

Molecular Definition of Distinct Cytoskeletal Structures Involved in Complement- and Fc Receptor-mediated Phagocytosis in Macrophages

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Summary

It has long been known from the results of ultrastructural studies that complement- and immunoglobulin G (IgG)-opsonized particles are phagocytosed differently by macrophages (Kaplan, G. 1977. *Scand. J. Immunol.* 6:797–807). Complement-opsonized particles sink into the cell, whereas IgG-coated particles are engulfed by lamellipodia, which project from the cell surface. The molecular basis for these differences is unknown. We used indirect immunofluorescence and confocal microscopy to examine how cytoskeletal proteins associate with phagosomes containing complement-opsonized zymosan (COZ) particles or IgG beads in phorbol-myristate-acetate-treated peritoneal macrophages. During ingestion of COZ, punctate structures rich in F-actin, vinculin, α -actinin, paxillin, and phosphotyrosine-containing proteins are distributed over the phagosome surface. These foci are detected beneath bound COZ within 30 s of warming the cells to 37°C, and their formation requires active protein kinase C. By contrast, during Fc receptor-mediated phagocytosis, all proteins examined were uniformly distributed on or near the phagosome surface. Moreover, ingestion of IgG beads was blocked by tyrosine kinase inhibitors, whereas phagocytosis of COZ was not. Thus, the signals required for particle ingestion, and the arrangement of cytoskeletal proteins on the phagosome surface, vary depending upon which phagocytic receptor is engaged. Moreover, complement receptor (CR)-mediated internalization required intact microtubules and was accompanied by the accumulation of vesicles beneath the forming phagosome, suggesting that membrane trafficking plays a key role in CR-mediated phagocytosis.

Phagocytosis is an essential arm of the immune response, involved in the clearance and destruction of invading pathogens. In response to a bacterial infection, antibodies are produced that coat the microbe surface and stimulate its ingestion via Fc receptors (FcRs)¹ on the surface of macrophages (1). By contrast, complement proteins are constitutively present in serum and opsonize bacteria nonspecifically (2). Receptors for C3b and C3bi mediate phagocytosis of complement-opsonized particles, and the C3bi receptor (Mac-1, CD11b/CD18, $\alpha_M\beta_2$) is an integrin (3, 4). FcRs are constitutively active for phagocytosis (1, 5), whereas the CRs of resident peritoneal macrophages bind but do not internalize particles in the absence of additional stimuli (3–5). Although PMA, TNF- α , granulocyte/macrophage colony-stimulating factor (GM-CSF), or attachment to lami-

nin- or fibronectin-coated substrata can activate CRs for ingestion (6–10), their mechanism of action remains unclear (5). Opsonin-independent phagocytosis can also occur when sugar residues on the microbe surface bind mannose-fucose or β -glucan receptors on the macrophage plasma membrane (11, 12).

Although all types of phagocytosis require actin polymerization at the site of ingestion (5), results of electron microscopy (EM) studies demonstrate that IgG- and complement-opsonized particles are internalized differently by macrophages (13). During FcR-mediated phagocytosis, veils of membrane rise above the cell surface and tightly surround the particle before drawing it into the body of the macrophage (13). Furthermore, Silverstein and colleagues demonstrated that ingestion occurs by a zipper process, in which FcRs in the macrophage plasma membrane interact sequentially with IgG molecules distributed over the surface of the particle being ingested (5). On the other hand, EM data indicate that CR-mediated phagocytosis is a relatively passive process that occurs by a variation of the classic zipper model. Complement-opsonized particles appear to sink into the cell with elaboration of small, if any, pseudopodia (13). Moreover, the phagosome membrane is

¹Abbreviations used in this paper: CBS, bovine calf serum; COZ, complement-opsonized zymosan; EM, electron microscopy; FBS, fetal bovine serum; FcR, Fc receptor; GM-CSF, granulocyte/macrophage colony-stimulating factor; MARCKS, myristoylated alanine-rich C kinase substrate; MFR, mannose-fucose receptor; PKC, protein kinase C; PKC- α , α isozyme of PKC; PY protein, protein containing phosphotyrosine residues.

less tightly apposed to complement-opsonized particles, with point-like contact areas separating regions of looser membrane (13). The proteins that mediate these points of contact, as well as the signals required for their formation, have not yet been identified.

Podosomes are punctate contacts where the plasma membrane is attached loosely to the substratum via the actin cytoskeleton. This type of attachment is common to monocytes, osteoclasts, spreading macrophages, and other motile cells (14–16). Many of the proteins found in podosomes are also associated with nascent phagosomes containing unopsonized zymosan particles or IgG-coated SRBCs, including F-actin, talin, myristoylated alanine-rich C kinase substrate (MARCKS), substrates for tyrosine kinases, and protein kinase C (PKC) (17–20).

We show here that the components of the cortical cytoskeleton present on nascent phagosomes, as well as the arrangement of these proteins, vary depending upon which phagocytic receptor has been engaged. During CR-mediated phagocytosis, F-actin, vinculin, α -actinin, paxillin, and proteins containing phosphotyrosine residues were enriched in discrete regions or foci on the phagosome surface, whereas CD11b/CD18, talin, MARCKS, and the α isozyme of protein kinase C (PKC- α) were distributed more uniformly. These foci of cytoskeletal proteins may represent the point-like contact areas previously seen by using EM, and their formation was blocked by inhibitors of PKC, but not by inhibitors of protein tyrosine kinases. By contrast, all proteins examined were diffusely distributed on phagosomes containing IgG beads. These data provide molecular evidence for the hypothesis that macrophages use distinct mechanisms to phagocytose IgG-opsonized or complement-opsonized particles.

Materials and Methods

Materials. PMA, chelerythrine, herbimycin and lavendustin were purchased from LC Laboratories (Woburn, MA). FBS and bovine calf serum (CBS) were from HyClone Laboratories (Logan, UT). Bio-Gel P-100 was from Bio-Rad (Melville, NY). SRBCs and rabbit anti-SRBC IgG and IgM were from Cappel/Organon Teknika (Durham, NC). Unless indicated otherwise, all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Macrophage Cultures and Activation. Peritoneal macrophages from female ICR mice (Charles River Laboratories, Wilmington, MA) were plated on glass coverslips (Propper Manufacturing Company, Inc., Long Island City, NY) in MEM α containing 1% L-glutamine, 100 U/ml penicillin G, and 100 μ g/ml streptomycin (all from JRH Biosciences, Lenexa, KS) and were incubated overnight at 37°C. The CRs of resident peritoneal macrophages were activated for phagocytosis by treating cells with 200 nM PMA for 15 min at 37°C (6, 8, 9) in Hepes-buffered RPMI medium (JRH Biosciences) prior to addition of particles and initiation of phagocytosis (see below). Activated macrophages were harvested by peritoneal lavage 5–7 d after injecting 1 ml of 1% pyrogen-free Biogel P-100 into the peritoneal cavities of ICR mice and were cultured as described above.

