Flotillin-1-enriched Lipid Raft Domains Accumulate on Maturing Phagosomes*

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Flotillin-1 was recently shown to be enriched on detergent-resistant domains of the plasma membrane called lipid rafts. These rafts, enriched in sphingolipids and cholesterol, sequester certain proteins while excluding others. Lipid rafts have been implicated in numerous cellular processes including signal transduction, membrane trafficking, and molecular sorting. In this study, we demonstrate both morphologically and biochemically that lipid rafts are present on phagosomes. These structures are enriched in flotillin-1 and devoid of the main phagosomes membrane protein lysosomal-associated membrane protein (LAMP1). The flotillin-1 present on phagosomes does not originate from the plasma membrane during phagocytosis but accumulates gradually on maturing phagosomes. Treatment with bafilomycin A1, a compound that inhibits the proton pump ATPase and prevents the fusion of phagosomes with late endocytic organelles, prevents the acquisition of flotillin-1 by phagosomes, indicating that this protein might be recruited on phagosomes from endosomal organelles. A proteomic characterization of the lipid rafts of phagosomes indicates that actin, the α- and β-subunits of heterotrimeric G proteins, as well as subunits of the proton pump V-ATPase are among the constituents of these domains. Remarkably, the intracellular parasite Leishmania donovani can actively inhibit the acquisition of flotillin-1-enriched lipid rafts by phagosomes and the maturation of these organelles. These results indicate that specialized functions required for phagolysosome biogenesis may occur at focal points on the phagosome membrane, and therefore represent a potential target of intracellular pathogens.

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Lateral assemblies of lipids, termed lipid rafts, have been postulated to represent a general feature of the plasma membrane of eukaryotic cells (1, 2). Rafts apparently form because of the biophysical properties of sphingolipids and cholesterol, which pack tightly into liquid-ordered (lo)2 domains that partition away from the more disorganized glycerophospholipids in the bulk of the membrane (3). Lipid-modified proteins and some transmembrane proteins are concentrated in the rafts while other proteins are excluded. Lipid rafts have been implicated in many important cellular processes, such as polarized sorting of apical membrane proteins in epithelial cells and signal transduction (4). Recent evidence further indicates that a raft-based mechanism might be involved in the sorting of SNAREs to the plasma membrane and in their function in apical membrane docking and fusion events (5). As this is in no way an exhaustive list of the potential function of lipid rafts, it appears that membrane subdomains represent important sites conferring specialized properties to foci within biological membranes.

In the present study, we provide evidence showing that lipid rafts are present on phagosomes. These specialized regions, devoid of the major phagosomal protein LAMP1, are enriched in flotillin-1. The phagosomal lipid rafts are unlikely to be simply transferred from the plasma membrane to phagosomes during phagocytosis because early phagosomes display low amounts of flotillin-1. Instead, flotillin-1 is recruited to phagosomes during phagosome maturation, possibly through fusion with late endocytic organelles. The identification of lipid rafts on phagosomes suggests that specific functions occur at focal points on the phagosome membrane. Further proteomic characterization allowed us to identify sets of proteins indicating that phagosome lipid rafts might be involved in signal transduction, interaction with actin, and phagosome acidification.

EXPERIMENTAL PROCEDURES

Cell Culture and Phagosome Formation and Isolation—The murine macrophage-like cell line J774 was cultured in Dulbecco’s modified Eagle’s medium high glucose (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a 5% CO2 atmosphere. Cells were grown to 80% confluency in Petri dishes prior to each experiment, as described previously (6).

