A Role for MARCKS, the α Isozyme of Protein Kinase C and Myosin I in Zymosan Phagocytosis by Macrophages

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Summary

Myristoylated, alanine-rich C-kinase substrate (MARCKS) is a lipopolysaccharide-induced protein kinase C (PKC) substrate that has been proposed to regulate actin–membrane interactions, as well as actin structure at the membrane. We studied the distribution of MARCKS, the α isozyme of PKC (PKCα), and myosin I in lipopolysaccharide-treated peritoneal macrophages ingesting zymosan particles. MARCKS, PKCα, and myosin I colocalized with F-actin and talin in the cortical cytoplasm adjacent to forming phagocytic cups. After particle ingestion was completed, myosin I, F-actin, and talin were no longer enriched in the vicinity of the phagosome. By contrast, MARCKS and PKCα remained associated with the phagosome membrane until after acquisition of the lysosomal marker Lamp-1. Vinculin was not detected on phagosomes at any time point examined. Phagocytosis of zymosan was accompanied by rapid and sustained phosphorylation of MARCKS. Inhibitors of PKC reduced zymosan binding to the macrophage surface and blocked the focal accumulation of F-actin, talin, phosphotyrosine-containing proteins, MARCKS, and PKCα beneath attached particles. We propose that PKC-dependent phosphorylation is an early signal required for zymosan phagocytosis and that MARCKS and PKCα have a role in phagosome maturation. The colocalization of F-actin and MARCKS at the cytoplasmic face of the nascent phagosome reinforces the hypothesis that MARCKS regulates actin structure at the membrane. Our data also suggest that myosin I functions as a mechanical motor during particle uptake.

The ability of macrophages to phagocytose and kill microorganisms is an essential element of the immune response. Phagocytosis is initiated when ligands on the particle surface engage specific receptors in the macrophage plasma membrane (1). Subsequent internalization requires localized actin polymerization and the extension of membrane pseudopodia around the particle (1). Actin is disassembled from the phagosome once internalization is complete (1), and the phagosome matures, eventually fusing with lysosomes (1). It has been suggested that localized receptor-ligand interactions and actin polymerization provide the mechanical force for particle engulfment (2, 3). It is unknown whether one or more motor proteins interact with actin to drive pseudopod extension. Furthermore, the signals that recruit actin to the nascent phagosome, as well as the signals for actin assembly and disassembly, remain unclear. Phagosomes mature by recycling plasma membrane proteins to the cell surface (4), and by sequential fusion events with endosomes and lysosomes, culminating in the formation of mature phagolysosomes (5–7). Although phagosome–endosome fusion has been reconstituted in vitro (6), the processes that recruit endosomes and lysosomes to the phagosome are unknown.

The pseudopodia of nascent phagosomes are similar in many ways to lamellipodia at the leading edge of motile/spreading cells. Both structures have a high content of F-actin, talin (8, 9), and the tyrosine kinase substrate paxillin (10–12). In addition, both processes are initiated by receptor–ligand interactions, and they involve localized rearrangements of the actin cytoskeleton (1, 3). All of these events are highly regulated (1–3).

Myristoylated, alanine-rich, C-kinase substrate (MARCKS)[1] is an actin-binding protein that transduces signals in the calcium/calmodulin and protein kinase C (PKC)-dependent signalling pathways (13, 14). MARCKS binds to the sides of actin filaments and cross-links them, and this cross-linking activity is disrupted by both phosphorylation and by calcium/calmodulin (15). MARCKS is a peripheral membrane protein that binds to membranes via an NH_2-terminal myristoylated membrane binding domain, and by the interaction of the basic effector domain with acidic phospholipids (16–18). Phosphorylation introduces negative charges into the basic effector domain and displaces the protein from the membrane into the cytosol (19). Upon dephosphorylation, MARCKS reassociates

[1] *Abbreviations used in this paper:* MARCKS, myristoylated, alanine-rich C kinase substrate; PKC, protein kinase C; PKCα, α isozyme of protein kinase C.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/09/0829/12 $2.00
Volume 182 September 1995 829–840

with the membrane (19). In macrophages, MARCKS is distributed throughout the plasma membrane and is enriched in punctate podosomes at the substrate adherent surface of actively spreading cells (20), where it colocalizes with vinculin and talin, known components of focal contacts (20). Immunoelectron microscopy demonstrates the presence of MARCKS at points where actin filaments interact with the cytoplasmic surface of the plasma membrane in actively spreading macrophages (Allen, L., A. Rosen, J. Hartwig, and A. Aderem, manuscript in preparation). These data suggest that MARCKS regulates actin–membrane interactions and the structure of actin at the plasma membrane. Furthermore, in fibroblasts, MARCKS shuttles between the plasma membrane and lysosomes (21), suggesting that MARCKS contains recognition elements for both types of membranes. MARCKS is enriched at the leading edge of motile fibroblasts, and mutation of the PKC phosphorylation sites of MARCKS results in a defect in cellular movement in a wound-healing assay (22). The similarity between cell movement and phagocytosis, together with the observation that MARCKS shuttles between the plasma membrane and lysosomes, prompted us to investigate a potential role for MARCKS in phagocytosis and phagosome–lysosome fusion. We now show that MARCKS and the α isozyme of PKC (PKCα) associate with the nascent phagosome at the same time as F-actin, that MARCKS is phosphorylated during phagocytosis, and that MARCKS and PKCα remain on the maturing phagosome until after phagosome–lysosome fusion has occurred. We also show that myosin I is recruited to the forming phagosome, where it is likely to act as a mechanical motor during phagocytosis.

Materials and Methods

Materials. [^{32}P]orthophosphate (320 TBq/mmol) was obtained from New England Nuclear (Boston, MA). Leupeptin was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Re595 LPS from *Salmonella minnesota* was from List Biological Labs, Inc. (Campbell, CA). PMA, chelerythrine chloride, and calphostin C were purchased from LC Laboratories (Woburn, MA). Unless indicated otherwise, all other chemicals were obtained from Sigma Immunochemicals (St. Louis, MO).

Macrophage Cultures. Macrophages from the peritoneal cavities of female ICR mice (Charles River Laboratories, Wilmington, MA) were plated on acid-washed glass coverslips (Propper Manufacturing Company, Inc., Long Island City, NY) at a density of 50–100,000 cells per coverslip in MEMα supplemented with 1% L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin (all from JRH Biosciences, Lenexa, KS), and 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT). After 2 h at 37°C, nonadherent cells were removed by washing with PD (calcium- and magnesium-free PBS), and cells were cultured overnight in fresh medium. The next day, macrophages were switched to Hepes-buffered RPMI (JRH Biosciences, Lenexa, KS) containing the supplements indicated above, as well as 50 ng/ml LPS.

Zymosan particles (Sigma) were prepared as described (23), dispersed by sonication, and resuspended in Hepes-buffered RPMI at 4°C. To synchronize ingestion, macrophages were switched to zymosan-containing medium and centrifuged at 450 *g* for 2 min at 4°C. Unbound particles were removed by washing, and cells were incubated at 37°C to initiate particle uptake. After various times, cells were fixed and processed for microscopy as described below. The total time in LPS was 3–4 h.

In some cases, internalization of zymosan (phagocytic index) was examined by staining live cells with 0.4% trypan blue in PBS. Extracellular zymosan particles stained blue, whereas fully internalized particles did not.

