AP-1 and ARF1 Control Endosomal Dynamics at Sites of FcR-mediated Phagocytosis[□]

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Phagocytosis, the mechanism of ingestion of large material and microorganisms, relies on actin polymerization and on the focal delivery of intracellular endocytic compartments. The molecular mechanisms involved in the formation and delivery of the endocytic vesicles that are recruited at sites of phagocytosis are not well characterized. Here we show that adaptor protein (AP)-1 but not AP-2 clathrin adaptor complexes are recruited early below the sites of particle attachment and are required for efficient receptor-mediated phagocytosis in murine macrophages. Clathrin, however, is not recruited with the AP complexes. We further show that the recruitment of AP-1-positive structures at sites of phagocytosis is regulated by the GTP-binding protein ARF1 but is not sensitive to brefeldin A. Furthermore, AP-1 depletion leads to increased surface levels of TNF- α , a cargo known to traffic through the endosomes to the plasma membrane upon stimulation of the macrophages. Together, our results support a clathrin-independent role for AP complexes in endosomal dynamics in macrophages by retaining some cargo proteins, a process important for membrane remodeling during phagocytosis.

INTRODUCTION

Phagocytosis is a mechanism of internalization that allows the ingestion of large particulate material and is used by amoeba to feed on bacteria. Specialized immune cells, such as macrophages, polymorphonuclear granulocytes, and dendritic cells, also use phagocytosis to internalize, degrade, and eventually present antigens derived from large foreign particles and microorganisms, thereby contributing to immune responses (Aderem and Underhill, 1999; Blander and Medzhitov, 2006). The engulfment of particles is initiated by the interaction between ligands exposed on the particle and receptors present on the surface of the phagocyte. A large number of receptors have been implicated in this process,

including mannose receptors, scavenger receptors, and Toll-like receptors (TLRs), together with receptors for opsonins, namely receptors for the Fc portion of immunoglobulins (Ig; FcRs) and receptors for complement C3bi (CR3). Phagocytosis induced by the FcRs has been best characterized. It activates a signaling cascade that involves small GTP-binding proteins of the Rho and Arf families and eventually leads to actin polymerization, plasma membrane remodeling, and extension of pseudopods around the particle (Greenberg and Grinstein, 2002; Underhill and Ozinsky, 2002; Niedergang and Chavrier, 2004).

Plasma membrane extension around large particles is supported by a process of internal membrane delivery that involves the fusion machinery relying on vesicle-associated, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (v-SNAREs; Booth *et al.*, 2001; Aderem, 2002; Braun and Niedergang, 2006; Stow *et al.*, 2006). The recycling endosomes bearing the SNARE protein VAMP3/Cellubrevin (Bajno *et al.*, 2000; Niedergang *et al.*, 2003) and a subpopulation of late endosomes bearing the SNARE protein VAMP7/TI-VAMP (Braun *et al.*, 2004) have been shown to undergo focal exocytosis at the site of phagocytosis and to be required for efficient phagocytosis. In addition, the endoplasmic reticulum and the SNARE protein ERS24/Sec22b have also been implicated in the phagocytosis of large particles (Gagnon *et al.*, 2002; Becker *et al.*, 2005). Of note, the major part of the membrane forming a phagosome is of plasmalemmal origin (Touret *et al.*, 2005b) and different intracellular compartments might contribute differentially depending on the cell type, the size and the nature of the particles engulfed and the receptors engaged (for reviews, see Booth *et al.*, 2001; Gagnon *et al.*, 2005; Touret *et al.*, 2005a; Braun and Niedergang, 2006). Of interest, the Golgi appara-

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Abbreviations used: AP, adaptor protein; ARF, ADP-ribosylation factor; BFA, brefeldin A; FcR, crystallizable fragment receptor; Lamp1, lysosomal-associated membrane protein 1; LPS, lipopolysaccharide; RPE, R-phycoerythrin; SRBC, sheep red blood cell; TAPI-1, TNF- α protease inhibitor-1; TGN, *trans*-Golgi network; TNF- α , tumor necrosis factor α .

tus is considered not to contribute to early steps of phagosome formation, because FcR-mediated phagocytosis proceeds normally in the presence of brefeldin A (BFA; Zhang *et al.*, 1998; Becker *et al.*, 2005). This fungal metabolite induces profound perturbation of the Golgi apparatus structure by stabilizing a complex between some guanine-nucleotide exchange factors (GEF) and the GDP-loaded ARF protein and therefore inhibiting its GTP loading. As a consequence, ARF1, COPI, and clathrin coat adaptor proteins rapidly dissociate from Golgi membranes (Jackson and Casanova, 2000; D'Souza-Schorey and Chavrier, 2006). Therefore, ARF1 was also considered not to be involved in phagocytosis until a recent study reported that ARF1 was activated in macrophages at sites of FcR-mediated phagocytosis (Beemiller *et al.*, 2006). As a consequence, ARF1 could regulate Golgi-independent trafficking events during phagocytosis, as reported for the related ARF6 protein, which controls the focal delivery of VAMP3-positive endocytic compartments (Zhang *et al.*, 1998; Uchida *et al.*, 2001; Niedergang *et al.*, 2003).

Although some of the endosomal markers and some proteins of the fusion machinery involved in membrane fusion at sites of phagocytosis are identified, the mechanisms underlying the budding and transport of vesicles to the plasma membrane remain poorly characterized. Formation of transport vesicles carrying cargo proteins between the plasma membrane, the *trans*-Golgi network (TGN) and endosomes relies in part on coats containing clathrin and adaptor protein (AP) complexes. The AP family contains four members, AP-1 to -4, each composed of two large subunits ($\gamma/\beta 1$, $\alpha/\beta 2$, $\delta/\beta 3$, or $\epsilon/\beta 4$), a "medium" subunit ($\mu 1-4$), and a "small" subunit ($\sigma 1-4$). AP-2 is located specifically at the plasma membrane. AP-1 is predominantly found at the TGN but also on endosomes and specialized granules, depending on the cell type. AP-3 is present on endosomal compartments and is involved in the trafficking of cargo proteins to lysosomes and lysosome-related organelles (for reviews, see Bonifacino and Glick, 2004; Robinson, 2004; Raposo *et al.*, 2007). The association of AP-1 and -3, but not AP-2, with the membrane of endosomes or the TGN is mediated by ARF1 (D'Souza-Schorey and Chavrier, 2006). So far, only AP-1 has been connected to the phagocytic process. The implication of AP-1 has been reported in *Dictyostelium discoideum*, where AP-1 was shown to be necessary for efficient phagocytosis (Lefkir *et al.*, 2004). The efficiency of uptake in the $\mu 1$ mutants was dependent on the size of the particles to internalize, indicating that the defect of phagocytosis in the $\mu 1$ mutant cells was at the stage of pseudopod extension. Nevertheless, the precise function of the AP-1 complexes at the onset of phagocytosis remains elusive.

