Arrest of Mycobacterial Phagosome Maturation Is Caused by a Block in Vesicle Fusion between Stages Controlled by rab5 and rab7*

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Mycobacterium tuberculosis and the closely related organism Mycobacterium bovis can survive and replicate inside macrophages. Intracellular survival is at least in part attributed to the failure of mycobacterial phagosomes to undergo fusion with lysosomes. The transformation of phagosomes into phagolysosomes involves gradual acquisition of markers from the endosomal compartment. Members of the rab family of small GTPases which confer fusion competence in the endocytic pathway are exchanged sequentially onto the phagosomal membranes in the course of their maturation. To identify the step at which the fusion capability of phagosomes containing mycobacteria is compromised, we purified green fluorescent protein-labeled M. bovis BCG phagosomal compartments (MPC) and compared GTP-binding protein profiles of these vesicles with latex bead phagosomal compartments (LBC). We report that the MPC do not acquire rab7, specific for late endosomes, even 7 days postinfection, whereas this GTP-binding protein is present on the LBC within hours after phagocytosis. By contrast, rab5 is retained and enriched with time on the MPC, suggesting fusion competence with an early endosomal compartment. Prior infection of macrophages with M. bovis BCG also affected the dynamics of rab5 and rab7 acquisition by subsequently formed LBC. Selective exclusion of rab7, coupled with the retention of rab5 on the mycobacterial phagosome, may allow organisms from the M. tuberculosis complex to avert the usual physiological destination of phagocytosed material.

A significant attribute of a pathogen with an intracellular lifestyle is its avoidance of or resistance to the noxious environment of the secondary lysosome, a highly acidic compartment filled with hydrolyses. Some organisms, such as Listeria monocytogenes, lyse the phagosome and escape into the cytoplasm (1). In contrast, Leishmania forms a vacuole that fuses rapidly with the lysosome, and the organism appears not to alter but to tolerate the environment of the phagolysosome (2). Other organisms fall along the spectrum between these extremes (3–10). Mycobacterium tuberculosis and other members of the M. tuberculosis complex, (e.g. Mycobacterium bovis), whose infectious cycle depends on entry and multiplication within mononuclear phagocytic cells (11–14), prevent fusion of the mycobacterial phagosome with compartments containing lysosomal enzymes (15, 16). Phagosomes containing mycobacteria fail to acidify (17) at least in part because of removal or lack of recruitment of the proton ATPase (18, 19). Studies with known markers of the endosomal-lysosomal pathway indicate that phagosomes with mycobacteria retain markers from the plasma membrane and recruit other molecules (19–24), but little is known regarding the control of the acquisition and loss of proteins and membranes in such processes.

Phagocytosis sets in motion a series of fusion and fission events between the phagocytic vesicle and endocytic organelles resulting in a sequential acquisition and loss of markers as the phagosome matures toward the phagolysosome (25, 26). The docking and fusion machinery affecting these processes is not completely understood, but some progress has been made (27). In a report (26) addressing fusion events in phagocytosis, a precise temporal redistribution of rab(s), small ras-like GTP-binding proteins, was observed during maturation of latex bead phagosomal compartments (LBC). Specific rab proteins are known to be recruited to membrane positions in their active, GTP-bound form to regulate assembly of docking proteins, providing a proofreading mechanism that ensures specificity of membrane fusions (28–30). The known functions of rabs and their presence and succession on LBC strongly suggest a role for rab proteins in the control of homotypic and heterotypic vesicle fusion in the process of phagosome maturation (26).

In this work we investigated the composition of small GTP-binding proteins on mycobacterial phagosomes and compared M. bovis BCG phagosomal compartments (MPC) with LBC from J774 macrophages (26) to test whether the distribution of GTP-binding proteins on MPC was affected by the pathogen. We report that MPC acquire and retain rab5, a regulator of early endosome membrane fusion (31–33), but fail to associate with rab7, a GTP-binding protein specific for the late endosome. The lack of rab7 defines the stage between the early and late endosome as the defective checkpoint in mycobacterial phagosome maturation.