Opsonization of Particles. M-280 supraparamagnetic beads (IgG-

beads) coated with sheep anti-rabbit IgG, or sheep anti-mouse IgG, were purchased from Dynal (Lake Success, NY). Zymosan particles (Sigma, St. Louis, MO) were prepared as described (21). Zymosan was opsonized with complement components by nutating for 1 h at 37°C in fresh FBS or CBS as described (22, 23). Under these conditions, C3b is rapidly fixed onto zymosan, and ~80% of the C3b is converted to C3bi (23). Alternatively, zymosan was opsonized with IgG (Molecular Probes, Inc., Eugene, OR) as directed by the manufacturer. Immunofluorescence microscopy after staining with anti-C3c antibodies (23) and/or the appropriate fluorescent secondary antibodies (see below) demonstrated that C3bi was uniformly distributed on the surface of complement-opsonized zymosan (COZ) and that IgG was uniformly distributed on the surface of IgG beads or IgG-zymosan. SRBCs were opsonized with IgG, or IgM and serum (to deposit C3bi [23]), as described (13).

Phagocytosis. To synchronize internalization, particles were centrifuged onto macrophages at $450 \times g$ for 2 min at 20°C, and cells were incubated at 37°C to initiate particle uptake. After various times, cells were fixed and processed for microscopy.

Fluorescence Microscopy. Macrophages were fixed and permeabilized as previously described (20) and were blocked for 1 h at 25°C in PAB (PBS plus 0.2 g/l sodium azide and 5 g/l BSA). Blocked cells were stained as previously described (20). Confocal microscopy and indirect immunofluorescence microscopy were performed as described (20).

Antibodies. F-actin was detected with FITC-phalloidin or rhodamine-phalloidin (Molecular Probes). Affinity-purified rabbit anti-murine MARCKS antibody was prepared as previously described (24, 25) and was detected with a Texas red-conjugated goat anti-rabbit secondary antibody (Molecular Probes) or FITC-conjugated goat anti-rabbit (Fab')₂ IgG (Tago, Inc., Burlingame, CA). Mouse mAbs to vinculin (VIN-11-5), talin (8d4), and α -actinin (IgM, BM-75.2) were obtained from Sigma. Mouse anti-phosphotyrosine antibodies (4G10) and antibodies to PKC- α (M6) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Mouse mAbs to paxillin (ZO35) were purchased from Zymed Laboratories, Inc. (South San Francisco, CA). Antibodies to complement C3c were obtained from Accurate Chemical and Scientific Corp., (Westbury, NY). Mouse mAbs were visualized with a FITC-conjugated, affinity-purified, goat anti-mouse IgG plus IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) or Texas red-conjugated goat anti-mouse antibodies (Molecular Probes). Rat mAbs M1/70 and 2.4G2, which detect CD11b and Fc receptors Fc γ RII and RIII, respectively, were the generous gift of Dr. Ralph Steinman (Rockefeller University) and were detected with a FITC-conjugated goat anti-rat (Fab')₂ IgG (Tago).

Electron Microscopy. PMA-treated peritoneal macrophages plated on 60-mm dishes ingested COZ or IgG-opsonized zymosan for 2 min at 37°C as described above. Cells were fixed and processed for cryo-EM as previously described (26).

Inhibitor Studies. To inhibit PKC, macrophages were treated with 200 nM PMA for 15 min at 37°C; 0.5 μ M staurosporine, 12 μ M chelerythrine chloride, or vehicle (DMSO) was added; and, after an additional 15 min at 37°C, opsonized particles were centrifuged onto the cells. After 5–60 min at 37°C, cells were fixed and stained for indirect immunofluorescence microscopy. To inhibit tyrosine kinases, macrophages were incubated overnight with 10 μ M herbimycin or 50 μ M lavendustin. PMA (200 nM) was added 15 min before addition of particles, and phagocytosis was initiated as described above. To depolymerize microtubules, macrophages were treated with 2 μ g/ml nocodazole for 15 min

at 37°C, 200 nM PMA was added, and cells were incubated for an additional 15 min at 37°C before addition of particles and initiation of phagocytosis. For some studies, resident peritoneal macrophages were plated on coverslips coated with 0.1 mg/ml fibronectin to activate their complement receptors for phagocytosis (7, 8). Attachment indices were determined by use of phase contrast optics and are presented as particles bound per 100 macrophages. Phagocytic indices were determined by examining the same coverslips for F-actin polymerization and accumulation of

proteins containing phosphotyrosine residues or paxillin on nascent phagosomes as previously described (20). Alternatively, cells were fixed and stained with Diff-Quick (Baxter Scientific Products, McGraw Park, IL).

Results

Previous EM studies demonstrated that the morphology of nascent phagosomes is different when macrophages ingest IgG- or complement-opsonized particles (13), and we confirmed these data here (Fig. 1): during FcR-mediated phagocytosis, lamellipodia protrude from the macrophage surface and form a phagosome that is tightly apposed to the particle, whereas during CR-mediated phagocytosis, particles sink directly into the body of the macrophage. The molecular basis for these different internalization mechanisms is not yet understood.

Experimental Design. In this study, we compared how cytoskeletal proteins associate with phagosomes containing COZ particles or IgG beads in PMA-treated peritoneal macrophages. Although these particles are not identical, we chose this system for the following reasons. Complement-opsonized SRBCs could not be used, owing to hemoglobin autofluorescence, and because opsonized SRBC ghosts retained erythrocyte proteins that interfered with detection of cytoskeletal proteins on the phagosome surface (data not shown). Other types of particles were also somewhat autofluorescent and partially obscured the subtle staining patterns observed with complement-opsonized particles. By contrast, zymosan particles are nearly transparent by fluorescence microscopy (20). Thus, using COZ, we could clearly display the novel clusters of cytoskeletal proteins we observed on the phagosome surface during CR-mediated phagocytosis (see below). These structures were present, but more difficult to detect, on complement-opsonized magnetic beads or SRBCs (data not shown).

We have previously shown that unopsonized zymosan is constitutively phagocytosed by peritoneal macrophages (20), and this particle uptake is mediated by mannose-fucose receptors (MFRs) and/or β -glucan receptors in the macrophage plasma membrane (11, 12). By contrast, three lines of evidence demonstrate clearly that COZ ingestion was mediated by CRs. First, COZ was not phagocytosed by peritoneal macrophages in the absence of a CR-activating stimulus (Fig. 2). Second, internalization of COZ was inhibited \sim 80% in the absence of divalent cations, or in the presence of saturating amounts of antibody M1/70 (data not shown). Third, CD11b was enriched on phagosomes containing COZ, but not on phagosomes containing unopsonized zymosan or IgG-opsonized particles (Fig. 2 and data not shown). In all cases, zymosan opsonized with heat-inactivated serum (which lacks active complement components [2]) was indistinguishable from unopsonized zymosan (data not shown).