Here, we investigated the role of APs during FcR-mediated phagocytosis in murine macrophages, and we observed that AP-1, but not -2, is recruited and accumulates in the cytoplasm of macrophages at sites of phagocytosis. RNA interference (RNAi) experiments showed that AP-1 and -3 are necessary for efficient phagocytosis. In addition, we showed that the recruitment of AP-1 under phagocytic cups is controlled by ARF1, but unaffected by BFA. Interestingly, clathrin was not recruited together with AP-1 at phagocytic sites. Furthermore, we observed that AP-1 depletion lead to an increased surface delivery of tumor necrosis factor (TNF)- α upon lipopolysaccharide (LPS) stimulation of the macrophages. Altogether, these results suggest that, in macrophages, AP-1 could function without clathrin to retain cargo proteins rather than playing a role in their sorting through a budding process.

MATERIALS AND METHODS

Plasmids and Reagents

The pEGFP-expressing (enhanced green fluorescent protein) plasmid (Clontech, Palo Alto, CA) and pEGFP-VAMP3/Cellubrevin plasmids (Braun *et al.*, 2004) have been described previously. The pSR α -ARF1^{T31N}-HA and pSR α -ARF6^{T27N}-HA plasmids have been described previously (Dubois *et al.*, 2005). The Clathrin-DsRed-expressing plasmid was a kind gift from Dr. Thomas Kirchhausen (Harvard Medical School, Boston, MA) (Rappoport *et al.*, 2003).

BFA and LPS were from Sigma (St. Louis, MO). TNF- α protease inhibitor-1 (TAPI-1) is from Calbiochem (La Jolla, CA). The following antibodies were used: anti-clathrin heavy chain (clone 23), anti- γ -adaplin (clone 88), anti-GM130 (clone 35), anti-mouse TNF- α (rat monoclonal, clone MP6-XT22; Becton Dickinson, Mountain View, CA); anti-clathrin heavy chain (mouse monoclonal, clone X22; ABR Affinity BioReagents, Golden, CO); anti-mouse TNF- α (goat polyclonal, R&D Systems, Minneapolis, MN); Cy2-, Cy3-, and Cy5-labeled F(ab')₂ (Ig fragment after digestion with the enzyme papain) anti-mouse or anti-rabbit IgG, R-phycoerythrin (RPE)-labeled F(ab')₂ anti-rat IgG and fluorescein isothiocyanate (FITC)-labeled anti-goat IgG (Jackson ImmunoResearch, West Grove, PA); horseradish peroxidase-labeled anti-mouse IgG (GE Healthcare, Waukesha, WI). Alexa633- or Alexa488-conjugated transferrin and Alexa350-coupled phalloidin were from Molecular Probes (Eugene, OR). Anti-TGN38 was provided by Dr. George Banting (Department of Biochemistry, University of Bristol, Bristol, United Kingdom). Anti- α -adaplin (clone AP6) was from Dr. Frances Brodsky (UCSF, San Francisco, CA). Anti- δ -adaplin (rabbit serum used for Western blots and mouse monoclonal, clone KF4, used for immunofluorescence) were a kind gift from Dr. Andrew Peden (Genentech, San Francisco, CA).

Cell Culture, Transfection, and siRNA Treatment

RAW264.7 macrophages were grown and transfected as described (Niedergang *et al.*, 2003). Bone marrow-derived macrophages were prepared as described (Braun *et al.*, 2004).

Human monocytes were isolated from blood of healthy volunteers (Etablissement Français du Sang, Rungis, France) by density gradient sedimentation in Ficoll (GE Healthcare), followed by two washes in 1 $\times$ phosphate-buffered saline (PBS) supplemented with 2 mM EDTA and 1 mg/ml bovine serum albumin and a negative selection with magnetic beads (Monocyte negative isolation kit, DYNAL, Lake Success, NY).

HeLa cells (a gift from A. Dautry, Institut Pasteur, Paris) were grown in the same medium as macrophages (RPMI 1640 supplemented with 10% fetal calf serum [FCS], 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 50 μ M β -mercaptoethanol; all from Invitrogen, Carlsbad, CA).

RAW264.7 macrophages were transfected with small interfering RNA (siRNA) duplex (Eurogentec, Serain, Belgium or Prolog, Kyoto, Japan) and Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen; Elbashir *et al.*, 2001). GFP siRNA duplex used as control was described (Braun *et al.*, 2004). The sequence of the siRNA specific for the γ subunit of the AP-1 complex (γ -adaplin) was 5'-AGCUAUGAAUGAUUAUUATT-3', which is similar to the sequence used in Dugast *et al.* (2005). The sequence of the siRNA specific for the δ subunit of the AP-3 complex (δ -adaplin) was 5'-CAUCAAGAUCAUCAAGCUG-3'. After 24 and 48 h, cell lysates were analyzed by Western blotting as described (Braun *et al.*, 2004).

Transferrin (Tf) internalization was performed as described previously (Niedergang *et al.*, 2003).

Phagocytosis Assays

When indicated, cells were pretreated with BFA at 10 μ g/ml for 30 min at 37°C or with the same volume of methanol. Phagocytosis was performed as described (Niedergang *et al.*, 2003; Braun *et al.*, 2004) except that BFA or methanol was kept in the phagocytosis medium.