EXPERIMENTAL PROCEDURES

Cell Lines, Bacterial Strains, and Culture Conditions—Murine macrophage-like cell line J774 was cultured and maintained in Dulbecco's
modified Eagle’s medium (Cellgro) containing 200 mM l-glutamine, 5% heat-inactivated fetal bovine serum (Hyclone Laboratories) (DMEM 5% FBS) (34). M. bovis BCG harboring hsp60-gfp (35) was grown in 7H9 medium, homogenized in DMEM 5% FBS, and uniformity of the single-cell suspensions was verified (34).

Isolation of Mycobacterial Phagosomal Compartments—Macrophages (3 × 10⁷) were infected by exposure to 25 M. bovis BCG/macrophage for 1 h, washed, and incubated as described previously (34). No apparent damage to the macrophage monolayer was observed up to 12 days. Macrophages were lysed by approximately 30 passages through a syringe apparatus fitted with 22-gauge needles in homogenization buffer containing leupeptin, pepstatin A, chlороacetamidine-7-amino-2-heatonone, and E64 as described by Chakraborty et al. (36). After repeated centrifugation three times at 200 × g for 6 min at 4 °C, the postnuclear supernatant was subjected to velocity sedimentation (1,000 × g, 45 min, 4 °C) through 15% (w/w) sucrose cushion (36). Material collected at the 15–50% sucrose interface was diluted into 2 ml of 15% sucrose and overlaid on a preformed 11 ml linear 32–53% (w/w) sucrose gradient. After isopycnic centrifugation at 100,000 × g for 15 h in an SW40 rotor at 4 °C, 0.9-ml fractions were collected from the top of the gradient using a Buchler Auto Densi-Flow Fractionator (37). Radiolabeling contamination assays (36) indicated ≤10% contamination of MPC preparations with other cellular proteins.

Latex Bead Compartment Isolation—Dyed latex beads (0.8 mm, 10% solids; Sigma) were added (1:500 dilution in DMEM 5% FBS) to macrophage monolayers for 1 h, and monolayers were incubated as indicated. For LBC preparations, cells were lysed as described above, and LBC were isolated from postnuclear supernatant by flotation on discontinuous sucrose gradients as described by Desjardins et al. (26). In experiments with sequential phagocytosis of M. bovis BCG and latex beads, macrophages were infected with mycobacteria and incubated for 72 h, after which latex beads were added (1:500 dilution) for 1 h followed by a 1-h chase. The infected monolayers were lysed, and LBC were isolated. The LBC fraction contained less than 1% of the bacteria in the gradient.

Flow Cytometry and Analysis of β-Hexosaminidase Activity—Flow cytometry was carried out as described previously (35). β-Hexosaminidase activity was detected using 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (Calbiochem) as described by Green et al. (38), and the product was assayed in an SLM Aminco SPF-500C spectrofluorometer (excitation 365 nm, emission 450 nm).

Epifluorescence, Laser Scanning Confocal and Electron Microscopy—Coverslips containing M. bovis BCG-infected macrophages were prepared for epifluorescence or confocal microscopy as described previously (35). For electron microscopy, macrophages and subcellular fractions were fixed and washed as described (35), osmicated, contrasted with uranyl acetate, embedded in 2% agarose, dehydrated with acetone, and infiltrated overnight in Epon (35, 37). Thin sections of the oriented pellets were examined by transmission electron microscopy using a Phaga 301 microscope.

Two-dimensional Gel Electrophoresis, GTP Binding Overlay Assay, and Western Blot Analysis—Equivalent amounts of phagosomes were solubilized in lysis buffer as described previously (37). Proteins were separated by isoelectric focusing using Ampholines, pH 5–7 and pH 7–9 (1:1), at 1,200 V for 17 h followed by 12% SDS-PAGE and were transferred to Immobilon P membranes by electroblotting (37). GTP-binding proteins were detected in an overlay assay with 1 μCi/ml [α-32P]GTP (3,000 Ci/mM; DuPont NEN) in 50 mM phosphate buffer, pH 7.5, 5 mM MgCl₂, 1 mM EGTA, and 0.3% Tween 20 as described (37, 39) and visualized by autoradiography. GTP binding was quantitated in a PhosphorImager. For immunoblots, membranes were incubated with affinity-purified antibodies for rab4, Rab5, and rab7 prepared and characterized as described by Zerial et al. (40), or rat monoclonal antibodies to murine Lamp1 (clone 1D4B), Lamp2 (clone ARL-93) (Developmental Hybridoma Bank, NICHHD, Iowa City, IA) transferrin receptor (Bio-source International), or rabbit anti-actin (Sigma). The ECL Western blotting detection system (Amersham Corp.) was used for visualization of bound antibodies.