Since MFRs and FcRs are constitutively active for phagocytosis (5), we used IgG beads rather than IgG-zymosan to ensure that IgG-opsonized particles were not ingested via the MFR. Phagocytosis of IgG beads was 70% inhibited in

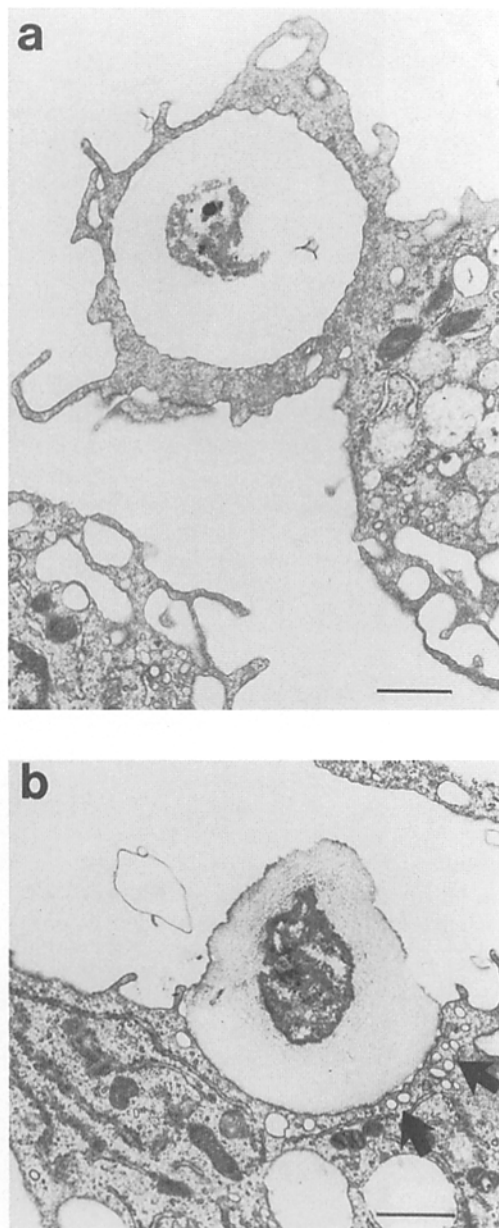


Figure 1. Complement or IgG-opsonized particles are internalized differently by macrophages. Cryo-EM sections of peritoneal macrophages that had ingested IgG-opsonized particles (*A*) or complement-opsonized particles (*B*) for 2 min at 37°C. Note that pseudopodia protrude from the macrophage surface to engulf the IgG-opsonized particle, whereas the complement-opsonized particle sinks directly into the macrophage. Arrows in *B* indicate vesicles directly beneath the forming COZ phagosome that are absent beneath the phagosome in *A*. Bars, 1 μ m.

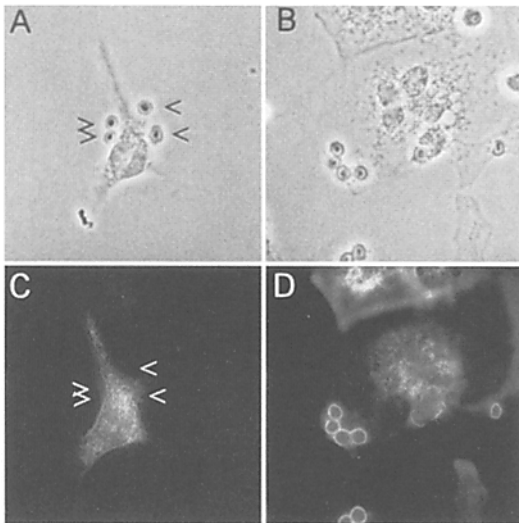


Figure 2. COZ particles are not ingested by peritoneal macrophages in the absence of a receptor-activating stimulus. Resident peritoneal macrophages plated on glass coverslips were cultured for 15 min at 37°C in the presence (B and D) or absence (A and C) of 200 nM PMA and were then allowed to ingest COZ for 5 min at 37°C. Cells were fixed and permeabilized and stained with antibodies to CD11b as described in *Materials and Methods*. A and B, phase contrast. C and D, CD11b, COZ, complement-opsonized zymosan. Arrowheads in A and C indicate COZ bound but not internalized in the absence of PMA.

the presence of the FcR-blocking antibody 2.4G2. Moreover, FcγRII and FcγRIII were enriched on phagosomes containing IgG beads, but not on phagosomes containing COZ or unopsonized zymosan (data not shown). PMA did not affect phagocytosis of IgG particles or unopsonized zymosan, or the association of cytoskeletal proteins with these structures (data not shown). Thus, a comparison of COZ and IgG beads was the best system in which to study the early cytoskeletal rearrangements associated with CR- and FcR-mediated phagocytosis.

In addition, we used PMA-treated peritoneal macrophages in this study rather than immune-activated macrophages for the following reasons. First, classically activated macrophages accumulate particles of *C. parvum* or myobacterium bovis BCG. Second, PMA-treated peritoneal macrophages responded more uniformly in our hands than did Bio-Gel-activated macrophages. Third, the ability to trigger phagocytosis of COZ with PMA facilitated the dissection of early cytoskeletal rearrangements associated with particle uptake. Fourth, it was easy to distinguish triggered, CR-mediated phagocytosis from the constitutive ingestion of unopsonized zymosan and IgG-beads in PMA-treated macrophages, whereas all particles were constitutively ingested by activated macrophages.

Vinculin and Paxillin Associate Differently with Phagosomes Containing Zymosan, IgG-opsonized, or Complement-opsonized Particles. When macrophages ingested IgG beads, both vinculin and paxillin were enriched in a diffuse pattern adjacent to nascent phagosomes (Fig. 3, C and D). By contrast, neither vinculin nor paxillin was enriched on phago-