To quantitate phagocytosis, the number of internalized sheep red blood cells (SRBCs) was counted in 50 cells randomly chosen on the coverslips, and the phagocytic index, i.e., the mean number of phagocytosed SRBCs per cell, was calculated. The index obtained for transfected cells was divided by the index obtained for control nontransfected cells and expressed as a percentage of control cells. We also counted the number of cell-associated (bound + internalized) SRBCs, calculated the association index (mean number of associated SRBCs per cell), and expressed it as percentage of control nontransfected cells. The index obtained for the siRNA-treated cells was divided by the index obtained for control (siRNA GFP) cells and expressed as a percentage of the latter.

To quantitate polymerized actin recruitment, we scored the presence or absence of F-actin around particles in 50 cells randomly chosen on the coverslips and calculated an accumulation index, i.e., the mean number of accumulations per cell.

To quantitate AP-1 recruitment, we scored the presence or absence of AP-1 accumulations as estimated by eye around particles in 50 cells randomly chosen on the coverslips and calculated an accumulation index, i.e., the mean number of accumulations per cell. In Figure 2, quantitation was performed from immunofluorescent images (see below).

Immunofluorescence Analysis

Cells were fixed in 4% paraformaldehyde/PBS and labeled (Niedergang *et al.*, 2003). Cells were examined under a motorized upright wide-field microscope (DMRA2, Leica, Deerfield, IL) equipped for image deconvolution as described (Braun *et al.*, 2004). Acquisition was performed using an oil immersion objective (100 $\times$ PL APO HCX, 1.4 NA) and a cooled CCD camera (Roper CoolSnap HQ, Tucson, AZ). Z-positioning was accomplished by a piezo-electric motor and deconvolution was performed (see Figures 1A and 5, A and C, and Supplementary Figure S2A). Alternatively, acquisitions were also performed using a motorized upright wide-field microscope (DMRXA2, Leica) equipped with oil immersion objective (63 $\times$ PL APO HCX, 1.32 NA) and a cooled CCD camera (Roper CoolSnap HQ; see Figure 4, A and C). Acquisitions were also performed using an inverted wide-field microscope (CTR 6000, Leica) equipped with oil immersion objective (100 $\times$ PL APO HCX, 1.4 NA) and a cooled CCD camera (Micromax, Princeton Instruments Research Instruments, Princeton, NJ; see Figures 2, A and B, and 5B, and Supplementary Figure S1, A and B). Z-positioning was accomplished by a piezo-electric motor and deconvolution was then performed (see Figures 5B and 6B). Image analysis was performed using the Image J (NIH) and Adobe Photoshop software (San Jose, CA).

Fluorescence Quantitation

Quantitation of fluorescence was performed using Metamorph software (Universal Imaging, West Chester, PA) on selected linear regions in one selected plane of the 16-bit stack acquired as described above with an inverted wide-field microscope (CTR6000, Leica) equipped with oil immersion objective (100 $\times$ PL APO HCX, 1.4 NA) and a cooled CCD camera (Micromax, Princeton Instruments). Primary fluorescence intensities of selected regions were background corrected by subtracting the mean value from a cell-free region. Ratio values obtained by dividing the fluorescence intensities in the phagocytic cups by fluorescence intensities in the cell body were calculated for AP proteins and for GFP, used as a free cytosolic probe. Results are expressed as a percentage of the cup/cell body ratio obtained for GFP.

Flow Cytometry Analysis

To measure surface TNF- α expression, when indicated, RAW 264.7 cells were incubated for 4 h with 100 ng/ml LPS and 10 μ M TAPI-1 at 37°C as described in Murray *et al.* (2005). TAPI-1 is an inhibitor of TACE, the enzyme that cleaves the transmembrane TNF- α protein and releases TNF- α in the extracellular medium. After incubation, cells were recovered from wells, incubated 1 h at 4°C with 10 μ g/ml anti-mouse TNF- α in 100 μ l of PBS supplemented with 2% FCS, and washed two times with 0.5 ml of PBS FCS 2%. The cells were then incubated 45 min at 4°C with RPE-anti-rat IgG and washed once with 0.5 ml of PBS FCS 2% and once with 0.5 ml of PBS to be finally resuspended in 0.5 ml of PBS. Flow cytometry was carried out in a FACS-calibur flow cytometer (Becton Dickinson). At least 10,000 live cells were acquired and the median RPE fluorescence was calculated. We could not select the depleted cells population. Therefore, the acquisition was performed on a mixed population of depleted and nondepleted cells. The values obtained for specific siRNA-depleted cells were expressed as a percentage of the value measured for control siRNA-depleted cells.

Immunoprecipitation

RAW264.7 cells or HeLa cells were grown until subconfluence. RAW264.7 cells were scrapped and HeLa cells detached with trypsin to be collected and washed once with prechilled PBS. Cells were then lysed on ice with 0.5 ml of lysis buffer (0.5% NP40, 20 mM Tris, pH 7.4, 150 mM NaCl, Complete Mini EDTA-free protease inhibitor cocktail [Roche, Alameda, CA], 1 mM sodium orthovanadate, and 50 mM sodium fluoride). As a preclearing step, lysates were incubated 30 min with protein G-Sepharose (4 Fast Flow, GE Healthcare). Lysates were then centrifuged and the supernatants were incubated for 4 h at 4°C with 1 μ g of anti- γ adaptin antibody and protein G-Sepharose. After four washes in cold wash buffer (0.5% NP40, 20 mM Tris, pH 7.4, 150 mM NaCl), the samples were analyzed by SDS-PAGE and Western blotting as described (Braun *et al.*, 2004).

Statistics

The statistical significance of the data were tested with an unpaired Student's *t* test, and calculated goodness-of-fit value (*p* value) is indicated in the figures legends.

Online Supplementary Materials

The plasmid encoding α -adaptin-GFP was a kind gift of Lois Greene (NIH, Bethesda, MD) and was described in Wu *et al.* (2003).