RESULTS

Isolation of M. bovis BCG Phagosomal Compartments—To investigate the small GTP-binding protein composition of mycobacterial phagosomes, vesicles containing M. bovis BCG had to be separated from other cellular compartments. To facilitate identification of MPC, M. bovis BCG was labeled in vivo by expressing green fluorescent protein (GFP) (Fig. 1A) encoded by the plasmid phs60-gfp (35). JJ74 macrophages were infected with GFP-labeled M. bovis BCG resulting in 1–4 bacilli/eukaryotic cell at the outset of the infection. The choice of JJ74 cells was based on the following considerations. (i) GTP binding profiles and several rab proteins have been defined on phagosomes in this cell line (26). (ii) JJ74 cells respond similarly to human monocyte-derived macrophages in phagocytosis studies (41) and have been used for studies of phagocytosis of various organisms and intracellular killing of mycobacteria (42, 43). (iii) The use of JJ74 allows isolation of subcellular organelles in sufficient quantities to permit biochemical analysis.

After infection of macrophage monolayers and internalization of mycobacteria (Fig. 1, A and B), MPC were separated (Fig. 2) from other subcellular as described under “Experimental Procedures.” The velocity sedimentation step eliminated Golgi and substantial amounts of endosomal markers from the postnuclear supernatant. Further purification was achieved by isopycnic centrifugation in sucrose gradients such that the majority (60%) of GFP-labeled phagosomes were located in fractions with a density of 1.16–1.19 g/ml (Fig. 3). These fractions contained 15% of total protein loaded on the gradient and appeared well separated from the remaining late endosomes and lysosomes that partitioned in fractions above the peak of green fluorescence and at the bottom of the gradient as assayed by β-hexosaminidase activity (Fig. 3). Fractions that displayed the highest GFP fluorescence were significantly enriched in M. bovis BCG-containing phagosomes were used in further studies as MPC. Morphological analysis by electron microscopy of thin sections of the oriented pellets, from top to bottom to ensure complete representation of the contents of the gradient fractions corresponding to MPC, showed that the phagosomal membranes were intact and indicated little contamination with
Comparison of GTP Binding Profiles in Mycobacterial and Latex Bead—Samples of MPC, and LBC preparations generated as described previously (26), were separated by two-dimensional gel electrophoresis, proteins transferred onto membranes under renaturing conditions, and GTP binding activity assessed by [32P]GTP overlay assay (26, 37). The MPC preparations showed several low Mr GTP-binding proteins (Fig. 4).

The equivalent fractions prepared from uninfected macrophages did not contain any of the GTP-binding proteins in the basic region of the two-dimensional gel (data not shown). Although GTP binding patterns of MPC relative to LBC appeared nearly identical at 1 h postinfection (not shown), dramatic differences began to emerge at later time points (Fig. 4). Interestingly, differences were prominent in the basic region of the gels where the rab proteins of the endosomal pathway are located (26, 44). Such proteins were persistently present in MPC preparations compared with the reduced GTP binding activity or its complete absence (168-h time point) in the basic region of the gels with LBC membranes (Fig. 4D). Taken collectively, these observations were consistent with differential accumulation of a specific subset of GTP-binding proteins on MPC relative to LBC membranes.