To form phagosomes, J774 macrophages were fed with 0.8 µm blue-dyed latex beads (Sigma) diluted 1:50 in culture medium. Depending on the experiment, cells were allowed to internalize beads for 30 to 90 min at 37 °C. Cells were then washed three times for 5 min with ice-cold PBS to remove non-internalized beads and were further incubated for increasing periods of time to obtain early and late phagosomes. Phagosomes were then isolated on sucrose step gradients as described previously (6). Purified phagosomes were resuspended in Laemmli or rehy-
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**Sensitivity to Bafilomycin A1**—To determine whether phagosome maturation is required for the acquisition of flotillin-1, we internalized latex beads in J774 macrophages for 30 min. Cells were then incubated for 3 h under conditions known to inhibit the vacuolar H^+ ATPase and prevents lysosome biogenesis (7), or without drug (MeSO vehicle only). To further determine whether the association of flotillin-1 present on phagosomes is modulated by luminal pH, we internalized latex beads for 60 min followed by a 3-h chase, a time point at which flotillin-1 is present in high amounts on phagosomes. This experiment was then extended for 60 min at 37 °C in the presence of inhibitors (80-20) to visualize the parasites within cells and with the anti-flotillin-1 antibody. After several washes in PBS, 1% bovine serum albumin, coverslips were incubated with an anti-rabbit IgG coupled to Alexa and with an anti-rat IgG coupled to Texas Red for 1 h. Coverslips were then washed and rehydrated on PBS two times for 5 min, two times for 10 min, and then blocked for 10 min with PBS, 2% bovine serum albumin (fraction V, Sigma, St.-Louis, MO), 0.2% gelatin. Coverslips were then incubated with the rabbit anti-flotillin-1 antibody and the rat anti-LAMP1 1D4B for 1 h. In the case of *Leishmania* infection, cells were incubated with the BFA-AE antibody directed against the major surface glycoconjugate of *Leishmania* (80-20) for 20 min. Fixation was then performed with 4% paraformaldehyde in PBS for 10 min and then permeabilized with 1% Triton X-100. Cells were then washed and rehydrated in PBS, 2% bovine serum albumin, and 0.1% Triton X-100. Treated cells were then blocked for 1 h with PBS, 1% bovine serum albumin, and 5% normal goat serum, and then incubated with the primary antibodies (see above). The same amount of protein was loaded for each sample. For a two-dimensional gel Western blot, the portion of the gel corresponding to the N terminus for coupling to carrier protein or for preparing an affinity purification column. Affinity purification was performed exactly as described previously (12). Appropriate second antibodies coupled to horseradish peroxidase were then used and the membranes were treated for ECL (Roche Diagnostics).

**RESULTS AND DISCUSSION**

In this study, we have provided evidence that lipid rafts are present on the phagosome membrane, a key organelle involved in the killing and degradation of intracellular pathogens (17). The existence of lipid subdomains on phagosomes was first suggested by a proteomic analysis indicating that proteins known to associate to lipid rafts, including flotillin-1, are present on this organelle (8). Here, we further demonstrate the enrichment of flotillin-1 on phagosomes by Western blot analysis in both one- and two-dimensional gels (Fig. 1, A–C). The association of flotillin-1 to the phagosome membrane was confirmed by Triton X-114 extraction showing that a great proportion of this protein partitioned in the detergent phase (Fig. 1D), and Promase proteolysis experiments indicating that flotillin-1 (at least the C-terminal end recognized by our antibody) is exposed on the cytoplasmic side of the phagosome membrane (Fig. 1E). The latter experiment rules out the possibility that flotillin-1 is simply present within the lumen of phagosomes for degradation. Although flotillin-1 was originally shown to accumulate in subdomains of the plasma membrane of adipocytes and neurons (18, 19), our studies using immunofluorescence analysis failed to detect noticeable levels of flotillin-1 on the plasma membrane of macrophages. Furthermore, biochemical (Fig. 2A) and morphological (Fig. 2B) analyses indicated that flotillin-1 is barely detectable on early phagosomes (derived from the plasma membrane), but accumulates on maturing phagosomes. Interestingly, our results showed that flotillin-1 associates to phagosomes at later time points than LAMP1, a marker normally used to define late endocytic/phagocytic structures (Fig. 2, A and B). In cells that had not internalized latex beads, observation at the confocal microscope revealed that although a small part of the flotillin-1 labeling is present on
housing the intracellular parasite showed by immunofluorescence its presence on phagosomes not simply associated with latex-containing compartments, we demonstrate that flotillin-1 is a general marker of phagosomes, and