Figure 1. AP-1 is recruited at sites of FcR-mediated phagocytosis but AP-2 is not. (A) RAW264.7 cells were incubated for 3 min with IgG-SRBCs, fixed, and stained with Cy2-anti-rabbit IgG to reveal external SRBCs (middle panels). The cells were then permeabilized and labeled with anti- γ -adaptin (top panels) or anti- α -adaptin (bottom panels) followed by Cy3-anti-mouse IgG to detect AP-1 and -2 complexes, respectively (left panels). Polymerized actin was labeled with Alexa350-phalloidin (right panels). Cells were analyzed by wide-field microscopy with deconvolution. Medial optical sections are shown. Bar, 5 μ m. (B) The number of accumulations of γ -adaptin and F-actin in nascent phagosomes was scored for 50 cells randomly chosen on coverslips. Results are expressed as percentage and are the mean $\pm$ SEM of three independent experiments. Differences between F-actin and AP-1 accumulations were not significant (*p* > 0.05).

RESULTS

AP-1 Complex Is Recruited to the Phagosome at Early Stages of Phagocytosis

To investigate the role of APs during phagocytosis, we first analyzed the localization of AP-1 and -2 during FcR-mediated phagocytosis in RAW264.7 macrophages as well as in bone marrow-derived macrophages. Macrophages were incubated with IgG-SRBCs for 5 or 10 min at 37°C and then fixed and stained to detect external SRBCs and intracellular AP-1 (γ -adaptin) or AP-2 (α -adaptin; Figure 1A). The AP-1 staining revealed a punctate pattern scattered in the cytoplasm of macrophages and concentrated in the perinuclear region (Figures 1 and 4C). We observed that AP-1 accumulated in the cytoplasmic regions located under the sites of phagosome formation, whereas AP-2 did not in the same conditions (Figure 1A). The lack of recruitment of AP-2 could be due to a defective recognition of the complex by the anti- α -adaptin antibodies, as a consequence of a potential cleavage of α or β 2 AP-2 large subunits, as described for AP-1 (see below and Figure 6A; Santambrogio *et al.*, 2005; Austin *et al.*, 2006). We therefore expressed an α -adaptin-GFP construct in RAW264.7 macrophages. The construct was properly localized in structures reminiscent of coated pits at the plasma membrane, but AP-2 was not found accumulated at sites of phagosome formation (Supplemental Figure S1A). Because actin polymerization is a rapid and transient event concomitant to phagosome formation (Swanson *et al.*, 1999; Coppolino *et al.*, 2002; Araki *et al.*, 2003; Henry *et al.*, 2004), we then scored the number of AP-1 recruitments around external particles and compared it to F-actin accumulations (Figure 1B). We observed that $90 \pm 4\%$ of actin cups also showed AP-1 cytoplasmic accumulations. To better estimate the enrichment of endogenous AP-1 in phagocytic cups, fluorescent intensities around the particles and in the cell body were quantified and compared with the intensities obtained for GFP, used as a freely diffusible cytosolic marker (Figure 2). AP-1 fluorescent intensities exhibited up to a twofold increase around the particles compared with the cell body. Compared with GFP, AP-1 was specifically enriched in the extensions around the particles

Figure 2. Quantitation of AP-1 enrichment at the phagocytic sites. (A) pEGFP transfected RAW264.7 cells were incubated for 3 min at 37°C with IgG-SRBCs, then fixed, permeabilized, and stained with anti- γ -adaptin (AP-1; left panels) followed by Cy3-anti-mouse F(ab')₂ and Alexa350-phalloidin to detect phagocytic sites (not shown). GFP fluorescence is shown in the right panels. Images are shown using the Rainbow2 (top panels) or Grayscale (bottom panels) look-up table. Values on the color scales in the left corners of the top panels indicate the fluorescence intensities. Scale bar, 5 μ m. (B) The profiles of γ -adaptin (left) and GFP (right) fluorescence intensities along the lines drawn at the phagocytic site (line 1) and in the cell body (line 2) are shown. (C) As described in A, except that GFP-expressing RAW264.7 cells were stained for α -adaptin (AP-2) after incubation for 3 min at 37°C with IgG-SRBCs (left panels). GFP fluorescence is shown in the right panels. A section on the dorsal

Figure 3. γ -adaptin is required for efficient FcR-mediated phagocytosis. (A) RAW264.7 cells were transfected either with γ -adaptin siRNA (AP-1) or with a nonspecific control siRNA (c). Twenty-four hours after transfection, cell lysates were analyzed by Western blot to detect γ -adaptin (bottom panel) and clathrin heavy chain (top panel). (B) γ -adaptin-depleted cells were incubated for 60 min at 37°C with IgG-SRBCs, then fixed, and stained with Cy2-anti-rabbit IgG to detect external SRBCs. The efficiencies of association (left) and phagocytosis (right) were calculated as described in *Material and Methods* in 50 γ -adaptin-depleted (AP-1) or control siRNA-treated cells (control). Results are expressed as a percentage of control cells. The means $\pm$ SEM of three independent experiments are plotted. Particle association ($p < 0.05$) and phagocytosis ($p < 0.005$) were significantly different between control siRNA- and AP-1-siRNA-treated cells.

(Figure 2, B and E), whereas a similar quantitative analysis confirmed that AP-2 was not accumulated at sites of phagosome formation (Figure 2, D and F). Taken together, these results show that AP-1-positive structures are specifically recruited at sites of phagosome formation.

AP-1 and -3 Are Required for Efficient FcR-mediated Phagocytosis in RAW264.7 Cells

We next assessed whether AP-1 is required for efficient FcR-mediated phagocytosis in macrophages (Figure 3). For this, we inactivated the AP-1 complexes using RNAi against the γ -adaptin subunit (Dugast *et al.*, 2005). Although the γ -adaptin siRNA only affects one subunit of the complex, previous reports have demonstrated that the depletion of one subunit of an AP complex decreases the stability of other

side of the cells has been analyzed to better detect AP-2 staining at the plasma membrane. (D) The profiles of α -adaptin (left) and GFP (right) fluorescence intensities along the lines drawn at the phagocytic site (line 1) and in the cell body (line 2) are shown. (E and F) The fluorescence intensities measured in the phagocytic cups were background subtracted and expressed as a percentage of the fluorescence intensities in the cell body. Data obtained for γ -adaptin (E) or for α -adaptin (F) were compared with and expressed as a percentage of values obtained for GFP in the same regions of interest. Data are the mean $\pm$ SEM of 15 independent measurements. Accumulation of AP-1 at phagocytic sites was significantly higher than that of GFP ($p < 0.0001$), but AP-2 was not enriched in nascent phagosomes as compared with GFP ($p > 0.1$).

subunits (Motley *et al.*, 2003; Rose *et al.*, 2005). We observed a diminished γ -adaptin expression in specific siRNA-treated cells compared with control cells treated with a nonspecific siRNA sequence (Figure 3A). The efficiency of particle binding and uptake via FcRs was monitored in AP-1-depleted cells identified by immunostaining with anti- γ -adaptin antibodies. Although the association of opsonized particles was hardly affected in AP-1-depleted cells ($93 \pm 5\%$ of control cells), particles internalization was reduced by $47 \pm 7\%$ (Figure 3B), indicating that AP-1 is required for efficient FcR-mediated phagocytosis.