Differential Accumulation of rab5 and Lack of rab4 and rab7 on M. bovis BCG Phagosomes—Next, we tested whether any previously characterized rab proteins were absent or retained on MPC compared with LBC membranes by immunoblotting (Fig. 5). Since the region on two-dimensional gels where the differences in GTP binding were most prominent coincided with the usual location of rab5 isomers and possibly of rab4 and rab7, corresponding antibodies were used to probe MPC and LBC membrane proteins separated by SDS-PAGE. A rab protein specific for the recycling endosomal compartment, rab4, was first tested. In these experiments, we could not detect rab4 on either LBC (consistent with the report by Desjardins et al. (26)) or MPC, whereas it was detected in postnuclear supernatants (Fig. 5A). Next we confirmed the identity of one of the prominent GTP-binding proteins on MPC as rab5a (Figs. 4C and 5C). The rab5 detected was not due to contaminating membranes based on the comparison of MPC 72 h postinfection (Fig. 5C, lane 1) with the corresponding fractions from uninfected cells (Fig. 5C, lane 2). MPC appeared not only to maintain levels of rab5a but also to gain additional amounts at longer incubation time points (Fig. 4C and 6B). The mean increase in GTP binding by rab5a from MPC membranes was...
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3-fold over 7 days as quantitated in a PhosphorImager. This was in contrast to LBC which, consistent with the findings of Desjardins et al. (26), showed a decrease of rab5a over time. When the amount of rab5a detected by Western blot analysis on LBC (Fig. 6B) was normalized for the amount of membranes loaded (Fig. 6A), rab5a content on LBC decreased between the 1-h and 168-h time points. Most importantly, very little rab7 was detected in MPC samples even 168 h postinfection, whereas LBC acquired rab7 by 1 h (Fig. 6C). These results indicated that MPC maintained or increased their rab5 content but did not significantly intersect with the rab7-containing compartment.

To address the possibility that infection with M. bovis BCG could affect the overall rab7 content of J774 cells, Western blots of whole cell extracts with antibodies against rab7 and actin (for standardization) were carried out and binding intensities quantitated by densitometry. The rab7 band in uninfected macrophage samples had an intensity of 157 ± 13.5 (for 3 days postinfection) arbitrary units compared with 141 ± 11.2 for BCG-infected macrophages (p value t test 0.4). In addition, no significant differences were observed in the distribution of rab7 across the gradient (Fig. 3) between BCG-infected and uninfected cells (data not shown). These results suggest that the presence of BCG does not alter the rab7 content of infected J774 cells and that the lack of rab7 on MPC reflects specific effects on the phagosomal compartment.

Analysis of Other Markers on MPC and LBC—Although MPC had no detectable rab4 (Fig. 5A), transferrin receptor was detected on MPC both at 1 h (Fig. 5B) and 72 h (data not shown). This observation can be best explained in light of the abundance of rab5 on MPC, compatible with the formal capacity for homotypic fusion within the early endocytic network. Lamps, the major membrane glycoproteins associated with lysosomes and late endosomes, were not present in appreciable quantities on MPC for the duration of the experiment, but they were present on LBC preparations at all time points tested as detected using anti-Lamp1 and anti-Lamp2 antibodies (data not shown and Fig. 6D). The very low level of Lamp proteins observed on MPC compared with LBC was in agreement with the observations by Clemens and Horwitz (20). In the case of rab7, ECL analysis of such blots indicated 146.8 ± 18.9 arbitrary units for LBC versus 15.8 ± 6.3 arbitrary units for MPC. These results are compatible with the interpretation that MPC have a severely reduced content of Lamp2 and rab7, an observation that could not be challenged by the purity of the preparations since any contamination with late endosomal and lysosomal membranes would contribute to the detection of rab7 and Lamp proteins (Fig. 6C).

M. bovis BCG Alters the Kinetics of rab Distribution in the Latex Bead Compartment in a Sequentially Infected Cell—To examine whether prior infection of host macrophage cells with M. bovis BCG can affect the distribution of rab proteins on subsequently formed phagosomes, we exposed macrophages sequentially to M. bovis and latex beads. Following infection with M. bovis and a 72-h incubation after internalization of the mycobacteria, macrophages were allowed to phagocytose latex beads for 1 h. After a chase period of 1 h, LBC were isolated, and rab profiles were examined by Western blots. Interestingly, LBC from preinfected cells showed different ratios of rab5 to rab7 than the matching LBC isolated from cells not previously exposed to M. bovis BCG (Fig. 7A, lanes 2 and 4). The abundance of rab7 relative to rab5 in the same samples changed from 129.4 ± 5.5% for beads alone (Fig. 7, lane 2) to 60.0 ± 8.9% in LBC from BCG-preinfected macrophages (Fig. 7, lane 4). Standard MPC preparations from infected cells with no addition of the beads were also analyzed at the same time (Fig. 7, lane 6). These results indicated that the preinfection with M. bovis BCG perturbed the physiological course of events and possibly delayed or reduced the acquisition of rab7 or increased the retention of rab5 by the latex bead phagosome in the cell infected previously with M. bovis. These findings suggest that given sufficient time, mycobacteria may exert broader effects on membrane trafficking and phagosome maturation in macrophages.
**DISCUSSION**