Fluorescence Microscopy. Macrophages were fixed for 7 min at 25°C in 10% neutral buffered formalin solution (Sigma) and permeabilized in −20°C acetone for 5 min. Cells were blocked for 1 h at 25°C in PD supplemented with 0.2 g/liter sodium azide and 5 g/liter BSA (PAB) and 10% horse serum (heat inactivated), and then incubated with primary antibodies for 1 h at 25°C in a humidified chamber. Coverslips were washed sequentially in eight medicine cups of PAB, incubated with secondary antibodies for an additional hour, and then washed eight more times in PAB. Coverslips were attached to microscope slides using Hydromount (National Diagnostics, Manville, NJ). Specificity of staining was assessed by omission of primary antibodies. All antibodies were diluted in PAB containing 10% horse serum.

MARCKS was visualized using an affinity-purified rabbit antimurine MARCKS antibody (20, 21, 24) and a 1:1,000 dilution of a Texas red–conjugated goat anti–rabbit secondary antibody (Molecular Probes, Eugene, OR). F-actin was detected using a 1:300 dilution of FITC-phalloidin (Molecular Probes). Talin and vinculin were detected using 1:400 dilutions of mouse mAbs 8d4 and VIN-11-5, respectively (Sigma) and a 1:100 dilution of FITC-conjugated, affinity-purified goat anti–mouse IgG plus IgM (Jackson ImmunoResearch Laboratories, West Grove, PA). Similarly, sites containing phosphotyrosine or PKCα were visualized using mouse mAbs 4G10 (UBI, Lake Placid, NY) and M6 (25) (generous gift from Dr. S. Jaken, UBI), respectively and the same secondary antibody as used for vinculin and talin. Lamp-1 was detected using a 1:300 dilution of rat mAb 1D4B (NIH Developmental Studies Hybridoma Bank, Baltimore, MD) and a 1:40 dilution of FITC-conjugated goat anti–rat (Fab')$_2$ IgG (Tago, Burlingame, CA). Mouse mAbs to myosin I (M2 an M5 [26]) were the generous gift from Dr. J. Albanesi, and were used at a 1:100 dilution. Confocal microscopy was performed using the Molecular Dynamics system and Image Space software in conjunction with an Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a 100× Plan-Apochromat objective.

Radiolabeling and Immunoprecipitation. Peritoneal macrophages (0.6–1.0 × 10^7 per 35-mm dish) were cultured overnight at 37°C in serum-free MEMα. Serum-starved cells were depleted of phosphate by a 90-min incubation in phosphate-free RPMI (GIBCO BRL, Gaithersburg, MD), and then labeled for 4 h in phosphate-free RPMI supplemented with 0.1 mCi/ml [^{32}P]orthophosphate in the presence of 50 ng/ml LPS. Zymosan particles were added to the culture medium during the final 1–90 min of the labeling period. Lysates were prepared by scraping cells into 200 μl of lysis buffer (10 mM Tris-HCl, pH 7.5, 15 mM EDTA, 50 mM KF, 50 mM NaH_2PO_4, 10 mM sodium pyrophosphate, and 1% NP-40) supplemented with protease inhibitors (0.09 TIU aprotinin, 0.5 mg/ml leupeptin, 1 mM PMSF, and 1 mM diisopropyl fluorophosphate). MARCKS was immunoprecipitated from postnuclear supernatants using a rabbit anti–mouse polyclonal antiserum as previously described (27). Immunoprecipitates were resolved by 8% SDS-PAGE. ^{32}Pi was visualized by autoradiography, and the incorporation of radiolabel into MARCKS was quantified using an Ultrascan enhanced laser densitometer (LKB Instruments, Helsinki, Finland) and by Cerenkov counting.

Inhibitor Studies. Macrophages on glass coverslips were treated

with 50 ng/ml LPS in Hepes-buffered RPMI for 3 h at 37°C, as described above. Cells were then treated with 1 μM staurosporine, 10 μM chelerythrine chloride, 5 μM calphostin C, or vehicle (DMSO) for 15 min at 37°C. Zymosan was added to the culture medium and centrifuged onto the cells as described above. After 3 min at 37°C, cells were fixed and stained for indirect immunofluorescence as described above. Cells were photographed using a Zeiss Axiophot microscope fitted with a 100× Plan-Neofluar objective and professional daylight slide film (Ektachrome; Eastman Kodak Co., Rochester, NY).

For radiolabeling experiments, peritoneal macrophages were labeled with ^{32}Pi in the presence of LPS as described above. Zymosan particles were added to the culture medium during the final 5 min of the labeling period. In some cases, macrophages were treated with 10 μM chelerythrine chloride, 5 μM calphostin C, or 1 μM staurosporine for 15 min before the addition of zymosan particles. Cells were lysed and MARCKS was immunoprecipitated as described above. Incorporation of radiolabel into MARCKS was visualized by autoradiography after SDS-PAGE and quantified as described above.

Results

MARCKS Associates with Nascent Phagosomes and Mature Phagolysosomes. We used indirect immunofluorescence and confocal microscopy to examine whether MARCKS associated with phagosomes containing zymosan particles in LPS-treated peritoneal macrophages. Phagocytosis was synchronized by centrifuging zymosan particles onto macrophages at 4°C, and internalization was initiated by warming cells to 37°C for various times. The fluorescence data shown are representative of eight independent experiments.

After 1 min at 37°C, MARCKS was concentrated in the vicinity of forming phagocytic cups (Fig. 1), where it colocalized with both F-actin (Fig. 1, *a–d*) and talin (Fig. 1, *e* and *f*). Free zymosan particles were not stained (data not shown). Significantly, vinculin, a protein found in adhesion plaques along with talin and actin (9, 28), was excluded from the nascent phagosome (Fig. 1, *g* and *h*), although it was associated with streak-shaped focal contacts at the substrate-adherent

