Increased Expression of Rab5a Correlates Directly with Accelerated Maturation of Listeria monocytogenes Phagosomes*

(Received for publication, January 29, 1999, and in revised form, February 19, 1999)

Carmen Alvarez-Dominguez‡ and Philip D. Stahl§
From the Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

Previous studies have shown that Listeria monocytogenes (LM) modulates phagocytic membrane traffic. Here we explore whether Rab5a, a GTPase associated with phagosome-endosome fusion, is related to phagosome maturation and to the intracellular survival of LM. Stable transfection of Rab5a cDNA into macrophages accelerates intracellular degradation of LM. Morphological studies confirmed that phagosome maturation and phagosome-lysosome fusion is enhanced by overexpression of Rab5a.

Down-regulation experiments using antisense oligonucleotides targeted to the Rab5a mRNA efficiently reduced Rab5a synthesis, reduced phagosome-endosome traffic, blocked phagosome-lysosome fusion, and extended intraphagosomal survival of LM. Down-regulation of Rab5a had no effect on LM internalization. Down-regulation of Rab5c had no effect on phagosome maturation and phagosome-lysosome fusion. The results indicate that Rab5a controls early phagosome-endosome interactions and governs the maturation of the early phagosome leading to phagosome-lysosome fusion.

Phagocytosis is a complex process required for host defense and tissue remodeling. The uptake of pathogens and the activation of membrane trafficking and other events that lead to killing and disposal is key to an efficient host defense strategy. Listeria monocytogenes (LM), a human pathogen that infects a variety of cell types including macrophages (MHö), has served as an excellent model for examining membrane trafficking events involved in intracellular killing (1). MHö, unlike other host cells, receives a common strategy for sustained intracellular growth. Various forms of this strategy can be found in Mycobacterium tuberculosis, Listeria monocytogenes, and Salmonella typhimurium among others.

Virulent strains of LM have been shown to access the cytoplasm where bacterial growth flourishes. However, using a nonhemolytic mutant of LM (LM<sub>blv</sub>), which is retained within the phagosome, we have made several observations that are pertinent to intracellular survival of the pathogen including delayed phagosome maturation and fusion with lysosomes. The choice of the LM<sub>blv</sub> mutant was fortuitous, since it allowed us to unmask bacterial targets, which modulate intracellular trafficking, that would not have been possible using the virulent organism (4).

Rab5, the rate-limiting GTPase for endocytosis (5–7), is expressed as three different isoforms (a, b, c) that appear to have overlapping intracellular distributions (8). Rab5 isoforms in the pathogen Trypanosoma brucei appear to have different localization and functions (9). Although Rab5a has been localized on phagosomes containing different particles (10–13), its role in mediating phagosome maturation has not been extensively investigated.

Our results indicate that Rab5a and Rab5c play different roles in the phagocytic pathway.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—MHö from normal donors were cultured and detached as described (14). J774 cells were cultured in RPMI 1640 medium, 5% fetal calf serum, 2 mM glutamine, 50 µg/ml gentamicin. The nonhemolytic LM mutant strain (DP-L2161) (LM<sub>blv</sub>) derived from the wild type strain (1040S) (15), was kindly provided by D. A. Portnoy (University of California). The following antibodies were used: mouse monoclonal anti-Rab5a (4F11) (1300 dilution) (4, 8, 11, 16); polyclonal rabbit anti-Rab5c (1:50 dilution) was developed by immunizing rabbits with rat serum albumin conjugated to the N terminus of a C-terminal peptide of Rab5c (CAPSRRNRGVDLQNSPSRSE). Phospho-Rab5a-antisense (5'-TGC GGC TCG ACT AGC CAT GT-3') and sense (5'-ACA TGG CTA GTC GAG GCG CA-3') oligonucleotides; Rab5c-antisense (5'-GC CTC CCC GAC CCG CCA TTG-3') and sense (5'-CA ATG GGC GTG CGG GGA GGG-3') (PT-oligonucleotides) (20-mer) purchased from Biosystems (Palo Alto, CA), included a pair of bases before the ATG to maximize hybridization and specificity (17, 18). Phosphodiester oligonucleotides were also designed for polymerase chain reaction purposes and used with Rab5a- and Rab5c-specific vectors to confirm the specificity of the oligonucleotides. Lipofectin and Opti-MEM medium were obtained from Life Technologies, Inc.