We also examined whether AP-3 could be involved in FcR-mediated phagocytosis. First, we stained phagocytosing cells with antibodies against the δ -adaptin subunit of AP-3 and observed that, although it was present in the cytoplasm under phagocytic sites during FcR-mediated phagocytosis in RAW264.7 macrophages (Supplementary Figure S2A), it was not quantitatively enriched (Supplementary Figure S2B). Using RNAi against the δ -adaptin subunit, we next assessed whether AP-3 was required for efficient FcR-mediated phagocytosis in macrophages (Supplementary Figure S2C). Expression of δ -adaptin was decreased in specific siRNA-treated cells compared with cells treated with a nonspecific siRNA sequence. We then measured the efficiencies of particles binding and uptake via FcRs in AP-3-depleted cells and compared them to control cells (Supplementary Figure S2D). We observed that in AP-3-depleted cells, particles internalization was reduced to $52 \pm 10\%$ of control cells, whereas the association of opsonized particles was not significantly affected. These results indicate that AP-3 is also important for efficient phagocytosis mediated by FcRs, although in contrast to AP-1 it is not accumulated in phagocytic cups.

AP-1 Recruitment is BFA Insensitive and Positively Regulated by ARF1

To get further insight into the function of AP-1 during phagocytosis in macrophages, we analyzed whether the recruitment of AP-1 in phagocytic cups occurred under the control of small GTP-binding proteins of the Arf family. For this, we treated the cells with BFA. As already described (Orci *et al.*, 1991; Donaldson *et al.*, 1992; Robinson and Kreis, 1992; Traub *et al.*, 1993), treatment of RAW264.7 macrophages with BFA for 30 min led to disassembly of the Golgi apparatus and scattering of the Golgi marker GM130 in the cytoplasm (Figure 4A). By contrast, BFA treatment did not affect association or phagocytosis of opsonized particles in macrophages (Figure 4B and Zhang *et al.*, 1998). Interestingly, the recruitment of AP-1 under phagocytic cups was unaffected by BFA treatment in contrast to its perinuclear (TGN) localization that was lost upon BFA treatment (Figure 4, C and D). We then tested if ARF1 and ARF6 could play a role in the accumulation of AP-1-positive structures at sites of phagosome formation. For this, we analyzed γ -adaptin recruitment in RAW264.7 macrophages transiently expressing dominant negative mutants of ARF6 or ARF1, ARF6^{T27N} or ARF1^{T31N}, respectively. Dominant negative ARF1^{T31N} expression inhibited AP-1 recruitment under opsonized particles by $69 \pm 1.7\%$, whereas ARF6^{T27N} expression only affected the AP-1 localization during phagocytosis by $20 \pm 5\%$ (Figure 4E), although it inhibited particle uptake (Bajno *et al.*, 2000; Niedergang *et al.*, 2003). As reported recently (Beemiller *et al.*, 2006), dominant negative ARF1^{T31N} expression lead to a decreased phagocytic activity of macrophages ($64 \pm 11\%$ of control cells).

Taken together, these results indicate that AP-1 recruitment under bound IgG-opsonized particles is controlled by ARF1, although it is BFA insensitive. Because Golgi-

associated ARF1-GEFs are inhibited by BFA (Jackson and Casanova, 2000; D'Souza-Schorey and Chavrier, 2006), these results also suggest that the pool of AP-1 that is present at sites of particle attachment does not originate from the Golgi compartment and could therefore derive from other structures.

AP-1 Is Associated with Endosomal Structures and Accumulated without Clathrin under the Particles

To address the origin of AP-1 recruited at the phagocytic cup, we first examined the steady-state localization of AP-1 in RAW 264.7 macrophages by immunofluorescence. In macrophages, AP-1 exhibited a punctate staining localized in a perinuclear region as well as scattered throughout the cytoplasm (Figures 1 and 5). The perinuclear staining colocalized with a TGN marker, TGN38 (Figure 5) and was BFA sensitive (Figure 4C). The cytoplasmic AP-1-positive structures partially colocalized with internalized Tf, a marker of early and recycling endosomes (Dautry-Varsat *et al.*, 1983) and with cellubrevin/VAMP3, a marker of recycling endosomes (Daro *et al.*, 1996; Bajno *et al.*, 2000; Niedergang *et al.*, 2003). The AP-1-positive structures also exhibited extensive colocalization with clathrin-DsRed (Figure 5).

These observations therefore tend to identify the AP-1-positive structures as part of the TGN and endosomal compartments labeled by clathrin in macrophages.

We then examined the localization of AP-1 in phagocytosing macrophages (Figure 5C). Surprisingly, although AP-1 accumulation was observed at sites of FcR-mediated phagocytosis (Figures 1A and 5C), clathrin was never detected in the same location (Figure 5C). Because antibodies against clathrin and AP-1 are mouse monoclonal antibodies, we could not use them to detect endogenous clathrin and AP-1 at the same time in phagocytosing cells and therefore used the clathrin-DsRed construct for colocalization experiments. The lack of detection of clathrin was not due to a potential processing of the protein and/or an impaired recognition by antibodies, because clathrin expressed as a fluorescent fusion protein or detected by specific antibodies labeled the same structures and were not recruited under the sites of particles attachment (Figure 5B). This suggests that the pool of AP-1 that is present around nascent phagosomes is not associated with clathrin.

These observations indicate that γ -adaptin, and thus the AP-1 complex, is present predominantly on endosomal compartments in macrophages. Although colocalization with clathrin could be observed in the cytoplasm, clathrin could not be detected at sites of phagocytosis together with AP-1.