A Around the membrane of this organelle (Fig. 3). To demonstrate this, flotillin-1 was cut off and transferred to nitrocellulose for immunoblotting with the anti-flotillin-1 antibody. Several spots at the same molecular mass with different pI were revealed. C Western blot from SDS-PAGE gels indicates that flotillin-1 is highly enriched on phagosomes (Phago) compared with total cell lysate (TCL). In each lane, equal amounts of protein were loaded. D Western blot analysis indicates that flotillin-1 is partially recovered in the detergent phase of a Triton X-114 phagosome extract, as expected for a membrane-associated protein. The presence of flotillin-1 in the aqueous phase could imply that this protein is loosely associated to phagosomal membrane. E, phagosome fractions were incubated for 30 min at 37 °C in the presence or absence of Pronase. This treatment degrades all proteins or portion of proteins exposed on the cytoplasmic side of phagosomes. The anti-flotillin-1 antibody, which recognizes the C-terminal portion of the protein, failed to reveal the protein in the fraction treated with Pronase, indicating that this part of the protein is present on the cytoplasmic side of phagosomes.

vesicles also labeled for LAMP1, most of the labeling does not colocalize to the same vesicle populations (Fig. 2C), suggesting that these markers are distributed on different vesicles of a common pathway. These results also indicate that flotillin-1 is a novel marker of late endocytic/phagocytic organelles that may accumulate on post-LAMP structures. This is supported by results showing that bafilomycin, a drug that inhibits the formation (or maturation) of lysosomes (7), also inhibits the accumulation of flotillin-1 to phagosomes (Fig. 2D).

At high magnification, double immunofluorescence labeling clearly indicates that flotillin-1 is present on patches of the phagosome membrane whereas LAMP1 forms a uniform ring around the membrane of this organelle (Fig. 3A). To demonstrate that flotillin-1 is a general marker of phagosomes, and not simply associated with latex-containing compartments, we showed by immunofluorescence its presence on phagosomes housing the intracellular parasite Leishmania (Fig. 3B). However, we observed that only a small proportion of Leishmania-containing phagosomes were positive for flotillin-1. Indeed, quantitative analysis indicates that over 90% of latex bead-containing phagosomes are positive for flotillin-1, whereas only 20% of phagosomes housing Leishmania parasites are labeled by the antibody (Fig. 3C). We have shown previously that the promastigote form of Leishmania parasites are able to inhibit phagosome fusion with late endocytic organelles (14). This inhibition is caused by the lipophosphoglycan (LPG), the major surface glycoconjugate of Leishmania, because mutants lacking LPG fuse extensively with late endocytic organelles (20). Accordingly, we performed additional experiments and measured the presence of flotillin-1 on phagosomes containing Leishmanialpg2−/− mutants. The results obtained indicate that 53% of phagosomes containing that mutant are positive for flotillin-1. This suggests that flotillin-1 might be necessary for, or acquired through, fusion with late endocytic organelles. Interestingly, LPG is a GPI-anchored molecule secreted by the parasite. Its mode of action in the inhibition of phagosome-endosome fusion was proposed to involve its insertion through the lipidic anchor in the phagosomal membrane (14, 21). Because GPI anchors have a strong affinity for lipid rafts (22), this process could interfere with the formation of lipid rafts on Leishmania-containing phagosomes or the association of flotillin-1 to these structures. Other Leishmania LPG-deficient mutants are currently tested in our system to further ensure the role of that molecule in the modulation of raft formation.