**Immunoprecipitations and PT-antisense Oligonucleotide Treatment**—Immunoprecipitation was carried out as described (18). PT-oligonucleotides (10 µg) were incubated with 10 µg of Lipofectin in 200 µl of Opti-MEM for 15 min at room temperature before being added to Opti-MEM-treated HMö for 4 h at 37°C. For electroporation, HMö (5 x 10<sup>6</sup>/ml) were incubated with 10 µg of PT-oligonucleotides on ice for 10 min. Electroporation was performed with a Baxter BTX-600 electroporator using 2-mm gap cuvette chambers with the following settings: 220 V, 800 microfarads, 72 Ω. Cells were placed on ice immediately, and complete medium was added. Cells were set onto culture plates for 2 h at 37°C and extensively washed before use.

**Overexpression of Rab5α in J774 Cells**—Rab5α:wt in cDNA was subcloned into pcDNA3 using EcoRI/BamHI sites. Cells (5 x 10<sup>6</sup>) were transfected with (20 µg) Rab5α:wt/pcDNA3 or with pcDNA3 vector (control cells) by electroporation (150 V, 800 microfarads, 129 Ω) and selected with G418 (0.8 mg/ml). Cells containing Rab5α:wt/pcDNA3 were cloned twice by limiting dilution before using.

**LM Phagocytosis**—LM infection was performed according to standard protocols (4, 11) at a 10:1 bacteria/cell ratio. Cells were incubated at 37°C for 15 min to allow for bacterial uptake followed by a 45-min


**FIG. 1. Overexpression of Rab5a accelerates phagosome maturation and enhances intracellular killing of L.M. A.** Rab5a overexpression in J774 cells was confirmed by immunoprecipitation with 4F11 antibody (A, upper panel). Infection with LMM<sup>−</sup> was performed as described previously (4). The ratio of live bacteria (i.e. cfu) recovered at time 0 divided by the cfu recovered at 8 h was used as an index of intracellular killing. CfU recovered at time 0 for control and for Rab5a-transfected cells were 2.5 × 10<sup>6</sup> ± 267 cfu and 9 × 10<sup>5</sup> ± 189 cfu, respectively. At 8 h, cfu recovered from control and from Rab5a-transfected cells was 1 × 10<sup>6</sup> ± 122 cfu and 2.7 × 10<sup>5</sup> ± 64 cfu, respectively. Results are the mean ± S.D. of four different experiments. B, J774 cells transfected with Rab5a or with pcDNA3 vector alone (control cells) were incubated with BSA-gold (10-nm particles, 1 mg/ml) for 10 min and chased overnight to label lysosomes. Another set of cells was incubated the following day with BSA-gold for 10 min to label endosomes. All cells were then infected with LMM<sup>−</sup> for 10 min. Total gold particles per cell were quantified in each case from a total of 200 cells, as well as the number of gold particles found in phagosomes. Results are expressed as the percentage of gold particles found in phagosomes compared with total gold. Results are representative of at least three different experiments. C, cells were infected with radiolabeled live LMM<sup>−</sup> (200,000 cpm/well) as reported previously (4), incubated at 37°C for 20 min, washed, and solubilized with 1% Triton X-100. Proteins were precipitated from cell lysates with 10% trichloroacetic acid on ice. Results correspond to bacteria uptake (counts/min) after a 20-min incubation. Results are the mean of triplicates ± S.D. of four different experiments.

---

**RESULTS AND DISCUSSION**

Previous studies have shown that LMM<sup>−</sup> interfered with phagocytic trafficking and phagosome maturation (4, 11). Here, we analyze the role of Rab5a in phagosome maturation. To demonstrate that Rab5a, or a Rab5a regulatory factor plays a role in phagosome maturation, we stably expressed Rab5a in the mouse MØ cell line, J774, and investigated whether elevated levels of functional Rab5a could override the inhibitory effect of the bacterium. Stable transfection of J774 with Rab5a or with pcDNA3 vector alone (control cells) was performed as described with minor modifications (4, 11, 19). Bacteria were labeled with Tran<sup>35S</sup>-label. Dead (1 h, 60°C (11)) and live LM (3 × 10<sup>5</sup> cpm/well) were added to 2 × 10<sup>5</sup> HMØ pretreated with PT-oligonucleotides as described above. After 20 min of internalization, cells were washed and lysed to quantify LM uptake. To measure LM catabolism, cells were incubated for 1.6 h before lysis. Cells were solubilized in 1% Triton X-100 and proteins were precipitated with 10% trichloroacetic acid.
maturation was also confirmed by the acquisition of typical lysosomal proteins such as Lamp-1 and the mature form of cathepsin-D (Ref. 4 and data not shown). Rab5a appears to operate principally at the phagosomal compartment rather than in phagosome formation, since overexpression of Rab5b caused only a moderate increase in LM^{ly-} uptake (1.2–1.5-fold) (Fig. 1C).