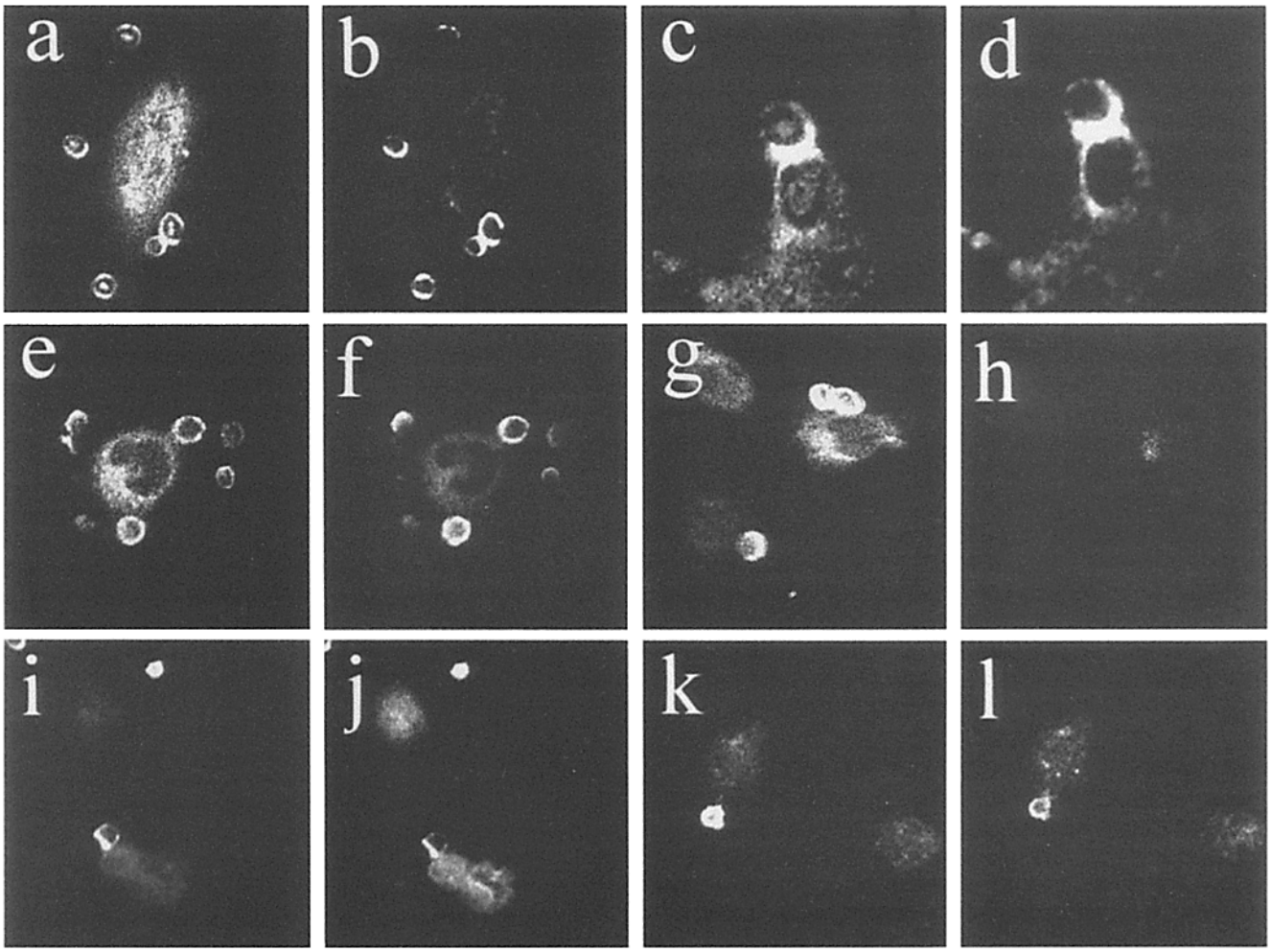

Figure 1. MARCKS associates with forming phagosomes. LPS-treated peritoneal macrophages were allowed to ingest zymosan particles for 1 min at 37°C. Fixed and permeabilized cells were double stained for MARCKS (*a*, *c*, *e*, *g*, *i*, and *k*) and F-actin (*b* and *d*), talin (*f*), vinculin (*h*), PKCα (*j*), or myosin I (*l*), as described in Materials and Methods. Each panel is a single section from the confocal microscope.

surface (data not shown). PKCα, which is associated with adhesion plaques in fibroblasts (25), also colocalized with MARCKS at the cytoplasmic face of the forming phagosome (Fig. 1, *i* and *j*). In addition, myosin I (Fig. 1, *k* and *l*), and phosphotyrosine-containing proteins (see Table 1 and Fig. 6, and data not shown) colocalized with MARCKS on nascent phagosomes. On the other hand, forming phagosomes lacked markers of late endosomal compartments, such as the lysosome-associated glycoprotein Lamp-1 (references 29–31 and data not shown). Similar data were obtained for control macrophages not treated with LPS, although the cellular levels of MARCKS were lower (data not shown). Cell-associated zymosan particles stained prominently with trypan blue after 1 min at 37°C, confirming that the particles were not completely internalized (32).

After 3 min at 37°C, ~90% of zymosan particles were completely internalized, as judged by the failure of these particles to stain with trypan blue (data not shown). Recently internalized particles retained enhanced staining for MARCKS, as well as F-actin, talin (Fig. 2 *a-f*), and phosphotyrosine-containing proteins (Fig. 6, *e* and *f*). PKCα (Fig. 2 *h*) and myosin I (Fig. 2 *f*) were also detected on phagosomes at this time. Consistent with the data obtained at earlier time points, vinculin was excluded from all phagosomes examined (data not shown). Recently internalized particles had not yet ac-

Table 1. *Inhibition of PKC Blocks Zymosan Phagocytosis*

	Control	Stauro.	Cheleryth.	Calph. C
Bound	208 ± 49	15 ± 5	14 ± 11	10 ± 5
Percentage of Actin +	90 ± 4	12 ± 6	1 ± 1	0 ± 0
Percentage of P-Y +	93 ± 2	3 ± 3	0 ± 0	0 ± 0
P-Y dist.	Phago., A.P.	Diffuse	Punctate	Diffuse
P.I.	185 ± 9	0 ± 0	0 ± 0	0 ± 0
Percentage of Inhib. PKC	(0)	88 ± 6	91 ± 4	90 ± 5

LPS-treated macrophages were cultured for 15 min at 37°C in the absence (control) or presence of 1 μM staurosporine, 10 μM chelerythrine chlorine, or 5 μM calphostin C. Zymosan particles were added, centrifuged onto the cells, and the dishes were incubated for 3 min at 37°C to allow internalization. Cells were fixed and stained for indirect immunofluorescence microscopy as described in Materials and Methods. "Bound" refers to the number of zymosan particles bound per 100 macrophages as judged by phase contrast microscopy. Bound zymosan particles were scored for the presence of F-actin and P-Y using immunofluorescence microscopy. Phagocytic indices (particles internalized per 100 macrophages), were scored in live cells using trypan blue. To demonstrate inhibition of PKC, macrophages were labeled with [^{32}P]orthophosphate with or without the indicated inhibitors, and the incorporation of label into MARCKS was quantified as described in Materials and Methods. Data are the average ± SD of three independent experiments. Stauro., staurosporine; Cheleryth., chelerythrine chloride; Calph. C., calphostin C; P-Y, phosphotyrosine; dist., distribution; Phago., phagosomes; A.P., adhesion plaques; Inhib., inhibition; P.I., phagocytic index.