AP-1 Controls the Surface Delivery of TNF- α

The observation that AP-1 is necessary for efficient phagosome formation and that AP-1-positive vesicles present in phagocytic cups are devoid of clathrin led us to investigate further the role of AP-1 in membrane trafficking during phagocytosis. It has recently been shown that AP-1 complexes undergo a caspase-dependent cleavage in their hinge regions in immature dendritic cells, resulting in the removal of domains responsible for the interaction with clathrin and other endocytic effectors (Santambrogio *et al.*, 2005). It was therefore possible that a similar cleavage of AP-1 existed in macrophages. Western blot analysis after γ -adaptin immunoprecipitation in RAW264.7 cells revealed the presence of two bands for AP-1: a high-molecular-weight form presumably corresponding to non-cleaved γ -adaptin and a lower molecular weight product

Figure 4. Recruitment of γ -adaptin at sites of phagocytosis is BFA-insensitive and controlled by ARF1. (A) RAW264.7 cells were incubated in the presence BFA (bottom panel) at 10 μ g/ml or the equivalent volume of methanol (top panel) for 30 min at 37°C, fixed, and stained with an anti-GM130 antibody followed by Cy3 anti-mouse-IgG. Cells were analyzed by wide-field microscopy with deconvolution. Medial optical sections are shown. Bar, 5 μ m. (B) Cells pretreated or not with BFA were incubated for 60 min at 37°C with IgG-SRBCs, then fixed, and stained with Cy2-anti-rabbit IgG. The efficiencies of association (left) and phagocytosis (right) were determined as described in Figure 2. The means $\pm$ SEM of three independent experiments are plotted. (C) γ -adaptin recruitment during phagocytosis was evaluated in the presence of brefeldin A (BFA, bottom panels) or in the presence of the equivalent volume of methanol (control, top panels). RAW264.7 cells were fixed after 3 min of contact with IgG-SRBCs and stained with Cy2 anti-rabbit IgG to detect external SRBCs (middle panels). The cells were then permeabilized and labeled with anti- γ -adaptin followed by Cy3 anti-mouse IgG (left panels) and Alexa350-phalloidin (right panels). Cells were analyzed by wide-field microscopy with deconvolution. Medial optical sections are shown. γ -adaptin exhibits a perinuclear localization in control cells (arrow), which is lost in BFA-treated cells. By contrast, γ -adaptin is enriched in phagocytic cups (arrowheads) in control as well as in BFA-treated cells. Bar, 5 μ m. (D) BFA-treated and control cells were scored for γ -adaptin accumulations around bound particles. Results are expressed as a percentage of control cells. Means $\pm$ SEM of $n = 7$ independent experiments are plotted. (E) RAW264.7 cells transiently transfected to express ARF1_{T31N} or ARF6_{T26N} were incubated with IgG-SRBCs for 3 min at 37°C. Accumulation of γ -adaptin at sites of particles attachment was scored in 50 ARF1_{T31N}-positive cells or 50 ARF6_{T26N}-positive cells as well as 50 negative control cells on the same coverslips. Results are expressed as a percentage of control cells. Means $\pm$ SEM of $n = 6$ independent experiments are plotted. Both ARF1_{T31N} and ARF6_{T26N} significantly inhibited AP-1 accumulations at phagocytic sites ($p < 0.005$). (F and G) RAW264.7 cells transfected with ARF1_{T31N} were incubated for 60 min at 37°C with IgG-SRBCs, then fixed, and stained with Cy2-anti-rabbit F(ab')₂ to detect external SRBCs. The efficiencies of association (F) and phagocytosis (G) were calculated as described in *Material and Methods* in 50 transfected or control cells (control). Results are expressed as a percentage of control cells. The means $\pm$ SEM are the result of four independent experiments. ARF1_{T31N} significantly inhibited phagocytosis ($p < 0.05$) but not association of particles ($p > 0.05$).

Figure 5. Colocalization of AP-1 with subcellular markers in RAW264.7 macrophages. (A) When indicated, RAW264.7 cells were incubated with Alexa488-coupled transferrin internalized for 10 min at 37°C (D–F) or transiently transfected to express GFP-VAMP3 (G–I). The cells were then fixed and stained with anti- γ -adaptin followed by Cy3 anti-mouse IgG (B, E, H, and K) together with anti-TGN (A) or anti-clathrin (J) antibodies. Cells were analyzed by wide-field microscopy with deconvolution. Medial optical sections are shown. Combined images are presented (C, F, I, and L). Insets are shown in D–L. Images were converted numerically using the AMIRA software to calculate the volume rendering and visualize the colocalization between the TGN (green) and γ -adaptin (red; C). Bar, 5 μ m. (B) RAW264.7 cells transiently expressing clathrin-DsRed were incubated for 3 min with IgG-SRBCs, fixed, permeabilized, and labeled with anti-clathrin heavy chain followed by Cy2 anti-mouse F(ab')₂ (green in merged image) and Alexa350-phalloidin (blue in merged image) to detect polymerized actin at phagocytic sites. Cells were analyzed by wide-field microscopy with deconvolution. Combined image (right panel) shows no recruitment of clathrin at phagocytic sites. Bar, 5 μ m. (C) RAW264.7 cells transiently expressing Clathrin-DsRed were incubated for 3 min with IgG-SRBCs (blue in merge), fixed, permeabilized, and labeled with anti- γ -adaptin followed by Cy3 anti-mouse F(ab')₂ to detect AP-1 complexes (middle panel, red in merge). Cells were analyzed by wide-field microscopy with deconvolution. Although AP-1 is recruited in the phagocytic site, clathrin is not, as shown in combined image (right panel). Bar, 5 μ m.

(Figure 6A). Of note, we never observed such degradation products in HeLa cells in the same conditions, but freshly

isolated human monocytes expressed a lower molecular weight form as well (Figure 6A).