The dramatic effect of Rab5a overexpression on phagosome maturation is also reflected in the morphological characteristics of the LM^{ly-} phagosomal compartment. LM^{ly-} phagosomes in control cells appear as swollen, "endosomal-like" compartments (see Fig. 2a). In Rab5a-transfected cells, LM^{ly-} phagosomes display tightly apposed limiting membranes with dense material and membrane inclusions, typical of multivesicular and lysosomal compartments (BSA-gold-marked lysosomes are shown in Fig. 2, b and c, and BSA-gold marked endosomes are shown in Fig. 2d). Interestingly, this compartment resembled phagolysosomes containing dead bacteria (4). All of these findings point to a linkage between phagosome maturation and LM^{ly-} killing in MØ and to Rab5a as an essential regulator of both processes.

Conclusions based on overexpression of proteins alone may not rule out compensatory expression of other factors that could account for the dramatic effects observed above. To corroborate our findings with Rab5a overexpression, we carried out experiments to determine whether down-regulation of Rab5a would alter the killing capabilities of the MØ and allow for LM^{ly-} survival.

Antisense phosphothioate oligonucleotides (PT-oligonucleotides) were chosen due to their longer intracellular half-lives (17, 18, 20). Transfection with PT-oligonucleotides was carried out by two methods with similar results (Lipofectin treatment or electroporation) (21). Transfection with antisense PT-oligonucleotides directed to the AUG translation initiation codon of the mRNA sequence of Rab5a and Rab5c was carried out on HMØ, because the sequences of human Rab5 isoforms (Rab5a and Rab5c) are known, whereas the mouse sequences are not available. The intracellular degradation of LM^{ly-} in HMØ resembles that found in the mouse MØ cell line, J774 (22–24). Several PT-oligonucleotides, designed to hybridize with specific Rab5a mRNA sequences (e.g., effector domain, 3′-untranslated regions, or translation initiation codon), were examined. However, only those oligonucleotides hybridizing with the translation initiation codon of Rab5a mRNA blocked Rab5a synthesis (approximately 85–90%, as determined by immunoprecipitation (Fig. 3A)). Rab5c, which shares more than 80% identity with Rab5a (8, 25), was unaltered by Rab5a-antisense treatment indicating that the PT-oligonucleotide effects were highly specific. Rab5a-antisense-treated HMØ permitted intracellular growth of LM^{ly-} mutant, while in sense or control cells, the bacteria were destroyed (Fig. 3B) (biosynthetic levels of Rab5a uptake experiments). Cells were incubated at 37°C for 100 min for catabolism experiments (lower panel). Cells were solubilized in 1% Triton X-100 and proteins precipitated from cell lysates with 10% trichloroacetic acid on ice. Results are expressed as the percentage of cell-associated radioactive bacteria remaining after 20 min of uptake and 100 min of chase carried out in triplicate. Results are representative of four independent experiments. B. HMØ were treated with Rab5a-PT-oligonucleotides (S, sense lanes; A, antisense lanes) as in A–C. Cells were offered BSA-gold (10 nm, 1 mg/ml) for 10 min, washed, and chased overnight to label lysosomes. Cells were infected with live LM^{ly-} (Dead-LM or dead LM^{ly-}) for 2 h (1-h infection period plus another hour of incubation without bacteria). The number of live LM^{ly-} was 1–2 × 10^{5} cfu. Total gold particles per cell were quantified in each case from a total of 200 cells, as well as the number of gold particles found in phagosomes. Results are expressed as the percentage of gold particles found in phagosomes compared with total gold. Results are representative of at least three different experiments.

![Diagram](image-url)
are shown as an inset in Fig. 3B. LM<sup>Δly</sup>-growth occurred inside the phagosomes, since this mutant lacks the protein necessary for lysis of the phagosomal membranes (as detected by electron microscopy, data not shown). The growth of LM<sup>Δly</sup> in Rab5a-antisense-treated cells was heterogeneous (Table I). Approximately 35% of the cells were free of bacteria after a 2-h pulse. The remaining 65% of the cells contained 1–2 bacteria per cell. Following a 4-h incubation, 20% of the infected cells contained 1–2 bacteria per cell, whereas nearly half of the cells contained 3–5 bacterial profiles per cell. At 24 h, 15% of the infected cells contained 3–5 bacteria per cell, and 37% of the cells contained >5 bacteria per cell, reflective of significant bacterial growth.