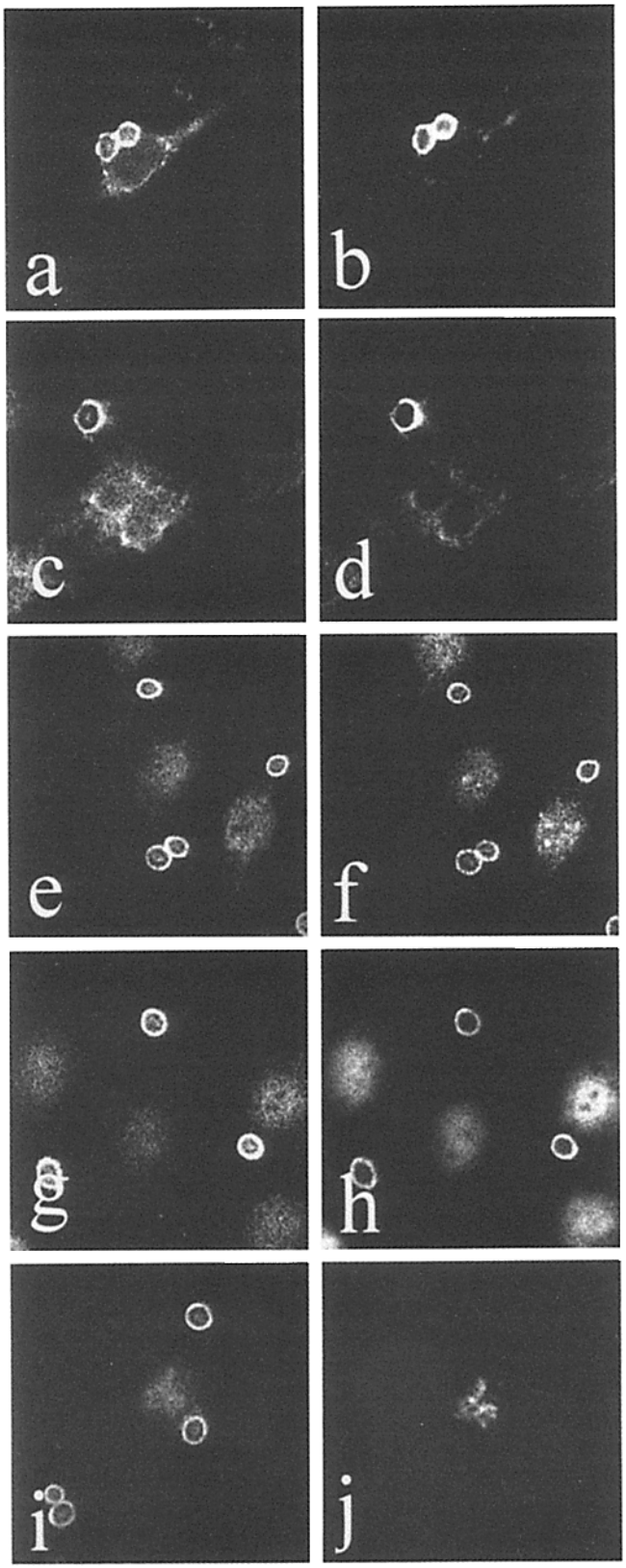

Figure 2. MARCKS is retained on recently internalized phagosomes. LPS-treated peritoneal macrophages were fixed and permeabilized for immunofluorescence microscopy after ingesting zymosan particles for 3 min at 37°C. Cells were double-stained for MARCKS (*a*, *c*, *e*, *g*, and *i*) and F-actin (*b*), talin (*d*), myosin I (*f*), PKCα (*h*), or Lamp-1 (*j*), as described in Materials and Methods. Each panel represents a single section from the confocal microscope.

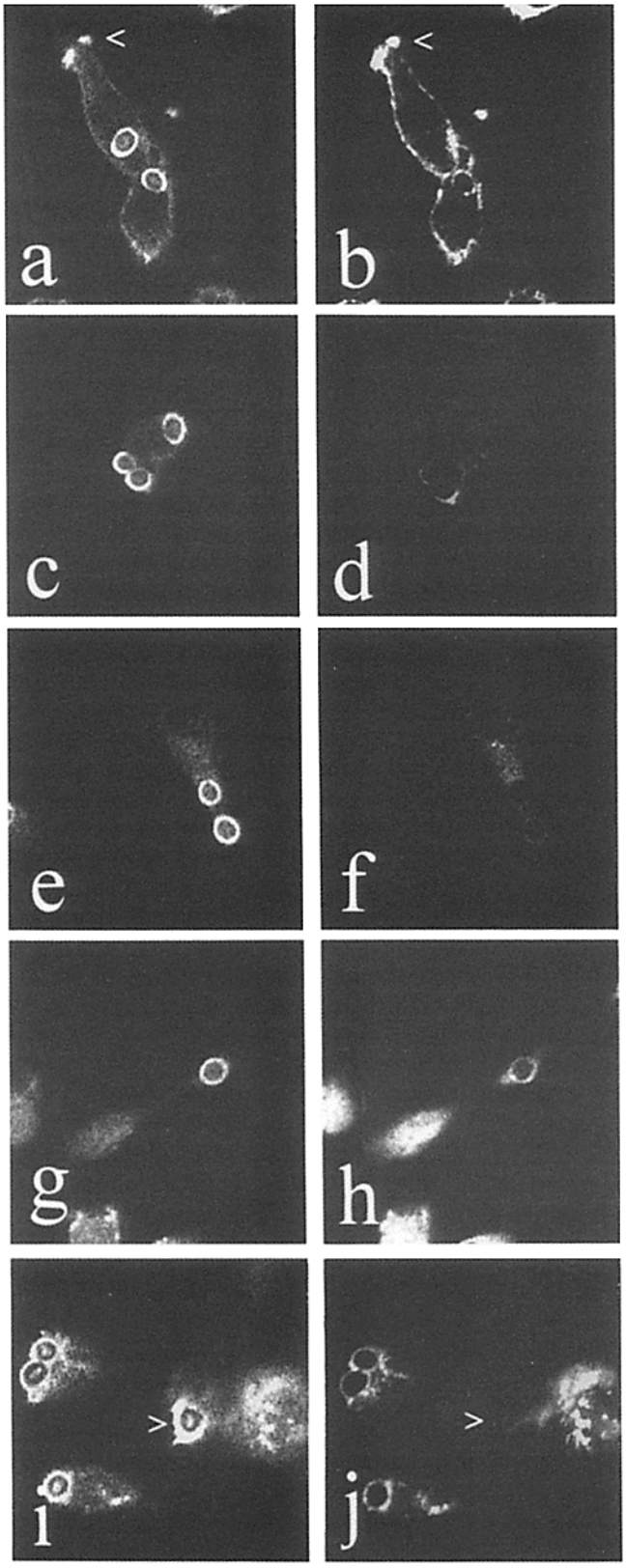

Figure 3. Maturing phagosomes containing zymosan particles retain MARCKS and PKCα. LPS-treated peritoneal macrophages ingested zymosan particles for 15 min at 37°C before processing for immunofluorescence microscopy. Fixed and permeabilized cells were double-stained for MARCKS (*a*, *c*, *e*, *g*, and *i*) and F-actin (*b*), talin (*d*), myosin I (*f*), PKCα (*h*), or Lamp-1 (*j*), as described above. Arrowheads in *a* and *b* indicate colocalization of MARCKS and F-actin in the leading edge of the plasma membrane. Arrowheads in *i* and *j* mark phagosomes prominently stained for MARCKS which have not yet acquired Lamp-1. Each panel is a single section from the confocal microscope.

quired markers associated with late endosomes and lysosomes, such as Lamp-1 (Fig. 2 *j*).

As previously described for phagosomes containing IgG-coated particles (33), further maturation of zymosan phagosomes (7–15 min at 37°C) was associated with a disappearance of staining for F-actin, talin, and phosphotyrosine-containing proteins (Fig. 3 *b–d* and data not shown). Myosin I was also undetectable on zymosan phagosomes after 15 min at 37°C (Fig. 3 *f*). By contrast, MARCKS (Fig. 3, *a*, *c*, *e*, *g*, and *i*) and PKCα (Fig. 3 *h*) were retained on the maturing phagosome. Although F-actin was nearly undetectable on phagosomes after 15 min, MARCKS and F-actin were colocalized in the leading edge of polarized cells (Fig. 3, *a* and *b*, *arrowheads*). A subset of phagosomes had acquired Lamp-1 staining after 15 min of internalization (Fig. 3 *j*), suggesting that some phagosome-lysosome fusion had occurred by this time point.

After 90 min at 37°C, MARCKS colocalized with both PKCα and Lamp-1 on the phagosomal membrane (Fig. 4 *g–j*), whereas actin, talin, and myosin I were not associated with phagosomes at this time (Fig. 4 *a–f*). MARCKS and Lamp-1 remained associated with phagosomes for at least 8 h (data not shown). Taken together, our data suggest that MARCKS is associated with the phagosomal membrane from its inception through the formation of mature phagolysosomes.

MARCKS Is Phosphorylated during Phagocytosis. Since PKCα and MARCKS, a known PKC substrate, were colocalized on phagosomes, we examined whether uptake of zymosan particles induced the incorporation of ^{32}Pi into MARCKS. MARCKS phosphorylation increased more than threefold within 1 min of zymosan binding, remained high during particle ingestion (3–15 min), and was slightly elevated even after phagosome–lysosome fusion was completed (90 min), (Fig. 5, *a* and *b*). The maximum level of MARCKS phosphorylation induced by zymosan was ~70% of the amount seen when PKC was activated by PMA (data not shown).

