffer). After fixation for 10 min, the samples were centrifuged for 15 min at 12000 g and the pellets were processed for transmission electron microscopy.

**RESULTS**

**β-COP binds to highly purified phagosomes**

To study the interaction of phagosomes with COP proteins, phagosomes of high purity were prepared with a discontinuous sucrose-density gradient as described in the Experimental section. Electron microscopic analysis of the highly purified phagosomes showed essentially no contamination by other organelles (Figure 1). The efficiency of phagosome isolation was assessed biochemically by measuring different subcellular markers. As shown in Table 1, 31% of the antibody-coated 125I-labelled *S. aureus* originally located in the PNS was recovered in the pellet obtained after centrifugation of the PNS through a sucrose cushion. Plasma membrane and other endomembranes of similar density (e.g. endosomes and Golgi vesicles) were present only in trace amounts in the purified phagosomal fraction. Only 0.3% of a plasma membrane marker (Nph-β-glucuronidase–anti-Nph IgG complex bound to the cell surface) and 0.3% of a Golgi membrane marker (galactosyltransferase activity) of the totals present in the PNS were found in the phagosome fraction (Table 1).

It has been shown that GTP-binding proteins are important in the interaction between COPs and Golgi membranes [28–30]. Therefore, to study the recruitment of COP proteins to phagosomal membranes, we incubated purified phagosomes with cytosol (COPs source) in the presence of guanosine 5′-γ-

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**Table 1** Analysis of the distribution of phagosomal, Golgi and plasma membrane markers in subcellular fractions obtained during the phagosome isolation protocols

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Phagosomal marker (% of total)</th>
<th>Golgi marker (% of total)</th>
<th>Plasma membrane marker (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNS</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Purified phagosomes</td>
<td>31.5 ± 13.2</td>
<td>0.3 ± 0.5</td>
<td>0.3 ± 0.2</td>
</tr>
</tbody>
</table>

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**Figure 1** Electron micrographs of purified phagosomes

Electron micrographs showing purified phagosome preparations at low (A) and high (B) magnifications. Scale bars, 0.67 μm (A) and 0.21 μm (B).
The binding of β-COP to phagosomes was assessed (see the Experimental section) after the incubation of purified phagosomes with cytosol for 20 min at 37 °C in the absence or presence of 25 μM GTP[S] (GTP-S), 200 μM [S]ppG (GDP-S), 1 mM Mg-ATP, 100 μM AlNH₄(SO₄)₂ plus 10 mM KF, or 100 μM AlNH₄(SO₄)₂ plus 10 mM KF plus 1 mM GTP.

Figure 3  Inhibition of GTP[S]-enhanced binding of β-COP to phagosomes by Gβγ subunits

Phagosomes and cytosol were preincubated with GTP[S] (GTP-S) (20 μM) or Gβγ subunits (500 nM) for 6 min at 37 °C. Subsequently, either Gβγ subunits or GTP[S] was added to the samples and incubated for another 12 min. The samples were then processed as indicated in the Experimental section.

The binding of β-COP to phagosomes was analysed by SDS/PAGE and Western blot with a specific monoclonal antibody. As shown in Figure 2, cytosolic β-COP associated strongly with purified phagosomes in the presence of GTP[S] (lane 2). GTP also stimulated binding, although to a smaller extent than GTP[S] (Figure 2, lane 4). Lane 3 shows that guanosine 5′-[γ-thio]diphosphate ([S]ppG), a GDP analogue that locks GTP-binding proteins into the GDP-bound form, was a strong inhibitor of β-COP recruitment to phagosomes. As with β-COP, binding of β-COP to phagosomal membranes was also observed in the presence of GTP[S] (results not shown).

AlF₄⁻, an agent widely used to study heterotrimeric G-proteins [31], was also tested. As shown in Figure 2, AlF₄⁻ stimulated the association of β-COP with phagosomes (lane 5) and the binding was increased markedly by the simultaneous addition of both GTP and AlF₄⁻ (lane 6). These results suggest that one or more GTP-binding proteins are involved in the interaction of β-COP with phagosomes and phagosomes.