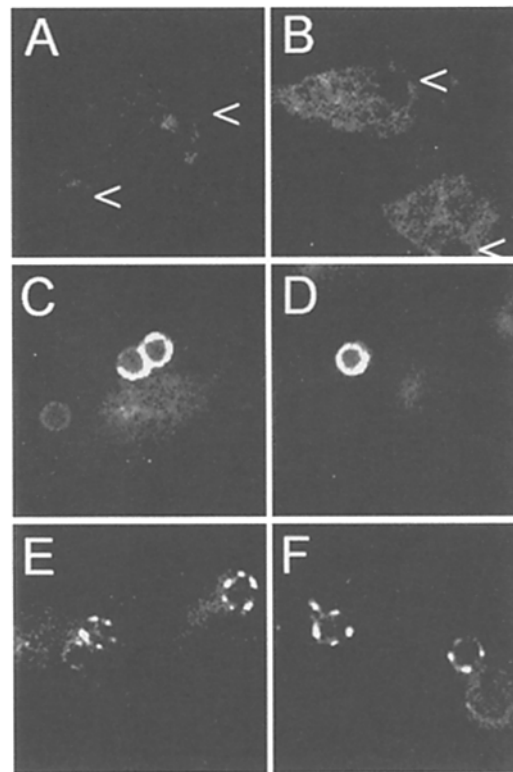


Figure 3. Vinculin and paxillin associate differently with different types of phagosomes. Peritoneal macrophages were treated with 200 nM PMA for 15 min at 37°C and then allowed to ingest unopsonized zymosan (A and B), IgG beads (C and D), or COZ (E and F) for 3 min at 37°C prior to processing for indirect immunofluorescence and confocal microscopy. Fixed and permeabilized cells were stained with antibodies to vinculin (A, C, and E) or paxillin (B, D, and F) and a FITC-conjugated secondary antibody. Each panel is a single section from the confocal microscope. Arrowheads in A and B indicate zymosan phagosomes that are not stained with anti-vinculin or anti-paxillin antibodies, respectively.

somes containing unopsonized zymosan (Fig. 3, A and B), or zymosan opsonized with heat-inactivated serum, which lacks active complement proteins (2) (data not shown). Surprisingly, yet another result was obtained for phagosomes containing COZ. In this case, vinculin and paxillin were detected in discrete spots, or foci, on early phagosomes (Fig. 3, E and F). Moreover, the punctate distribution of vinculin and paxillin appeared scattered over the entire phagosome surface (Fig. 4). These data suggest that signals generated by FcRs and CRs, but not receptors for unopsonized zymosan, such as the MFR and the β-glucan receptor (11, 12), recruit vinculin and paxillin to the periphagosomal cytoplasm.

Foci Containing F-Actin and Other Cytoskeletal Proteins Form on the Surface of COZ Phagosomes, but Not on Phagosomes Containing IgG Beads. The observation that vinculin and paxillin were distributed in foci on the surface of COZ phagosomes suggested that other cytoskeletal proteins might be distributed in a similar pattern. Indeed, F-actin, proteins containing phosphotyrosine residues (PY proteins), and α-actinin were also detected in discrete spots

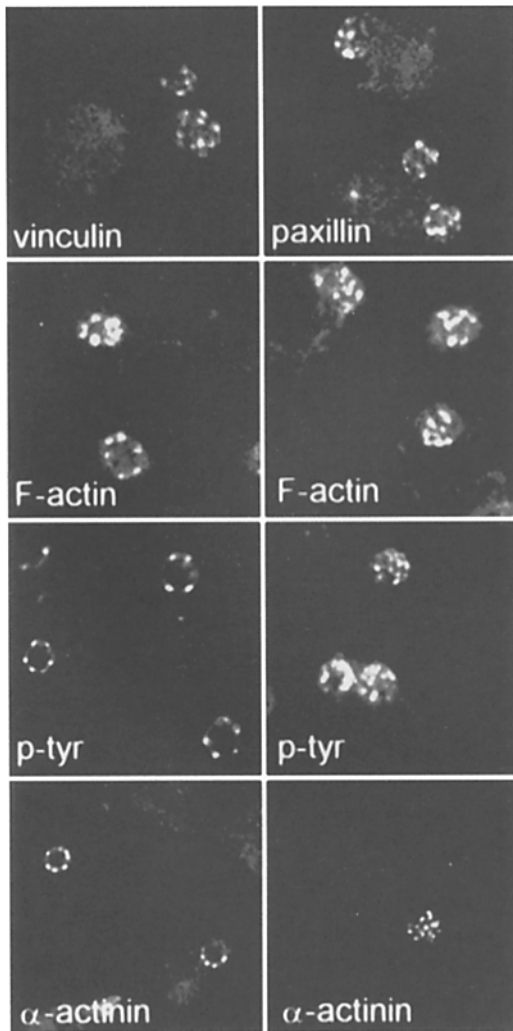


Figure 4. Vinculin, paxillin, F-actin, α -actinin, and PY proteins are enriched in discrete foci on the surface of phagosomes containing COZ. PMA-treated macrophages ingested COZ for 3 min at 37°C as described above. Fixed and permeabilized cells were stained with FITC-phalloidin to visualize F-actin, or with antibodies to vinculin, paxillin, phosphotyrosine residues, or α -actinin as described in *Materials and Methods*. Each panel is a single section from the confocal microscope. Note that the left panels for F-actin, p-tyr, and α -actinin are optical cross-sections of phagosomes, whereas the remaining panels are views of the phagosome surface. P-tyr, phosphotyrosine.

on the phagosome surface during CR-mediated phagocytosis (Fig. 4). Results of double-labeling experiments (Fig. 5) demonstrated that F-actin colocalized with PY proteins, vinculin, paxillin, and α -actinin in these punctate structures.

To examine whether the punctate distribution of cytoskeleton-associated proteins we observed was present beneath bound COZ at very early stages of particle ingestion, COZ particles were bound to the surface of PMA-treated macrophages, and after 30 s at 37°C, cells were processed for confocal microscopy. Vinculin, paxillin, F-actin, and PY proteins were present in discrete spots beneath bound

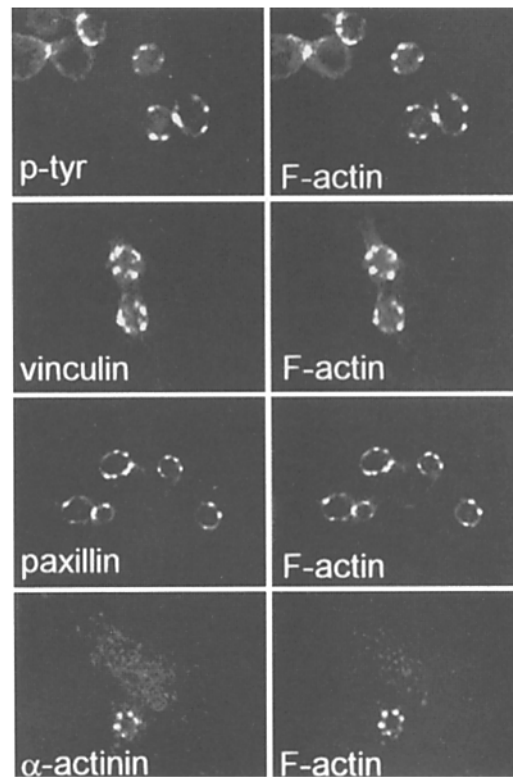


Figure 5. F-actin colocalizes with vinculin, paxillin, α -actinin, and PY proteins in punctate structures on the surface of phagosomes containing COZ. Peritoneal macrophages were pretreated with 200 nM PMA for 15 min at 37°C and then allowed to ingest COZ for 3 min at 37°C before processing for confocal microscopy. Fixed and permeabilized cells were double-stained with rhodamine-phalloidin, to visualize F-actin, and with antibodies to phosphotyrosine, vinculin, paxillin, or α -actinin as indicated. Each section represents a single section from the confocal microscope. P-tyr, phosphotyrosine.