PKC Inhibitors Block Zymosan Binding and Internalization. Since PKCα and MARCKS were colocalized on nascent phagosomes, and since MARCKS was phosphorylated during phagocytosis, we examined whether activation of PKC was required for particle ingestion. Both specific (calphostin C and chelerythrine chloride [34, 35]) and relatively nonspecific inhibitors of PKC (staurosporine [36]) were tested. All three inhibitors blocked zymosan internalization, reduced the number of zymosan particles bound to the macrophage surface (Table 1), and disrupted the focal accumulation of F-actin, phosphotyrosine, MARCKS, PKCα, and talin in the cortical cytoplasm adjacent to bound particles (Table I, Figs. 6

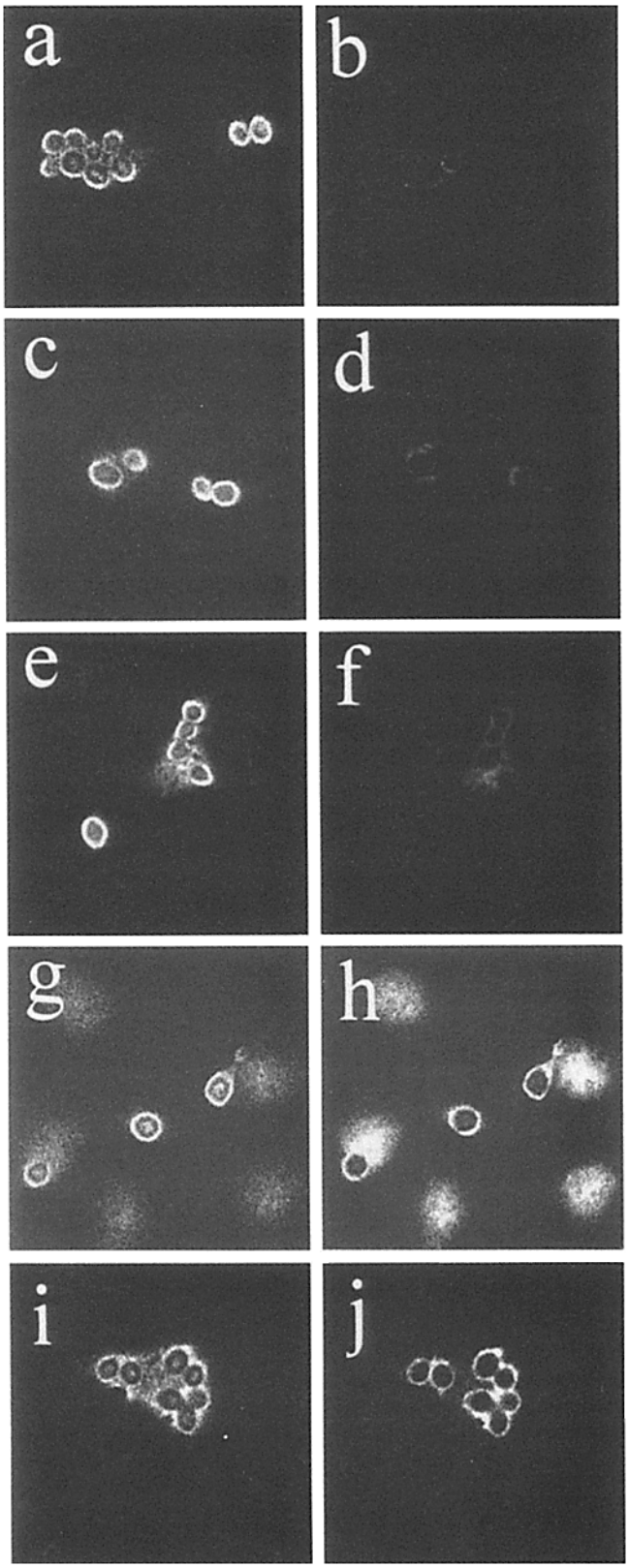

Figure 4. MARCKS colocalizes with Lamp-1 and PKCα on mature phagolysosomes. LPS-treated macrophages that had ingested zymosan particles for 90 min at 37°C were fixed and processed for immunofluorescence microscopy as described above. Macrophages were double-stained for MARCKS (*a*, *c*, *e*, *g*, and *i*) and F-actin (*b*), talin (*d*), myosin I (*f*), PKCα (*h*), or Lamp-1 (*j*). Each panel is a single section from the confocal microscope.

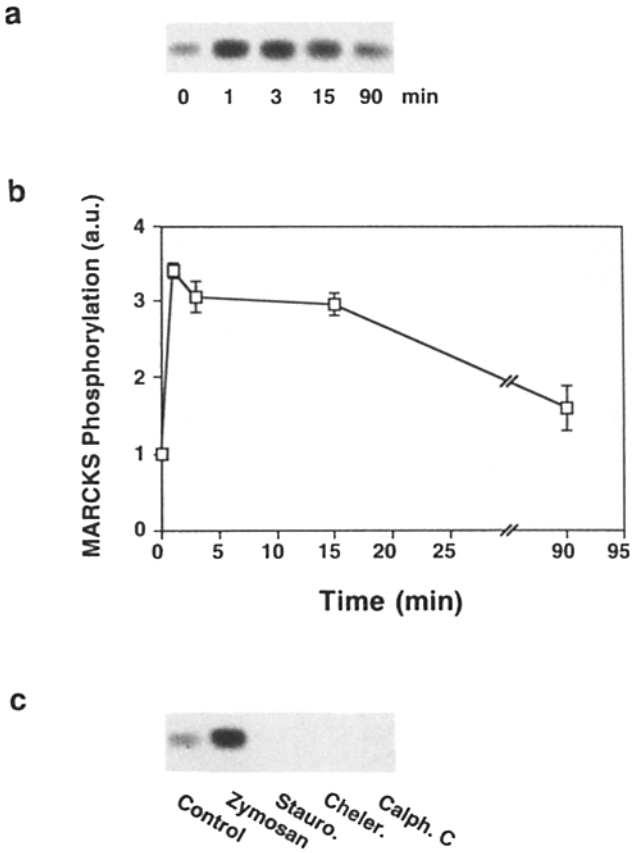

Figure 5. Zymosan induces MARCKS phosphorylation by activating PKC. (*a* and *b*) Time course of MARCKS phosphorylation. Macrophages were pretreated with LPS and labeled with ^{32}Pi before ingesting zymosan particles for 0–90 min. MARCKS was immunoprecipitated from cell lysates and quantified as described in Materials and Methods. (*a*) Autoradiograph showing MARCKS phosphorylation. (*b*) Time course of MARCKS phosphorylation in macrophages ingesting zymosan particles. Error bars indicate the range of duplicate samples. *a.u.*, arbitrary units. (*c*) PKC inhibitors block MARCKS phosphorylation in the presence of zymosan. Macrophages were pretreated with LPS and labeled with ^{32}Pi. In some cases, cells were treated with 1 μM staurosporine, 5 μM calphostin C, or 10 μM chelerythrine chloride for 15 min before addition of zymosan for 5 min. MARCKS was immunoprecipitated from cell lysates as described above. Basal MARCKS phosphorylation in the absence of zymosan (*Control*). *Stauro*, staurosporine; *Cheleryth.*, chelerythrine chloride; *Calph. C*, calphostin C.