Because AlF₄⁻ is an activator of trimeric G-proteins, the effect of AlF₄⁻ suggests that this family of GTP-binding proteins is involved in the interaction of β-COP with phagosomes. Gβγ subunits of heterotrimeric G-proteins inhibit the function of these GTP-binding-proteins [32,33]. Therefore, if phagosomes and cytosol are preincubated with Gβγ subunits before adding GTP[S], the β-COP binding should not be stimulated by this nucleotide. When this sequential incubation was performed, a significant inhibition of β-COP binding to phagosomes was observed (Figure 3, lane 5). In contrast, when the membranes were first incubated with GTP[S] before the addition of Gβγ subunits, the binding of β-COP to phagosomes was not altered (Figure 3, lane 2).

These results confirm the role of heterotrimeric G-proteins on the recruitment of β-COP to phagosomal membranes.

β-COP binding to phagosomal membrane requires membrane-associated proteins

To determine whether a membrane component was required for β-COP binding, phagosomes were treated with different agents. Membranes were stripped of peripherally associated proteins by incubation with 0.8 M NaCl or 100 mM Na₂CO₃. As shown in Figure 4, both treatments moderately decreased β-COP binding (lanes 2 and 3), although binding was not affected by washing the membranes in a buffer containing 50 mM EDTA (lane 4), suggesting that Ca⁴⁺ was not required for binding. In contrast, trypsin completely abolished the ability of the membranes to support β-COP binding [compare trypsin treatment (Figure 4, lane 5) with mock trypsin treatment (lane 6)].

Taken together, the results suggest that a membrane-associated factor is required for the interaction of β-COP with the phagosomal membranes.

BFA blocks the binding of β-COP to phagosomal membranes: role of ARF1

It has been shown that the binding of coatomer to the Golgi depends on the interaction of the small GTP-binding protein ARF with the membrane [29]. A process that is blocked by BFA [10,11]. We therefore tested the effect of BFA on the binding of β-COP to phagosomes. As shown in Figure 5(A), BFA blocked the stimulatory effect of GTP[S] on the binding. The specificity of the effect of BFA is shown by the fact that the inactive analogues BFA-17 and BFA-31 did not inhibit the binding (Figure 5B).

The effect of BFA suggests that the binding of β-COP to phagosomes might be regulated by ARF. We therefore incubated phagosomes and cytosol in the presence of recombinant wild-type ARF1 (WT) or the GTPase-defective mutant (Q71L). As shown in Figure 6, WT bound to and stimulated the binding of β-COP to phagosomes when the protein was activated by adding GTP[S] to the assay (lane 2). As expected, the addition of [S]ppG...
blocked the recruitment of both ARF1 and β-COP to phagosomal membranes (Figure 6, lane 3). GTP did not support ARF1 and β-COP binding. In contrast, Q71L, the ARF mutant defective in GTP hydrolysis, bound more efficiently than WT and supported the binding of β-COP to phagosomes independently of added nucleotide (Figure 6, lanes 5–8), confirming the participation of active ARF1 in the process.

Figure 6  Effect of WT and mutant ARF1 on the binding of β-COP to phagosomes
Phagosomes and cytosol were incubated with WT (ARF1wt) or the ARF1 mutant ARF1Q71L (each at 100 μg/ml) in the absence or presence of GTP[S] (20 μM), GDP[S] (25 μM) or GTP (200 μM). After 20 min at 37 °C, the samples were processed as indicated in the Experimental section.
BFA, suggesting that a BFA-sensitive factor was involved, at least in part, in the process.

DISCUSSION

Here we present evidence that \(\beta\)-COP, one of the components of the protein complex coatomer [34], is recruited to highly purified phagosomes. We have observed that both GTP[S] and AIF, promote the association of \(\beta\)-COP with phagosomal membranes, suggesting that one or more GTP-binding proteins participate in the binding of \(\beta\)-COP to the membranes. This is consistent with our previous observation that several heterotrimeric G-proteins are present on highly purified phagosomal membranes [35]. The results obtained with AIF and G\(\gamma\) suggest that heterotrimeric G-proteins are likely to be involved in the recruitment of cytoplasmic proteins (e.g. \(\beta\)-COP) to make a particular type of vesicle, possibly by sending signals across the phagosomal membrane. Indeed, we have evidence that AIF inhibits the recycling of proteins from the phagosomal compartment (M. T. Damiani, A. Savina and M. I. Colombo, unpublished work), suggesting that the formation of recycling vesicles might be regulated by heterotrimeric G-proteins.