COZ. Typically, one to four foci were detected directly below each particle (Fig. 6 and data not shown). No enrichment of cytoskeletal proteins was detected below COZ bound to the macrophage surface in the absence of PMA (data not shown), suggesting that signals generated by active complement receptors were required for their formation.

Nascent IgG bead phagosomes protruded from the cell surface and were highly enriched in F-actin, talin, vinculin, PY proteins, and MARCKS (Fig. 7 A and data not shown). As particles were internalized, F-actin, PY proteins, and α -actinin (Fig. 7 B), like vinculin and paxillin (Figs. 3 and 7 B), were uniformly distributed on or near the phagosome surface and were not detected in foci.

Taken together, these data reinforce the results of EM studies demonstrating that IgG and complement-opsonized particles are internalized differently by macrophages. It is tempting to speculate that the foci of cytoskeletal proteins we observed on the surface of COZ phagosomes occur at points where the phagosome membrane is attached to the particle via the cytoskeleton.

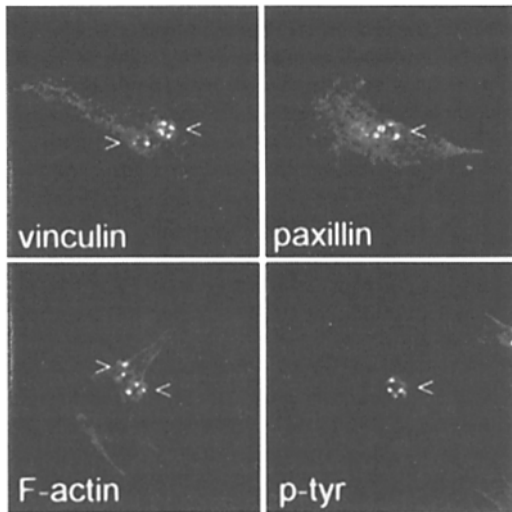


Figure 6. Focal concentrations of cytoskeletal proteins are present beneath bound COZ particles after 30 s at 37°C. Peritoneal macrophages were pretreated with PMA for 15 min at 37°C. COZ particles were centrifuged onto the cell surface, and macrophages were incubated at 37°C for 30 s, before processing for confocal microscopy. Fixed and permeabilized cells were stained with rhodamine-phalloidin to visualize F-actin, or with antibodies to vinculin, paxillin, or phosphotyrosine as indicated. Arrowheads indicate foci of cytoskeletal proteins directly below bound COZ particles. Each panel is a single section from the confocal microscope. P-tyr, phosphotyrosine.

MARCKS, PKC- α , and Talin Are Distributed Diffusely on Both COZ and IgG Bead Phagosomes. Unlike the other proteins examined thus far, CD11b, the α subunit of the C3bi receptor (3, 4), was detected as a diffuse patch beneath COZ after 30 s at 37°C and was distributed fairly uniformly on early phagosomes containing COZ (Figs. 2 and 8 A). Similarly, both PKC- α (Fig. 8 A) and talin (Fig. 8 B) were distributed in a uniform pattern near the surface of early phagosomes containing COZ and were enriched in diffuse patches below bound COZ 30 s after warming to 37°C.

We have previously shown that MARCKS is enriched on phagosomes containing unopsonized zymosan (20), and MARCKS was also enriched on phagosomes containing IgG beads (Fig. 7 B). Double staining demonstrated that MARCKS and talin were colocalized on COZ phagosomes after 3 min at 37°C (Fig. 8 B). However, MARCKS was not detected below bound COZ after 30 s at 37°C (Fig. 8 B, arrowheads), suggesting that this protein was recruited to the forming phagosome after talin and PKC- α . Alternatively, this difference may reflect the different affinities of the anti-talin and anti-MARCKS antibodies.

Collectively, the data suggest that microdomains containing different cytoskeletal and signaling proteins may be established on the phagosome surface during CR-mediated phagocytosis: CD11b, talin, MARCKS, and PKC- α were distributed uniformly on the phagosome surface, whereas F-actin, vinculin, paxillin, α -actinin, and PY proteins were enriched in discrete foci. By contrast, all of the above proteins, with the exception of CD11b, which was not de-

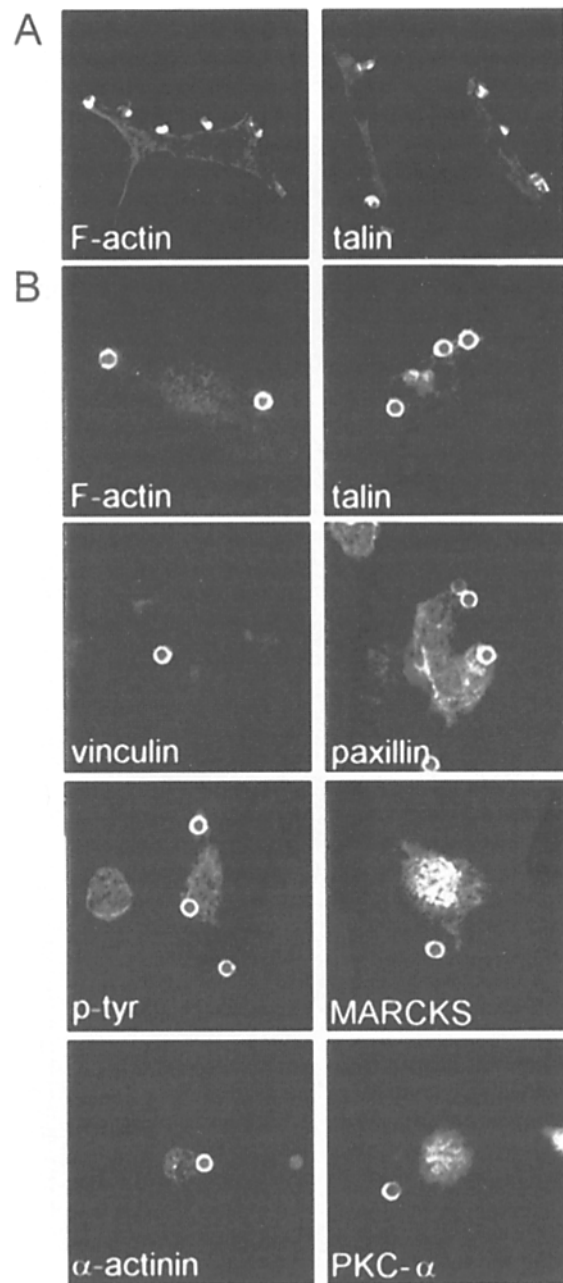
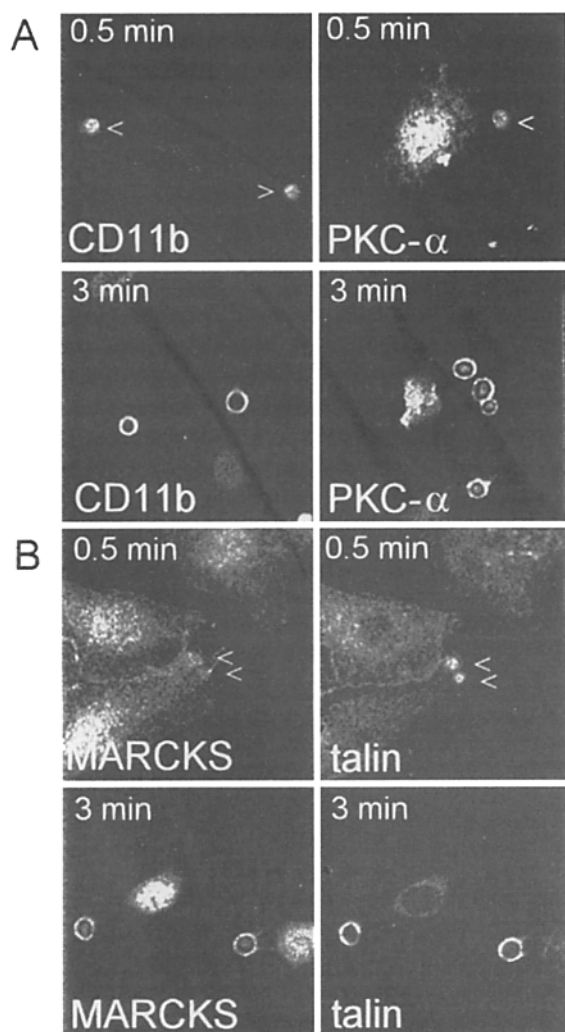