and 7, and data not shown). Unexpectedly, treatment with PKC inhibitors also altered intracellular staining patterns for phosphotyrosine. In macrophages allowed to ingest zymosan particles for 3 min at 37°C, phosphotyrosine was detected on nascent phagosomes as well as in streak-shaped focal contacts in the substrate-adherent plasma membrane (Fig. 6, *e* and *f*, and data not shown). In macrophages preincubated with chelerythrine chloride, phosphotyrosine staining was

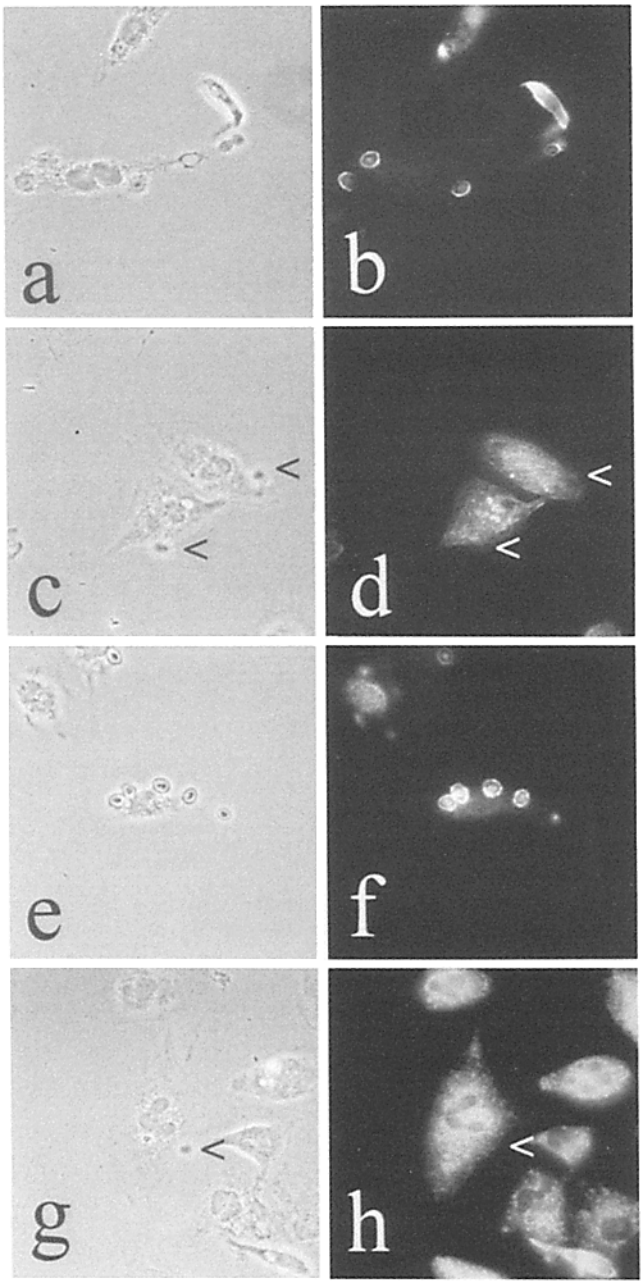

Figure 6. Chlelerythrine chloride disrupts the association of phosphotyrosine and F-actin with the zymosan phagosome. LPS-treated macrophages were incubated in the presence (*c*, *d*, *g*, and *h*) or absence (*a*, *b*, *e*, and *f*) of 10 μM chelerythrine chloride for 15 min at 37°C, before addition of zymosan particles for 3 min at 37°C. Fixed and permeabilized cells were stained for F-actin (*b* and *d*) or phosphotyrosine (*f* and *h*), as described in Materials and Methods. (*a*, *c*, *e*, and *g*) phase contrast. Arrowheads indicate bound zymosan particles not associated with staining for F-actin (*c* and *d*) or phosphotyrosine (*g* and *h*). Cells were photographed on a Zeiss Axiophot microscope fitted with a 100× Plan Neofluar objective.

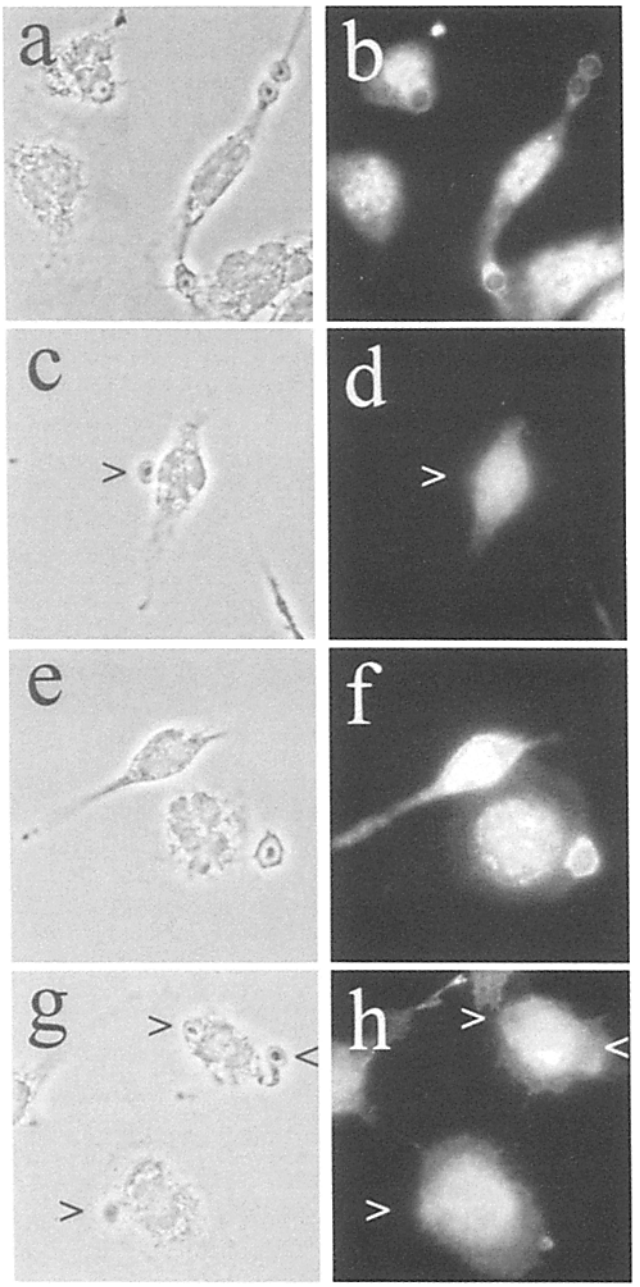

Figure 7. Inhibition of PKC blocks the association of MARCKS and PKC with the zymosan phagosome. LPS-treated macrophages were incubated in the presence (*c*, *d*, *g*, and *h*) or absence (*a*, *b*, *e*, and *f*) of 10 μM chelerythrine chloride for 15 min at 37°C, before addition of zymosan particles for 3 min at 37°C. Fixed and permeabilized cells were stained for PKCα (*b* and *d*) or MARCKS (*f* and *h*), as described in Materials and Methods. (*a*, *c*, *e*, and *g*) Phase contrast. Arrowheads in *c* and *d* indicate bound zymosan particles that lack detectable staining for PKCα. Arrowheads in *g* and *h* indicate bound zymosan particles that lack detectable staining for MARCKS. Cells were photographed as described in the legend to Fig. 6.