These observations indicate that a fraction of γ -adaptin is cleaved in macrophages as observed in dendritic cells and that truncated AP-1 complexes could play a role in organizing endosomal sorting by interacting with cargos rather than participating in the formation of clathrin buds. To test this hypothesis, we set out to determine if the surface delivery of cargos could be altered in AP-1-depleted cells. For this, we chose to follow the export of TNF- α , which was recently shown to be trafficked from the Golgi apparatus to the recycling endosomes before being delivered to the plasma membrane in a VAMP3-dependent manner (Murray *et al.*, 2005). Microscopic analysis showed an induction of TNF- α after activation of macrophages with LPS (not shown), as reported (Murray *et al.*, 2005). In our hands, LPS was more potent than phagocytosis to trigger the surface delivery of TNF- α . We observed a partial colocalization of TNF- α with AP-1 in the perinuclear region as well as in cytoplasmic vesicles close to the plasma membrane, where the staining sometimes coincided with fluorescently internalized Tf that labels endosomes (Figure 6B). Therefore, we followed by flow cytometry the surface expression of TNF- α after LPS stimulation of macrophages in cells where AP-1 was depleted and compared it to control siRNA-treated cells. Interestingly, although we could not specifically select the AP-1-depleted cell population, knocking down AP-1 lead to an increased TNF- α surface delivery ($144 \pm 6\%$, $n = 4$) compared with control cells (Figure 6, C and D). This observation indicates that AP-1 could play a role in retention of cargo proteins such as TNF- α at the level of endosomes.

DISCUSSION

In this work, we show that the adaptor complexes AP-1 but not -2 is recruited below phagocytic sites and is necessary for efficient phagocytosis mediated by FcRs in murine macrophages. Recruitment of AP-1 is controlled by ARF1 in a BFA-independent manner, suggesting that activation of ARF1 does not occur at the level of Golgi membranes. AP-1 complexes were indeed present on endosomal structures but clathrin was not accumulated together with AP-1 under phagocytosed particles. In addition, depletion of AP-1 lead to an increase in the surface delivery of TNF- α , which trafficks from the TGN to the plasma membrane via endosomes. These results support a role of AP complexes in cargo sorting at the level of endosomal compartments.

Control of AP-1 Recruitment by ARF1

We show here that AP-1 recruitment in phagocytic cups is positively regulated by ARF1, but not by ARF6. This result was somehow unexpected because ARF6 controls endosomal membrane delivery at the onset of phagocytosis (Niedergang *et al.*, 2003), whereas ARF1 was supposed not to be involved in phagocytosis, because phagocytosis is not impaired by BFA (Zhang *et al.*, 1998; Becker *et al.*, 2005). A recent report using elegant *in vivo* fluorescence microscopy however shows that ARF1 and ARF6 are indeed activated early during phagosome formation and proposes that both ARF proteins coordinate different functions at the onset of phagocytosis (Beemiller *et al.*, 2006). Given that ARF1, but not ARF6, controls the recruitment of AP-1-positive structures in phagocytic cups (Figure 4) and given that ARF6 is implicated in the control

Figure 6. AP-1 is cleaved in macrophages, and its depletion leads to increased surface detection of TNF- α . (A) HeLa cells, RAW264.7 macrophages and freshly isolated human monocytes were lysed, immunoprecipitated with anti- γ -adaptin antibody, run onto SDS-PAGE, and analyzed by Western blotting with anti- γ -adaptin antibodies. For HeLa and RAW264.7 cells an aliquot of the total cell lysate was also analyzed. (B) RAW264.7 cells were incubated with Alexa633-coupled transferrin internalized for 30 min at 37°C. The cells were then fixed, permeabilized, and stained with anti- γ -adaptin and anti-TNF- α followed, respectively, by Cy3 anti-mouse F(ab') $_2$ and Cy2 anti-rat F(ab') $_2$. Cells were analyzed by wide-field microscope with deconvolution. A Z projection of eight planes is shown. Bar, 5 μ m. (C) Surface TNF- α was detected by flow cytometry in AP-1-depleted cells (thin line) and compared with control siRNA-depleted cells (bold line). Cells stained with the secondary antibodies alone were used as a control (dotted line). (D) Mean fluorescence intensities of surface TNF- α detection in AP-1-depleted cells were expressed as a percentage of control siRNA-depleted cells. Data are the means $\pm$ SEM of four experiments. Depletion of AP-1 lead to a significant increase in the surface TNF- α on macrophages compared with control siRNA-treated cells ($p < 0.0005$).

of membrane tethering and fusion steps (Donaldson, 2003; D'Souza-Schorey and Chavrier, 2006), our results are consistent with subsequent roles of ARF1 and ARF6: ARF1, which is not activated in a particular region under the nascent phagosome (Beemiller *et al.*, 2006), would control sorting at the level of endosomes located in the cytoplasm underneath attached particles, whereas ARF6, which is specifically activated at the tips of the pseudopods (Beemiller *et al.*, 2006), would control a later step at the leading edge of the phagocytic cup (see Figure 7).

Nature of the AP-1-positive Vesicles and Absence of Clathrin

Association of ARF1 and ARF6 with membranes is stabilized by GDP/GTP exchange, and this step is catalyzed by GEFs. We observed that phagocytosis and AP-1 recruitment under the sites of phagocytosis were unaffected by the presence of BFA. Because BFA inhibits Golgi-associated ARF1-GEFs, the activation of ARF1 during phagocytosis most likely involves GEFs of the ARNO family associated with non-Golgi compartments (Jackson and Casanova, 2000; D'Souza-Schorey and Chavrier, 2006). Interestingly, a recent study shows that activated GTP-bound ARF6 recruits ARNO/cytohesin GEFs at the plasma membrane by binding to their pleckstrin homology domain. This in turn activates ARF1 (Cohen *et al.*, 2007). In addition, our fluorescent and ultrastructural analyses demonstrate that at steady state AP-1 is localized on endosomal structures scattered throughout the cytoplasm in macrophages. In other cell types, AP-1 strongly accumulates in a perinuclear region corresponding to the TGN, but it has also been found on endosomes and specialized granules (Dittie *et al.*, 1996; Le Borgne *et al.*, 1996; Stoorvogel *et al.*, 1996; Futter *et al.*, 1998; Klumperman *et al.*, 1998; Mallard *et al.*, 1998; Theos *et al.*, 2005; Popoff *et al.*, 2007).