Reports that *M. tuberculosis* resides in macrophage compartments that display limited fusion capacity with the lysosome have a relatively long history (15–23). A link has been postulated (13, 15, 18, 21) between this intriguing phenomenon (15, 16), or other potential pathways of intracellular survival (42), and the capacity of pathogenic mycobacteria to survive and replicate in host monocytes. Several recent studies have extended the original microscopic observations (15, 16) with information at the molecular level concerning specific markers retained from the plasma membrane or blocked from access to the mycobacterial phagosome (18–21, 23, 24). However, very little is known about the regulatory mechanisms controlling phagosome maturation and whether they are affected by mycobacteria.

Since GTP-binding proteins of the rab family have been implicated in the regulation of transport in the endocytic and exocytic pathways and in conferring specificity of vesicle fusion (28–30, 45, 46) the objective of our present study was to compare the GTP-binding proteins of mycobacterial phagosomes with those of other phagosomal membranes. A seminal analysis by Desjardins et al. (26) of the succession of GTP-binding proteins on phagosomes containing latex beads from J774 macrophage-like cells was used as a starting point for our investigation of mycobacterial phagosomes. In the present study, we have examined the time course of changes in GTP-binding protein profiles of mycobacterial phagosomes and compared them with the latex bead phagosome. Initially, the phagosomes containing *M. bovis* BCG resemble the GTP binding pattern of the vesicles containing latex beads; but with continued incubation, the two profiles diverge dramatically: (i) MPC appear to accumulate rab5 proteins over time, whereas LBC lose rab5 proteins; and (ii) rab7 is not detected on MPC, whereas LBC lose rab5 proteins and lose rab7 upon extended incubation.

The small GTPases rab4, rab5, and rab7 control trafficking through the endocytic pathway. Rab5 regulates transport from the plasma membrane to early endosomes and the homotypic fusion of early endosomes (31, 32). Overexpression of both wild type rab5 and its GTPase-deficient mutant (rab5 Q79L) increases the rate of endocytosis and causes the appearance of enlarged early endosomes (32, 45). Rab4 regulates trafficking from the early endosomes through the recycling pathway (47), whereas rab7 regulates transport from the early to late endosomes (48). Overexpression of wild type rab7 (as high as 50-fold over the endogenous levels) has no significant effect on the morphology of the endocytic compartment (48), but its GTPase-deficient mutant (rab7 Q67L) accumulates partially on the lysosomes, a compartment downstream from late endosomes (49). A striking difference in the effects of the overexpression of wild type and mutant rab5 and rab7 proteins suggests different mechanisms of action of these proteins in the control of endocytosis. Rab5 appears to regulate a rate-limiting step in early endosomal fusion and may be responsible for the maintenance and self-preservation of the early endosomal sorting compartment, whereas both rab4 and rab7 regulate membrane transport out of the early endosomal compartment and therefore may regulate sorting and/or targeting steps.

During phagosome maturation, interactions have been observed between phagosomes and early and late endosomes but not recycling endosomes (26). We did not detect rab4 on MPC or LBC. During phagosome-endosome interactions, the delivery of rab5 extends the capacity for homotypic fusion with early endosomes to the maturing phagosome. However, subsequent delivery of rab7 to the phagosome may not reflect homotypic fusion with the late endosome but could represent a targeting signal for transport toward the lysosome. If this is the case, this event could be detrimental for the survival of the phagocytosed pathogen, and fusion with rab7-bearing compartments should be avoided. Lack of recruitment or selective removal of rab7 would enable mycobacteria to survive and multiply inside the macrophage while escaping the precarious rab7-regulated pathway leading out of the early endosomal sorting compartment toward the lysosome.