associated with cytoplasmic vesicles and was not detected adjacent to bound zymosan particles or in focal contacts (Fig. 6, *g* and *h*, and data not shown). Moreover, phosphotyrosine staining was diffuse in macrophages treated with either calphostin C or staurosporine (Table 1 and data not shown). The effects of all three inhibitors were completely reversed within 15 min of drug removal (data not shown). Consistent with recent reports (35, 37), the activity of calphostin C was also abolished if cells were incubated in the dark, but

normal room light was sufficient to activate the inhibitor (data not shown). To confirm that PKC activity was blocked in macrophages treated with 1 μM staurosporine, 5 μM calphostin C, or 10 μM chelerythrine chloride, we quantified the incorporation of [^{32}P]orthophosphate into MARCKS. Relative to controls, the PKC inhibitors reduced MARCKS phosphorylation by ~90% (Fig. 5 *c* and Table 1). Lower concentrations of inhibitors that only partially inhibited PKC activity (as judged by a 50% decrease in MARCKS phosphorylation) reduced zymosan binding ~60% and slowed zymosan uptake, but they did not block the association of any of the above markers with the nascent phagosome (data not shown). Taken together, our data suggest that activation of PKC is an early signal required for attachment and internalization of zymosan particles, and that MARCKS may integrate signals from PKC into dynamic changes in the actin cytoskeleton required for zymosan internalization.

Discussion

Although a number of proteins are known to associate with nascent phagosomes, the signals that mediate actin polymerization and particle internalization have not been characterized fully. We now show that MARCKS, an actin-binding protein and PKC substrate, is recruited to the forming phagosome along with PKCα and myosin I. The kinetics of myosin I association with and dissociation from the phagosome parallel those of F-actin and talin, whereas MARCKS and PKCα are retained until after phagosomes have acquired the late endosome/lysosome marker Lamp-1. Inhibition of PKC blocks zymosan internalization, reduces particle binding, and prevents the focal accumulation of MARCKS, F-actin, PKCα, and phosphotyrosine-containing proteins beneath attached particles. Taken together, these data suggest roles for myosin I, MARCKS, and PKC in particle internalization, as well as continuing roles for MARCKS and PKCα in phagosome maturation. A schematic diagram depicting the temporal association of the proteins described in this study with the zymosan phagosome is shown in Fig. 8.

Phagocytosis has many features in common with cell motility and adhesion. All of these processes are initiated by receptor–ligand interactions, and they involve surface rearrangements driven by the actin cytoskeleton. Many proteins associated with early phagosomes are also found at the leading

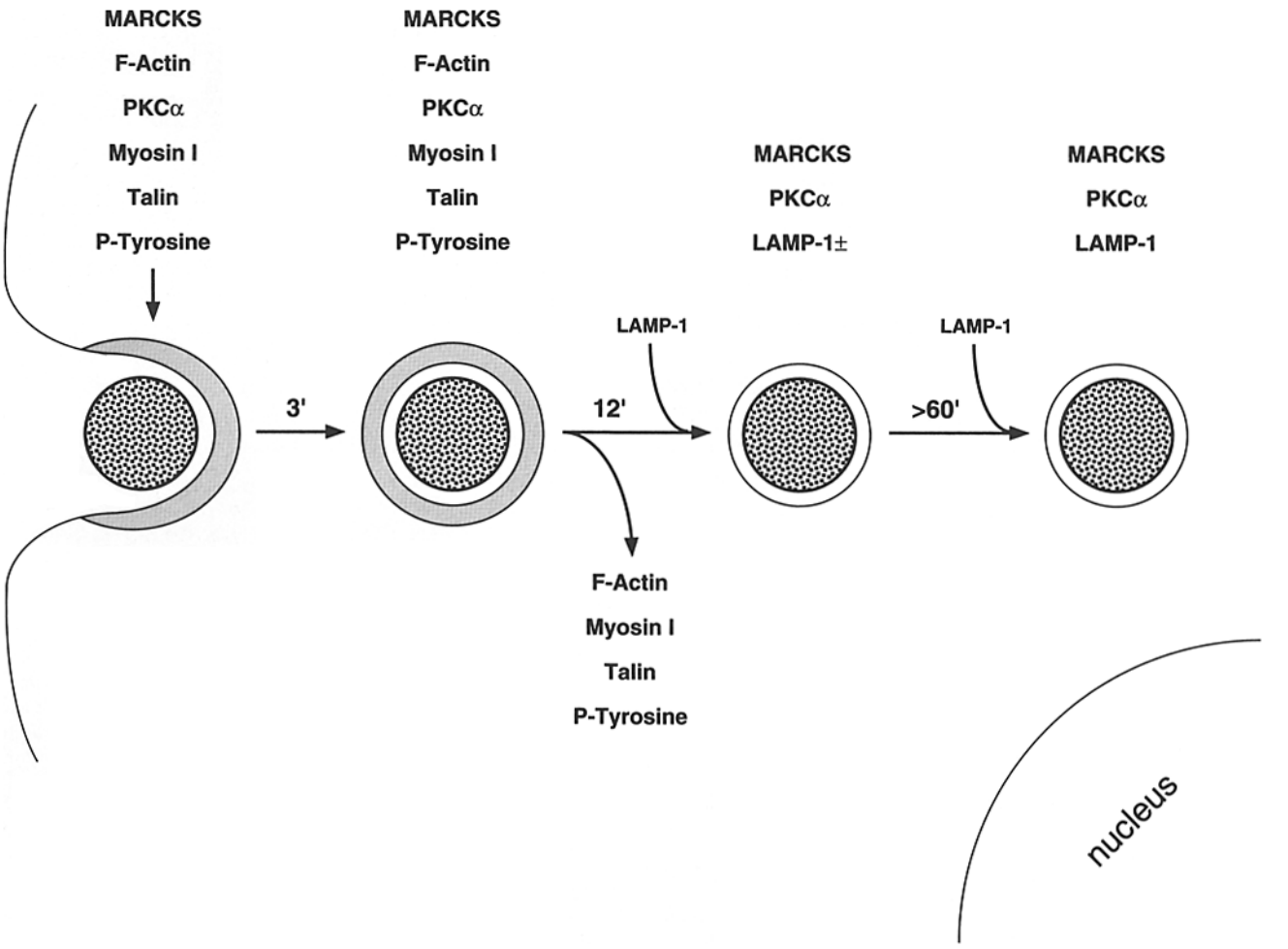

Figure 8. Maturation of the zymosan phagosome. Schematic diagram depicting the temporal association of the proteins described in this study with the zymosan phagosome.

edge of motile cells and in focal contacts, including F-actin, talin, and substrates for tyrosine kinases (3, 8, 33). However, the tyrosine kinase syk is activated during Fc receptor–mediated phagocytosis (10), whereas FAK is associated with focal contacts (11). This suggests that similar yet distinct signaling pathways are used during phagocytosis and adherence to the substratum. The transient contacts made with the substratum in actively moving cells more closely resemble the forming phagosome than do the stable contacts made by quiescent cells. Although both types of contacts contain F-actin and talin, vinculin is acquired late by adhesion plaques, after talin and integrins (38), and is not associated with the transient contacts formed by motile fibroblasts (39). This is consistent with our observation that vinculin is not associated with nascent phagosomes, and may reflect the transient nature of this structure.

PKCα and MARCKS, an actin binding protein and PKC substrate, are recruited to the forming phagosome with kinetics indistinguishable from those of F-actin. Thus, MARCKS may have a role in modulating actin cross-linking during particle internalization, especially since the actin cross-linking activity of MARCKS is regulated by PKC (15). Indeed, fibroblasts expressing mutant MARCKS molecules, in which the serines normally phosphorylated by PKC have been mutated to alanines, exhibit defects in motility in a wound-healing assay (22).