We have observed that GTP increased the \(\beta\)-COP binding stimulated by AIF. Because AIF activates the trimeric G-proteins only in the GDP-bound form [36], GTP might activate other GTP-binding proteins. As with the results observed with Golgi membranes [10], GTP[S] promoted the association of \(\beta\)-COP to phagosomal membranes and prevented the inhibitory effect of BFA. It has been shown that BFA prevents the assembly of coatomer on the Golgi membrane by inhibiting the factor that exchanges GTP bound to ARF [10–12,28]. Several BFA-sensitive guanine nucleotide exchange factors for ARFs have been identified [37–39]. Our results therefore suggest that a BFA-sensitive member of the ARF family is involved in the binding of \(\beta\)-COP to phagosomes. Indeed, our results indicate that ARF1 participates in the binding of \(\beta\)-COP to phagosomes.

ARF family members have been implicated in several events of intracellular membrane trafficking [40]. Published evidence indicates that ARF is involved in the endocytic pathway. It has been shown that an ARF protein regulates endosome fusion reconstituted in a system in vitro [21,22]. Moreover, in intact cells the overexpression of an ARF6 mutant defective in GTP binding altered transferrin recycling and caused the accumulation of non-clathrin-coated structures on peripheral tubes [23,24]. Apparently, in HeLa cells ARF6 is related to a plasma membrane–juxtanuclear compartment recycling pathway that is different from the conventional one characterized by the presence of the transferrin receptors [41]. Nevertheless, in CHO cells, a partial co-localization of ARF6 with transferrin receptor suggests that the ARF6-containing compartment might partly overlap with a recycling endosomal compartment [42]. It has been shown that ARF6 also has a role in Fc receptor-mediated phagocytosis through the regulation of actin- and cytoskeleton changes that underlie phagocytosis [43]. However, it has recently been shown that ARF1 and not ARF6 is required for COP recruitment to endosomes and for the formation of endosomal carrier vesicles/multivesicular bodies from endosomal membrane in vitro [25].

A function for coatomer in the endocytic pathway has been proposed [17–20]. Microinjection of anti-\(\beta\)-COP antibodies inhibited the entry of viruses via the endocytic pathway [17]. These results, and the observation that \(\epsilon\)-COP-deficient CHO cells exhibit defective endocytosis [44,45], suggest that coatomer or a related protein complex might have a role in the endocytic system. Indeed, a purified fraction of endosomes was shown to recruit in vitro a coat containing \(\alpha\)-, \(\beta\)-, \(\gamma\)-, \(\epsilon\)- and \(\zeta\)-COP but not \(\gamma\)- or \(\delta\)-COP [17,18]. Interestingly, recruitment of these sets of proteins on endosomes was stimulated by GTP[S] and was sensitive to BFA [17]. These results are in agreement with our observation that the binding of \(\beta\)-COP to phagosomes is enhanced by GTP[S] and blocked by BFA.

Phagosome maturation is a complex process that involves the influx and efflux of material from phagosomes that gradually acquire lysosomal characteristics. The influx of material is a consequence of the interaction between phagosomes and endocytic compartments [3,46–52]. Protein and membrane recycling from phagosomes represents the efflux of material during the transformation of phagosomes to phagolysosomes. It has been shown that phagosomal proteins recycle rapidly to the plasma membrane [3,53]. Recycling seems to involve the formation of tubules and transport vesicles that selectively include molecules to be recycled back to the plasma membrane [3]. It is therefore possible that \(\beta\)-COP binds to phagosomes to allow the recycling of phagosome components to the cell surface. Indeed, the inhibition of normal recycling of either low-density lipoprotein or transferrin receptors to plasma membrane was observed in a temperature-sensitive \(\epsilon\)-COP-deficient CHO cell mutant [19,44,45,54], suggesting that this COP protein is involved in endosome recycling.

It has recently been shown that the removal of transferrin receptor from phagosomes is partly inhibited in \(\epsilon\)-COP-deficient or BFA-treated cells [55]. The authors suggest that transferrin receptor recycling is in part mediated by a COPI-dependent process. These results are in agreement with our observation that BFA significantly inhibited protein recycling from phagosomes to the cell surface.

Our observations suggest that COPI participates in delivering phagosome components towards the cell surface, a process that is essential for phagosome remodelling and antigen presentation.

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