Our results suggest that MPC can gain and retain early endosomal characteristics (e.g., rab5 and transferrin receptor) but appear isolated from the late endosomal compartment as evidenced by the lack or loss of rab7 and Lamp proteins. It is important to note that multiple rab proteins are recruited independently onto competent membranes (50). The presence of mycobacteria appears to alter or interfere selectively with the appropriate machinery or processes leading to the recruitment of rab7. This effect may be confined initially to the local environment of the mycobacterial phagosome, as others have noticed in coinfection experiments that each vacuole follows its own fate depending upon the pathogen or particle that it contains (19, 20). Intriguingly, in our experiments with sequential phagocytosis of *M. bovis* BCG and latex beads, spaced by a long incubation time of 72 h, we observed a change in the rab protein profiles of LBC relative to LBC from uninfected control monolayers. These observations suggest that mycobacteria may exert, upon establishment of their residence in the macrophage or in the course of such processes, more general effects on the host cell.

Altered phagosome acidification (17, 18) could be a contributing mechanism to the intracellular survival of pathogenic mycobacteria. Interestingly, whereas ammonia and chloroquine both raise intralysosomal pH, only the former amine has been found to inhibit phagosome-lysosome fusion in the context of mycobacterial interactions with the macrophage (51, 52). These and other studies addressing the relationship of acidification and endosome-lysosome fusions (53) suggest that additional mechanisms or properties of *M. tuberculosis* may be of significance. It is also of significance to note that *M. bovis* BCG avoids phagosomal-lysosomal fusion similarly to the more virulent *M. tuberculosis* strains (42) and that these variants differ mostly at the subsequent levels of interaction with the immune system including CD4+ and CD8+ T cells (54). Future investigations may address potential differences, if any, among strains in the context of the observations reported here. Although it is not known whether and how mycobacteria modulate the membrane constituents of the phagosome to block phagosome mat-
uration events, molecules controlling vesicular trafficking have been implicated as targets for several toxins from different bacterial pathogens. For example, Clostridium tetani, Clostridium botulinum, and Helicobacter pylori produce toxins that affect or change vesicle fusion capacity (55, 56). Furthermore, bacterial toxins affecting small GTP-binding proteins via ADP-ribosylation or transfer of glucose from UDP-glucose have been identified in C. botulinum (57, 58) and Clostridium difficile (59). Based on the local effects within MPC and the more generalized effects of M. bovis BCG infection on subsequently formed LBC, it is possible that some mycobacterial (51, 52, 60, 61) or other putative activities directly or indirectly interfere with the regulation of membrane sorting processes. Regardless of the precise mechanism of action, the uncovering of the exclusion of rab7 from MPC offers the definition of a discrete vesicular trafficking checkpoint that has been compromised, thus denying competence to the mycobacterial phagosome for fusion with the lysosomal compartment.

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REFERENCES
1. Falkow, S., Isberg, R. R., and Portnoy, D. A. (1992) Annu. Rev. Cell Biol. 8, 333–363
2. Mossner, D. M., and Rosenthal, L. A. (1994) in Strategies for Intracellular Survival of Microbes (Russel, D. G., ed) Vol. 2, pp. 191–212, Bailliere Tindall, London
3. Sibley, L. D., Weidner, E., and Krabbenbuhl, J. L. (1985) Nature 315, 416–419
4. Andrews, N. W., Abrams, C. K., Slatin, S. L., and Griffiths, G. (1990) In The Mycobacterial Phagosome, pp. 93–122, King’s College London
5. Armstrong, J. A., and Hart, P. D. (1971) J. Exp. Med. 134, 713–740
6. Armstrong, J. A., and Hart, P. D. (1975) J. Exp. Med. 142, 1–16
7. Crowle, A., Dahl, R., Ross, S., and May, M. (1991) Infect. Immun. 59, 1823–1831
8. Sturgill-Koszycki, S., Schlesinger, P. H., Chakraborty, P., Haddix, P. L., Collins, H. L., Feil, E. A., Allen, P., and Russell, D. G. (1994) Science 263, 678–681
9. Xu, S., Cooper, A., Sturgill-Koszycki, S., van Heyningen, T., Chatterjee, D., Orme, I., Allen, P., and Russell, D. G. (1994) J. Immunol. 153, 2568–2578
10. Joiner, K. A., Fuhrman, S. A., Miettinen, H. M., Jasper, L. H., and Mellman, I. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 937–942