Figure 7. Cytoskeletal and signaling proteins are distributed uniformly on phagosomes containing IgG beads. PMA-treated peritoneal macrophages were allowed to ingest IgG beads for 30 s (A) or 3 min (B) at 37°C before processing for confocal microscopy. Fixed and permeabilized cells were stained with FITC-phalloidin to visualize F-actin, or with antibodies to talin, vinculin, paxillin, phosphotyrosine, MARCKS, α -actinin, or PKC- α as indicated. Each panel is a single section from the confocal microscope. Note that the forming phagosomes in A appear to protrude from the macrophage surface.

ected, were distributed in a diffuse pattern on the surface of phagosomes containing IgG beads.

PKC Inhibitors, but Not Inhibitors of Protein Tyrosine Kinases, Block CR-mediated Phagocytosis. A number of recent reports suggest that signals from PKC and/or protein tyrosine



kinases are required for phagocytosis in a variety of systems (18–20, 27, 28, 29). Therefore, we examined the effects of various inhibitors on CR-mediated phagocytosis in peritoneal macrophages. The PKC inhibitors staurosporine and chelerythrine inhibited uptake of COZ or C3bi-SRBCs, and the accumulation of F-actin, paxillin, and PY proteins below particles bound to the cell surface (Table 1, Fig. 9, and data not shown). Phagocytosis of IgG beads and IgG-SRBCs was also inhibited under these conditions (Table 1 and Fig. 9). We have previously shown that these concentrations of staurosporine and chelerythrine inhibit PKC, as judged by their ability to block phosphorylation of the PKC substrate MARCKS, yet do not inhibit tyrosine kinases (20).

Consistent with previous reports (19), FcR-mediated phagocytosis was inhibited by ~80% in the presence of the tyrosine kinase inhibitors herbimycin and lavendustin (Table 1 and Fig. 9). On the other hand, these drugs did not block ingestion of complement-opsonized particles (Table 1), or the accumulation of F-actin and paxillin foci on the phagosome surface (Fig. 9 and data not shown). Similar data were obtained with resident peritoneal macrophages

Figure 8. CD11b/CD18, talin, and MARCKS are enriched in a diffuse pattern on phagosomes containing COZ. PMA-treated peritoneal macrophages were allowed to ingest COZ for 0.5 min or 3 min at 37°C before processing for confocal microscopy as described above. In *A*, fixed and permeabilized cells were stained with antibodies to CD11b or PKC- α as indicated. In *B*, cells were double stained with antibodies to MARCKS and talin as indicated. Arrowheads in *A* indicate diffuse patches of CD11b and PKC- α below COZ bound to the macrophage surface. Arrowheads in *B* indicate regions below bound COZ that are enriched in talin, but not MARCKS.

Table 1. Effects of Various Drugs on Binding and Ingestion of Complement- or IgG-opsonized Particles

Treatment	COZ		IgG beads		C-SRBCs	IgG-SRBCs
	Attachment index	Phagocytic index	Attachment index	Phagocytic index	Phagocytic index	Phagocytic index
Control	148 ± 55	139 ± 55	212 ± 52	185 ± 40	119 ± 12	118 ± 8
Chelerythrine	111 ± 23	3 ± 3	212 ± 12	2 ± 1	16 ± 4	25 ± 5
Staurosporine	138 ± 10	7 ± 3	199 ± 28	2 ± 2	11 ± 7	11 ± 3
Herbimycin	105 ± 13	96 ± 10	137 ± 20	28 ± 12	102 ± 12	15 ± 2
Lavendustin	107 ± 10	98 ± 10	147 ± 10	38 ± 14	104 ± 11	11 ± 1
Nocodazole	141 ± 25	23 ± 12	191 ± 36	178 ± 32	ND	ND

To inhibit PKC, macrophages were incubated with 200 nM PMA for 15 min at 37°C, and 0.5 μ M staurosporine or 12 μ M chelerythrine was added to the culture medium. After an additional 15 min at 37°C, IgG- or complement-opsonized particles were centrifuged onto the cell surface, and the dishes were incubated at 37°C for 5–60 min to allow ingestion to occur. To inhibit tyrosine kinases, macrophages were treated overnight at 37°C with 10 μ M herbimycin or 50 μ M lavendustin, and 200 nM PMA was added to the culture medium 15 min prior to the addition of opsonized particles. Particles were centrifuged onto the cells, and the dishes were incubated for 5–60 min at 37°C to allow internalization to occur. To depolymerize microtubules, macrophages were treated with 2 μ g/ml nocodazole for 15 min at 37°C prior to addition of PMA. Control macrophages were treated with PMA, but not with inhibitors. Attachment indices (particles bound per 100 macrophages) and phagocytic indices (particles internalized per 100 macrophages) were determined as described in *Materials and Methods*. Data are the average \pm SD of four independent experiments.