It has been reported that the Rab5 isoforms (a, b, and c) share similar functions and colocalize to the same compartment (8,25–27). However, recent results demonstrating differential expression of Rab5a following lymphokine signaling in MØs suggests specialized functions for the Rab5 isoforms (19).

Rab5c-antisense treatment was efficient and selective in blocking Rab5c synthesis (>88% inhibition, see Fig. 3A), since Rab5a synthesis remained unaltered. Analysis of LM<sup>Δly</sup>-infection in Rab5c-antisense-treated cells revealed no differences in LM<sup>Δly</sup> destruction (Fig. 3B). These findings suggest that Rab5a is the predominant regulatory Rab GTPase in the phagocytic pathway. Rab5c may play a minor role or function elsewhere.

Rab5a appears to play virtually no role in LM<sup>Δly</sup>-internalization, since transport from the plasma membrane to the phagosomes, as detected by following the internalization of radiolabeled LM<sup>Δly</sup> in antisense-treated cells (both Rab5a and Rab5cΔ), was unaffected. However, Rab5a is clearly involved in the degradation of pre-internalized dead LM<sup>Δly</sup>-. The percentage of radiolabeled bacteria remaining after a 20-min uptake and 100-min chase in Rab5a-antisense-treated cells was significantly higher (35% higher) than in Rab5a-sense-treated cells or control cells (Fig. 3C).

Overexpression of Rab5a only marginally increased bacterial phagocytosis. However, following phagosome formation, Rab5a appears to play at least two roles: (i) by mediating fusion events within the phagolysosomal-endosomal compartment and (ii) by facilitating or initiating phagosome maturation culminating in phagosome-lysosome fusion. The former is supported by in vitro reconstitution studies (11) and by the observations presented here that the intermingling of phagosomes and endosomes is reduced in Rab5a-antisense-treated cells. The latter is supported by the observation that fusion of phagosomes, containing dead LM<sup>Δly</sup>-bacteria, with lysosomes was impaired in Rab5a-antisense treated cells. The finding that phagosomes containing dead LM<sup>Δly</sup>-bacteria are unable to mature in Rab5a-antisense-treated cells and the observation that live LM<sup>Δly</sup>-phagosomes remain as immature endosomal-like compartments point to key, perhaps common, regulatory steps that are required to initiate phagosome maturation (28).

Table I

| Infection time | No. bc per cell | Cells containing no. bc: |
|----------------|-----------------|--------------------------|
|                |                 | 0  | 1–2 | 3–5 | ≥5  |
| h              | %               |    |     |     |     |
| 0              | 1.3 ± 0.3       | 35 ± 02 | 65 ± 06 | 0 | 0   |
| 4              | 2.3 ± 0.2       | 32 ± 04 | 20 ± 04 | 48 ± 02 | 0   |
| 8              | 3.2 ± 0.1       | 42 ± 06 | 5 ± 01  | 53 ± 04 | 0   |
| 24             | 8.0 ± 0.2       | 45 ± 03 | 1 ± 00  | 15 ± 03 | 37 ± 03 |

Phagosome maturation is a complex process, and results reported to date suggest that the nature of the internalized particle plays a role in modulating the rate and perhaps the quality of the process. For example, a recent report using latex beads as model particles in J774 cells demonstrated that phagosomes, which had been internalized for several hours, fused with early and late endosomes in vitro in a Rab5-dependent manner (13). Indeed, earlier work (29) demonstrated that the fusion capacity of phagosomes, containing Staphylococcus A particles internalized via the Fe receptor, is restricted to early endosomes in an in vitro phagosome-lysosome fusion. Thus, it is likely that both the receptor that mediates particle internalization and the nature of the internalized particle (e.g. digestible versus nondigestible, live versus dead etc.) play important roles in phagosome maturation and phagosome-lysosome fusion. Interestingly, interferon-γ, a lymphokine known to accelerate intracellular killing of pathogenic LM (LM<sup>Δly</sup>-) and other intracellular pathogens (30), specifically induces Rab5a biosynthesis and processing (18).