Although actin polymerization is required for both motility and phagocytosis, the driving force behind the extension of pseudopodia and lamellipodia remains unclear. Myosin I is a nonfilamentous form of myosin found in the leading edge of motile fibroblasts (26, 40). The distribution of myosin I has lead to speculation that this protein may maintain polarity by transporting vesicles to the leading edge (40). Others have also proposed that movement of myosin I along actin filaments may drive pseudopod extension by displacing the plasma membrane and creating space for additional actin polymerization (3, 41). We now show that myosin I is enriched in the cortical cytoplasm beneath entering zymosan particles, further reinforcing the parallels between motility and phagocytosis, and the notion that myosin I may provide the mechanical force for particle uptake. Moreover, the observation that myosin I is shed from internalized particles along with F-actin is consistent with recent data suggesting that maturing phagosomes move along microtubules rather than actin filaments (42, 43).

Several lines of evidence suggest that activation of PKC is an early signal required for zymosan phagocytosis: (*a*) PKCα colocalizes with MARCKS on the nascent phagosomal membrane and in the membrane beneath bound zymosan particles. (*b*) MARCKS is phosphorylated within 1 min of zymosan binding. (*c*) Inhibition of PKC blocks zymosan internalization and prevents the focal accumulation of F-actin, MARCKS, and phosphotyrosine in the cortical cytoplasm under the few zymosan particles that do bind to the macrophage surface. (*d*) Phosphotyrosine-containing proteins are observed in macrophages treated with chelerythrine chloride, indicating that tyrosine kinases are not inhibited under these conditions. Taken together, the data suggest a role for PKC in zymosan binding and internalization, perhaps by modulating the number of receptors for zymosan at the cell surface. The observation that zymosan particles are not internalized in the presence of PKC inhibitors further suggests that activation of PKC is required for zymosan engulfment. PKC may also have a role in localizing phosphotyrosine-containing proteins in the cortical cytoplasm at the site of internalization. This requirement for PKC does not preclude a requirement for tyrosine kinases in zymosan phagocytosis.

The effects of PKC inhibitors on zymosan phagocytosis are similar to the effects of these drugs on focal adhesions in fibroblasts. Inhibition of PKC blocked formation of focal adhesions, and dispersed talin and vinculin, but not integrins, from recently formed contacts (38). By contrast, inhibition of PKC had no effect on stable adhesion plaques that were several days old (38). This suggests that PKC has a role in the formation of contacts at the plasma membrane, and is consistent with our observation that activation of PKC is required for phagocytosis.

The observation that zymosan induces MARCKS' phosphorylation, and that MARCKS is enriched on the phagosome membrane, even though phosphorylated MARCKS is usually displaced from membranes into the cytosol, may result from a number of factors: (*a*) All three serines in the effector domain must be phosphorylated to release MARCKS from the membrane (reference 18 and data not shown). It is not clear whether all three serines are phosphorylated during phagocytosis. (*b*) In fibroblasts, PKC activation promotes the movement of MARCKS from the plasma membrane to lysosomes (21), indicating that phosphorylation can direct the targeting of MARCKS to specialized membrane compartments. (*c*) The protein composition of the forming phagosomal membrane differs somewhat from the bulk plasma membrane (5, 7), suggesting that the phagosomal membrane, as the lysosomal membrane, may have MARCKS-targeting determinants.

The literature contains conflicting reports on the requirement for PKC during phagocytosis. A direct comparison of the results of these studies is complicated by the fact that the investigations used different cell types (monocytes, resident macrophages, neutrophils, or activated macrophages), cells from different species (human and mouse), and most importantly, that different receptors were engaged for particle uptake. Zymosan particles are yeast cell wall components that bind to the mannose–fucose receptor and the β-glucan receptor, whereas the Fc receptor binds IgG (44, 45). Zheleznyak and Brown (46) found that activation of PKC is an early signal required for Fc receptor–mediated phagocytosis in human monocytes and that PKC is enriched on purified phagosomal membranes. PKC is also activated during complement receptor–mediated phagocytosis in neutrophils (47). In this study, we found that zymosan binding to the macrophage surface activates PKC, resulting in MARCKS phosphorylation, and that inhibition of PKC blocks particle binding and internalization. By contrast, Greenberg and co-workers (33) found that treating inflammatory macrophages with inhibitors of PKC has no effect on Fc receptor–mediated phago-

cytosis. It is possible that PKC was only partially inhibited in the study by Greenberg et al. In our hands, the concentrations of inhibitors they used only partially inhibited PKC activity, as judged by the incorporation of ^{32}Pi into MARCKS, and slowed but did not block zymosan uptake or the association of F-actin and other proteins with the phagosome (data not shown). It is also possible that in thioglycollate-elicited macrophages activation of PKC is not required for Fc receptor–mediated phagocytosis. We cannot reconcile these differences at this time.

In general, phagocytosis is accompanied by an increase in intracellular calcium (2, 32, 48–50), and single-cell imaging shows calcium transients in the vicinity the phagosome (48). The role of calcium in phagocytosis is controversial. Single-cell measurements have demonstrated a requirement for calcium during particle internalization, while studies by other investigators have demonstrated that calcium is not required for the internalization event (2, 32, 48–51). Calcium, however, is required to depolymerize actin from phagosomes containing yeast in neutrophils (49). MARCKS senses calcium through calmodulin, and calcium/calmodulin prevents the actin–cross-linking activity of MARCKS (15). Therefore, MARCKS may integrate signals from both PKC and calcium/calmodulin to alter actin structure around the phagosome.

Results of both in vivo and in vitro studies suggest that phagosomes mature via a series of membrane fission and fusion events. Most plasma membrane proteins are rapidly recycled to the cell surface (4), and further maturation involves sequential fusion with endosomes and lysosomes (5–7), resulting in the formation of mature phagolysosomes. In addition, actin depolymerization may be required for endosomes and lysosomes to gain access to the phagosome membrane, and calcium is required for phagosome–lysosome fusion in some systems (50). It is interesting that MARCKS and PKCα are retained on the maturing zymosan phagosome, and this suggests a role for these proteins beyond their potential interactions with actin. In fibroblasts, MARCKS cycles between the plasma membrane and lysosomes in a phosphorylation-dependent manner (21), and MARCKS accumulates on lysosomes when acidification of the endocytic pathway is blocked (21). Although the function of MARCKS on the lysosomal membrane is unknown, MARCKS clearly has recognition determinants for the lysosome membrane. It is tempting to speculate that both MARCKS and PKC have a role in phagosome–lysosome fusion. Furthermore, phagosome–lysosome fusion represents a major intersection of the microtubule-based and actin-based cytoskeletons used for organelle motility. Particle internalization has an absolute requirement for actin polymerization (1), whereas lysosomes (52, 53) and perhaps maturing phagosomes (42, 43) move primarily in association with microtubules. MARCKS has been associated both with actin-based motility (20, 22) and with membrane trafficking (21), and the current study suggests that MARCKS integrates signals from PKC and calcium/calmodulin to regulate actin structure around the phagosome and perhaps phagosome–lysosome fusion.

The authors thank Drs. Albanesi and Jaken for the generous gift of antibodies.

This work was supported by grants from the Paul Erlich Foundation for Biomedical Research and National Institutes of Health (NIH) to A. Aderem (AI25032). L. Allen is the recipient of a postdoctoral fellowship from the NIH (AI08618). A. Aderem is an established investigator of the American Heart Association.

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Received for publication 3 April 1995 and in revised form 5 May 1995.

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