The properties governing the association of flotillin-1 to phagosome lipid rafts are unknown. The presence in its structure of a Prohibitin Homology (PHB) Domain, also referred to as Stomatin, Prohibitin, Flotillin, HflC, and K (SPFH) Domain
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Flotillin-1 is present in subdomains of the phagosome membrane. A, J774 macrophages were fed with latex beads for 60 min followed by a 3-h incubation in normal medium. Double-immunofluorescence was then performed to localize LAMP1 and flotillin-1. The results indicate that whereas LAMP1 covers the whole surface of latex bead-containing phagosomes, flotillin-1 is present on distinct regions of the phagosome membrane (arrows). B, J774 macrophages were infected with the intracellular pathogen L. donovani for 60 min followed by a 3-h incubation in normal medium. Double-immunofluorescence was then performed to localize flotillin-1 and the leishmanial molecule LPG. The results indicate that pathogen-containing phagosomes also display a punctate labeling for flotillin-1 (arrow), although only a very small proportion of phagosomes are labeled. C, quantitative analysis shows that 20% of L. donovani wild-type-containing phagosomes are positive for flotillin-1. In contrast, 53% of the lpg2−/−Leishmania-containing phagosomes and 90% of latex beads-containing phagosomes are labeled for flotillin-1. This result represents the mean of three separate experiments.

(23), might allow its association to lipid rafts. As the name implies, this domain is also present on prohibitin and stomatin, two membrane proteins known to associate with Triton X-100-insoluble rafts (24, 25). Interestingly, both prohibitin and stomatin have been identified on latex bead-containing phagosomes by mass spectrometry (8). Alternatively, results by Western blot analysis on two-dimensional gels showing that flotillin-1 migrates as a series of spots of different pI suggest that flotillin-1 might be hyperphosphorylated (Fig. 1B). This feature may also provide a potential mechanism of association to lipid rafts, as shown for p56

(26). The presence of lipid rafts on phagosomes is surprising because these structures have been described mainly in the Golgi apparatus and the plasma membrane. Despite the phagosomal distribution of flotillin-1 in raft-like structures, it was important to establish whether these structures correspond to the biochemical definition of rafts, which is the insolubility in Triton X-100 and low density in sucrose gradients. To address this point, we performed raft isolation from purified phagosomes and tested them for the presence of flotillin-1 by Western blotting (Fig. 4A). Our results clearly showed an enrichment of flotillin-1 in the phagosome-rafts fraction compared with total phagosomes (Fig. 4B). In contrast, LAMP1, a major membrane protein of phagosomes, was not detected in the phagosome-rafts fraction, demonstrating the specificity of our extraction procedure. Our results also showed that a significant portion of flotillin-1 present on phagosomes is solubilized by the Triton X-100 treatment (Fig. 4A), suggesting that some of the phagosomal flotillin-1 is not associated with lipid rafts. It is also possible that flotillin-1-enriched rafts are partially solubilized by the detergent because it was shown that lipid rafts displaying different sensitivities to solubilization, depending on the detergent used, can coexist in the same cells (27).

SDS-PAGE analysis indicated that several proteins are enriched in the phagosome-rafts fraction compared with total phagosomes (Fig. 4C). To identify some of the proteins present in lipid rafts, we used a proteomic approach. The proteins recovered in phagosome lipid rafts isolated from [35S]methionine-labeled cells were separated by two-dimensional gel electrophoresis. The gels were dried and exposed for 6 weeks and then analyzed and compared with gels of total phagosomes. This allowed identification of phagosome proteins present in
the rafts. Using our data base of identified phagosomal proteins (8), we were able to show that actin, the α, β1, and β2 subunits of heterotrimeric G-proteins, as well as the A, B, and possibly the E subunits of the vacuolar proton pump ATPase were among the major proteins of the Triton X-100 insoluble lipid rafts (Fig. 4D). At least 9 as yet unidentified proteins were also enriched in the phagosome lipid rafts preparations (3 of which are highlighted by question marks in Fig. 4C). Subunits of heterotrimeric G-proteins have been identified in lipid rafts in other studies (28). Their identification was instrumental to the proposal that rafts are specialized sites for signal transduction (4). Our findings suggest that signal transduction could also take place through specialized regions of the phagosome membrane. Subunits of the proton pump ATPase have also been identified previously in Triton X-100 insoluble fractions (29), in association with proteins of the SNARE complex, suggesting that control of fusion events (see below), through acidification, could involve lipid rafts.