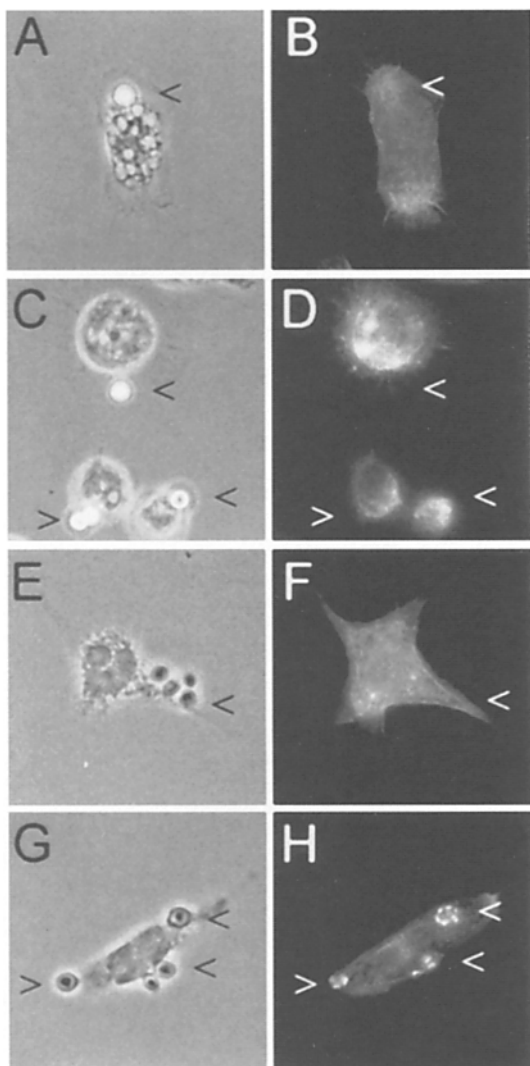


Figure 9. FcR- and CR-mediated phagocytosis show different sensitivities to inhibitors of tyrosine kinases and PKC. Macrophages were treated with 12 μ M chelerythrine and 200 nM PMA (A, B, E, and F), or 10 μ M herbimycin and 200 nM PMA (C, D, G, and H) as described in *Materials and Methods*, and were then allowed to ingest IgG beads (A–D) or COZ (E–H) for 5 min at 37°C. Fixed and permeabilized cells were stained with FITC-phalloidin to detect F-actin. Phase contrast: A, C, E, and G. F-actin fluorescence: B, D, F, and H.

whose CRs were activated for phagocytosis by plating on fibronectin-coated coverslips, or by using Bio-Gel-activated macrophages (data not shown). Taken together, these data suggest that PKC is required for CR-mediated phagocytosis, whereas FcR-mediated phagocytosis requires signals from both PKC and protein tyrosine kinases.

Consistent with previous data (30), CR-mediated phagocytosis was blocked by microtubule-destabilizing agents such as nocodazole, whereas phagocytosis of IgG beads was not (Table 1). These data reinforce the hypothesis that IgG- and complement-opsonized particles are ingested by means of different mechanisms.

Discussion

The results of this study demonstrate that the signals required for particle ingestion, as well as the arrangement of cytoskeletal proteins on the phagosome surface, vary depending upon which phagocytic receptor is engaged. Our data both confirm and extend the results of previous ultrastructural studies suggesting that complement-opsonized particles sink into the body of the macrophage, whereas ingestion of IgG-opsonized particles requires membrane to actively protrude from the cell surface to engulf the particle (13). We show here that during CR-mediated phagocytosis discrete foci containing F-actin, vinculin, paxillin, α -actinin, and PY proteins are distributed over the phagosome surface as particle uptake proceeds. Formation of these structures requires both active CRs and active PKC, since cytoskeletal proteins do not accumulate below COZ bound to Bio-Gel-activated macrophages in the presence of PKC inhibitors, or below COZ particles bound to resident peritoneal macrophages in the absence of PMA. Moreover, these foci do not reflect localized deposition of opsonin, since C3bi was uniformly distributed on the surface of COZ. By contrast, during FcR-mediated phagocytosis, nascent phagosomes rich in F-actin protrude from the macrophage surface, and F-actin, vinculin, paxillin, α -actinin, and PY proteins are enriched in a diffuse pattern, on or near the phagosome membrane during particle internalization. These data are consistent with the results of previous studies showing that F-actin, paxillin, and PY proteins are enriched on phagosomes containing IgG-SRBCs (19, 27).

CR- and FcR-mediated phagocytosis can also be distinguished by their sensitivity to inhibitors of protein tyrosine kinases and microtubule-destabilizing agents. Ingestion of complement-opsonized particles requires intact microtubules (reference 30 and this study), but not active protein tyrosine kinases. Conversely, FcR-mediated phagocytosis requires tyrosine kinases, but not intact microtubules (references 19 and 30 and this study). Nevertheless, these two types of phagocytosis do have common features. Both PKC- α and MARCKS are recruited to phagosomes containing COZ or IgG beads, and active PKC is required for phagocytosis of both types of particles. These data are consistent with the fact that PKC is enriched on phagosomes containing IgG-SRBCs, and that PKC inhibitors block FcR-mediated phagocytosis in human monocytes (18).

In macrophages, phagocytosis of unopsonized zymosan particles is mediated by MFRs or β -glucan receptors (11, 12). This is a primitive recognition system (5), and we show here that vinculin and paxillin are not recruited to phagosomes containing unopsonized zymosan, but are enriched on phagosomes containing IgG beads or COZ. The reason for this difference is unclear. Nevertheless, these data reinforce the hypothesis that each phagocytic receptor recruits a unique group of cytoskeletal proteins to the phagosome surface.

Several observations suggest that activation of PKC is required for particle ingestion during CR-mediated phagocytosis. PKC is activated during phagocytosis (20, 29), and