Interestingly, although AP-1 and clathrin were colocalized in resting cells, we found AP-1 but not clathrin under the sites of particles attachment. In addition, AP-1 was found on punctate endosomal structures in the cytoplasm underneath the forming phagosome, but did not decorate the plasma membrane or the membrane of the phagosome. This is consistent with the localization of AP-1 in mammalian phagocytes as reported previously in Lefkir *et al.* (2004) and is different from the situation in *Dictyostelium*, where AP-1 was found on phagosomal membranes and could be identified on purified phagosomal fractions. The pattern of accumulation we observed below phagocytosed particles in murine macrophages is very different from the pattern of VAMP3 recruitment on forming phagosomes (Bajno *et al.*, 2000; Niedergang *et al.*, 2003), which indicates that the AP-1-positive membranes do not fuse with the plasma membrane, whereas VAMP3-positive membranes do so (see Figure 7).

We did not observe enriched clathrin or AP-2 under the sites of attachment of IgG-opsonized SRBCs. These observations were made when we stained the cells with antibodies against clathrin or AP-2 or when we expressed clathrin-DsRed or alpha-adaptin-GFP, which rules out a problem of detection by the antibodies used. This observation differs from some studies reporting the presence of clathrin in phagocytic cups (Aggeler and Werb, 1982; Takemura *et al.*, 1986; Perry *et al.*, 1999; Castellano *et al.*, 2000; Lefkir *et al.*, 2004). Many differences in the experimental systems might explain these discrepancies. In *Dictyostelium*, clathrin was found associated with purified phagosomes together with AP-1 (Lefkir *et al.*, 2004). Another difference is the size of the particles. Indeed, particles internalized in Aggeler *et al.* were latex beads of 0.1 μ m only. These beads may therefore be internalized by a classical clathrin-coated pit. FcR clustering

Figure 7. Schematic representation of the possible role of adaptors during phagocytosis. This model proposes that AP-1 complexes exist as two pools in macrophages. The uncleaved proteins would be recruited on Golgi membranes after activation of ARF1 by a BFA-sensitive GEF, whereas the cleaved proteins would be recruited on endosomal membranes close to sites of particle attachment after activation of ARF1 by a BFA-insensitive GEF. In addition, no clathrin is associated with the AP-1 complexes in the cytoplasm under the sites of phagosome formation. The cleaved complexes without clathrin would help segregate proteins away from recycling to the plasma membrane during phagocytosis, a process that is necessary for phagocytosis to be efficiently completed. ARF6 would be important for later steps of VAMP3-dependent membrane fusion and pseudopod extension.

on coverslips during frustrated phagocytosis might also favor the accumulation of clathrin-coated regions (Takemura *et al.*, 1986). Coated pits were also observed in phagosomes induced by a system mimicking receptor-mediated phagocytosis by direct recruitment of active Rac1 under the plasma membrane (Castellano *et al.*, 2000). Because ligation of receptors connected to active Rac1 was induced at 37°C before beads addition, and because active Rac1 was shown to block clathrin-mediated endocytosis (Lamaze *et al.*, 1996), this system might be prone to accumulate clathrin-coated pits. However, in our experimental system, the lack of accumulation of AP-2 and clathrin in phagocytic cups is consistent with data showing that specific inhibition of clathrin-mediated endocytosis by a dominant negative Eps15 construct (Niedergang *et al.*, 2003) or by RNAi against clathrin (Tse *et al.*, 2003) does not impair FcR-mediated phagocytosis. Therefore, in the case of FcR-mediated phagocytosis, clathrin is not required for internalization to proceed. This suggests another role for AP-1 at the onset of phagocytosis than promoting clathrin coat formation and vesicle budding.

AP-1 Cleavage and Role for APs without Clathrin in Cargo Sorting

The absence of clathrin associated with AP-1 was correlated with the presence of two forms of the γ -adaptin subunit of AP-1 in macrophages: a higher and a lower molecular weight protein of a size compatible with cleavage of the ear domain, as reported previously in dendritic cells (DCs; Santambrogio *et al.*, 2005). Such a cleavage of the ear domain was proposed to modify the properties of the AP-1 complex, which would be still recruited efficiently onto membranes but, because of a lack of interaction with clathrin and some accessory proteins, would be unable to promote the budding of a vesicle (Santambrogio *et al.*, 2005). In immature DCs, cleaved AP-1 would retain proteins such as the major histocompatibility complex proteins in endosomes, whereas in mature cells, intact

AP-1 would allow vesicle budding and surface delivery of the proteins. Cleavage of large subunits of AP-1 in immature DCs relies on a caspase-dependent mechanism (Wong *et al.*, 2004; Santambrogio *et al.*, 2005). We therefore tested if the mechanism was similar in macrophages, but treatment of macrophages with a pan-caspase inhibitor did not prevent the cleavage of the γ -adaptin subunit. In addition, this cleavage was not regulated during phagocytosis (data not shown). Therefore, it seems that two pools of AP-1 complexes coexist in macrophages. Based on the lack of clathrin recruitment at sites of particles attachment, we propose that the complexes associated with the vesicles present under phagocytic cups are the cleaved complexes. We could not directly test this hypothesis because we had no possibility to differentially purify the AP-1-positive vesicles located below the forming phagosomes. We therefore speculate that these complexes could serve as a platform of sorting and retention for some cargos (see Figure 7). In line with this hypothesis, we observed an increase in TNF- α surface expression when AP-1 γ -adaptin was depleted. Similarly, the surface delivery of Lamp-1 was augmented in cells depleted for AP-3 (Dell'Angelica *et al.*, 1999; Peden *et al.*, 2002 and our unpublished observations). Membrane delivery at sites of phagosome formation would therefore involve subdomains of endosomes devoid of AP-1 but bearing the SNARE proteins such as VAMP3 (Bajno *et al.*, 2000; Niedergang *et al.*, 2003; Figure 7).

Because AP-1 and -3 complexes are required for efficient phagocytosis to proceed, our results lead us to suggest that AP complexes, by acting as retention platforms at the level of endosomes, control the outcome of a trafficking event that is the hallmark of specialized cells, phagocytosis. Whether AP complexes use similar mechanisms to modulate intracellular recycling pathways that are crucial for cell growth, migration, or spreading will be of major interest for future studies.

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