Although flotillin-1 was recently shown to be involved in insulin signaling at the plasma membrane of adipocytes (30), the functions of this protein and, more generally, of lipid rafts on phagosomes are currently unknown. An interesting feature of phagosomes is that it is an organelle unable to perform its main task, the killing and degradation of microorganisms, immediately after its formation at the plasma membrane. Indeed, the acquisition of phagosome functional properties depends on complex sets of interactions with various cellular organelles, leading to the biogenesis of phagolysosomes (31). Studies of this complex process in the last few years has put forward at least two types of interaction required for phagolysosome biogenesis. First, phagosomes must bind and move along cytoskeletal elements, both microtubules and actin filaments, to encounter and interact with other endocavular organelles (6, 32). Second, phagosomes must recognize and fuse with these endocavular organelles to allow the transfer of important microboidal molecules to the phagosome lumen. Interestingly, data from the study of phagolysosome biogenesis, as well as analyses of lipid raft composition and function suggest the idea that specialized subdomains of the phagosome membrane might play key roles in both types of interactions. Biochemical analyses have shown that actin and actin-binding proteins are closely associated with phagosomes (11) and that this organelle has the ability to induce the nucleation of actin at certain foci on its membrane (32). Interestingly, the latter study demonstrated that late phagosomes are more efficient at inducing actin nucleation, in accordance with a potential role for flotillin-1 and lipid rafts in this process, which accumulate on maturing phagosomes. Proteomic analysis of phagosome lipid rafts indicated that actin is a major protein of these structures, in accordance with recent results showing that lipid rafts are the sites of actin accumulation and polymerization (33). Allen and Aderem (34) have also published results clearly showing the focal recruitment of the actin-associated molecules vinculin and paxillin to the phagosome membrane. Subunits of the proton pump ATPase have also been identified previously in Triton X-100 insoluble fractions (29), in association with proteins of the SNARE complex, suggesting that control of fusion events (see below), through acidification, could involve lipid rafts.

The presence of molecules of the fusion machinery in membrane subdomains indicates that specialized regions of biological membranes might also favor membrane fusion (4, 35). There is increasing evidence that fusion between phagosomes and endosomes might take place preferentially at certain sites on the membrane of these organelles. Stahl and co-workers (36) have shown that phagosome-endosome fusion is initiated at “hot spots” on membranes where Rab5 accumulates. Focal distribution of EEA1, a Rab5 effector of endosome/phagosome fusion, was also observed at the surface of early endosomes (37). Interestingly, phagosome-endosome fusion also appears to involve transient interactions of parts of their membranes, a process referred to as kiss and run fusion (6, 31). According to the kiss and run hypothesis, fusion between these organelles is initiated by the formation of a fusion pore that allows transient exchange of luminal molecules. However, the expansion of the pore is limited and does not lead to the complete fusion of the organelles. Instead, the fusion pore closes allowing the separation of phagosomes and endosomes. Confirmation that transient fusions occur between phagosomes and endosomes was shown by the fact that molecules of different sizes present in the same endosomes are not transferred to phagosomes simultaneously (38). Instead, small molecules are transferred whereas larger molecules remain in the endosomes (39). Similar results are also observed between endosomes along the endocytic pathway (40). Remarkably, the kiss and run fusion is regulated, in part, by the small GTPase Rab5, as shown by the loss of size selectivity in the transfer of solute materials from endosomes to phagosomes in Raw 264.7 macrophages expressing the active GDP-bound form of Rab5 (13). Interestingly, current models of the fusion pore predict that lipidic pores could either expand irreversibly or remain open for several seconds and then close if slight changes in the membrane lipid composition were to occur (41). In this context, the presence of lipid microdomains on the phagosome membrane could rapidly provide the lipid changes required for the fusion pore closure.

This study extends current models of lipid raft microdomain formation to the membrane of phagosomes. Segregation of lipids and proteins within the phagosomal membrane may provide focal points on which complexes of signaling proteins or proteins of the fusion machinery can assemble, and where specialized functions may occur. Phagosomes have considerable advantages in the study of the function of lipid rafts because these organelles can be formed and isolated at will under various cellular conditions, and experimentally manipulated in vitro assays.

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