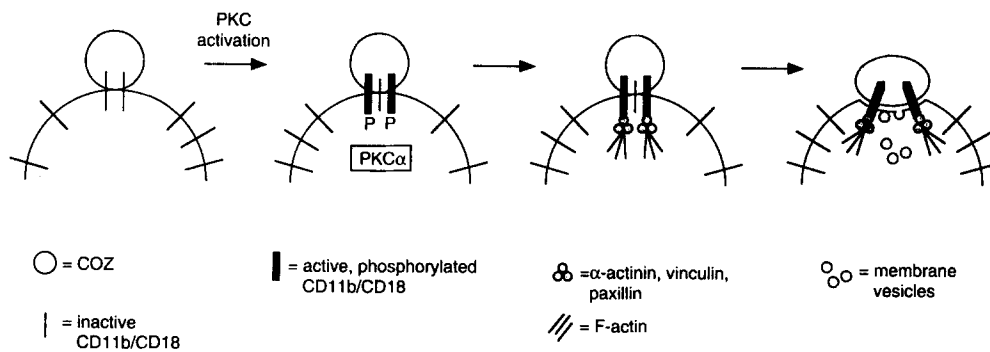


Figure 10. A model for CD11b/CD18-mediated phagocytosis in macrophages. (1) C3bi molecules on the surface of complement-opsonized particles bind to CD11b/CD18 molecules in the macrophage plasma membrane (3, 4). (2) PKC is activated, allowing CD11b/CD18 to cluster beneath bound particles (reference 43 and this study). (3) PKC- α is recruited to the cortical cytoplasm beneath bound COZ, where it colocalizes with CD11b/CD18. (4) Serine resi-

dues in the cytoplasmic tail of CD18 are phosphorylated, perhaps by PKC (31, 32). (5) α -actinin is recruited to the site of ingestion, where it binds a subset of CD18 molecules (perhaps those that have been phosphorylated). Vinculin, actin, and paxillin are also recruited to these sites and assemble, along with α -actinin, into focal structures on the phagosome membrane. (6) Actin polymerization (alone or in combination with one or more motor proteins [20, 44, 45]) draws the particle into the macrophage without protrusion of large lamellipodia (13). (7) As internalization proceeds, membrane vesicles are transported along microtubules to the site of ingestion. These vesicles fuse with the phagosome membrane, providing sufficient membrane to allow particle engulfment. (8) As the particle is drawn into the cell, additional C3bi molecules on the particle bind to CD11b/CD18, and the cycle is repeated until internalization is completed.

PKC inhibitors block phagocytosis of complement-opsonized particles (references 28 and 30 and this study) and the accumulation of cytoskeletal proteins beneath attached particles. In addition, treatment of macrophages with PMA causes the rapid and sustained phosphorylation of CD18 on serine residues (31, 32), activates CD11b/CD18 for phagocytosis (6, 9, 10), and leads to clustering of this receptor beneath bound particles (this study).

Four lines of evidence suggest that the foci containing α -actinin, vinculin, paxillin, and F-actin we observe on COZ phagosomes are the structures that mediate particle internalization. First, these foci are composed of proteins that also associate with podosomes, the punctate complexes of cytoskeletal proteins at the substrate-adherent surface of highly motile cells (14–16). Second, these same proteins are associated with phagosomes containing IgG beads. Third, CD11b/CD18 is a member of the integrin family of receptors, which link the actin cytoskeleton to the plasma membrane at the substrate-adherent surface (3, 4). Fourth, these foci are actin rich, and actin polymerization is required for particle engulfment (5).

The presence of F-actin in these foci also suggests that these complexes of cytoskeletal proteins are assembled on active rather than inactive receptors. Although both talin and α -actinin can bind β integrins (33), and both these proteins are found on the surface of COZ phagosomes, α -actinin is found only at sites that nucleate actin, whereas talin is more widely distributed. This may indicate that talin binds to inactive receptors that cannot mediate particle internalization. Consistent with this idea, Brown and co-workers demonstrated that 10–40% of the C3bi receptors at the plasma membrane are associated with the actin cytoskeleton, and that only this form of the receptor is important for phagocytosis (3, 34, 35). CR-mediated phagocytosis is accompanied by the phosphorylation of serine residues in the cytoplasmic domain of CD18 (31, 32). This suggests that receptor phosphorylation may be the feature

that distinguishes active and inactive receptors. It is also possible that phosphorylated CD18 binds α -actinin with higher affinity than talin. This notion is supported by the fact that α -actinin binds β 1 integrins with higher affinity than talin (33), and that α -actinin co-immunoprecipitates with CD18 from activated neutrophils, whereas talin does not (36). Nevertheless, how segregation of talin and α -actinin on the phagosome surface leads to assembly of F-actin at discrete sites is currently unknown.

Separation of actin foci by regions of actin-poor membrane may be required for CR-mediated particle internalization. Phagosomes containing complement-opsonized particles sink into the macrophage; thus, sites for membrane addition must exist in order for the phagosome membrane to enlarge as the particle is drawn into the cell. Membrane addition may occur by recruitment of membrane vesicles to the site of ingestion and their subsequent fusion with the phagosome membrane. Indeed, vesicles accumulate beneath forming phagosomes containing COZ (Fig. 1), and intact microtubules are required for phagocytosis of complement-opsonized particles (reference 30 and this study), supporting a role for vesicle trafficking in this process. Moreover, vesicles are likely to fuse with the plasma membrane at sites containing inactive CRs because actin filaments restrict access to the cytoplasmic face of the phagosome membrane at sites containing active CRs. There are many precedents for the ability of F-actin to restrict access of membrane vesicles to sites of membrane docking and fusion. For example, F-actin is shed from the phagosome surface before these membranes can fuse with endosomes (17, 20, 37), and, at the presynaptic junction, actin must be remodeled before synaptic vesicles can access the presynaptic membrane (for review see reference 38). By this reasoning, we propose that membrane is added to the phagosome in regions where CD11b/CD18 is inactive and F-actin foci are lacking. In addition, MARCKS may play a role in recruiting membrane to the forming phago-

some, since this protein has previously been implicated in membrane traffic (26, 39, 40). Our observation that the number of actin foci increases on the phagosome membrane as COZ particles are internalized also suggests that CD11b/CD18 molecules are continuously being activated as particle internalization proceeds. On the basis of the available data, we propose a model for CD11b/CD18-mediated phagocytosis in macrophages (Fig. 10).

It has long been known that actin polymerization is required for phagocytosis (5); however, the signals that mediate this process are unclear. Our data suggest that during CR-mediated phagocytosis, actin polymerization requires PKC, but is independent of tyrosine kinases. By contrast, Greenberg and co-workers have shown (19, 27), and we confirm here, that actin polymerization during FcR-mediated phagocytosis has an absolute requirement for tyrosine kinases. The role of PY proteins on COZ phagosomes is unclear, since tyrosine phosphorylation is not required for

particle ingestion. One possibility is that tyrosine phosphorylation increases the efficiency of ingestion, since we found that the rate of ingestion of COZ was slowed by half (10 min vs. 5 min to internalize) in the presence of herbimycin and lavendustin.

This is the first molecular description of cytoskeletal structures associated with phagosomes during CR-mediated phagocytosis. The system provides novel insights into the links between the actin cytoskeleton and integrins, and contrasts with FcR-mediated actin assembly during phagocytosis in its lack of requirement for tyrosine kinases. Moreover, FcR-mediated phagocytosis results in the production of arachidonic acid metabolites and reactive oxygen intermediates, whereas CR-mediated phagocytosis does not (41, 42). Therefore, an understanding of the differences between CR- and FcR-mediated phagocytosis has profound implications for our understanding of the inflammatory response.

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