Rab10 regulates phagosome maturation and its overexpression rescues *Mycobacterium*-containing phagosomes maturation

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Abstract

Phagosome maturation follows a defined biochemical program and, in the vast majority of cases, the microbe inside the phagosome is killed and digested. Although, an important number of pathogens, including Mycobacterium tuberculosis, which kills around two million people every year, have acquired the ability to survive, and even replicate by arresting phagosomal maturation. To identify more of the machinery involved in phagocytosis and phagosomal maturation, we investigated the function of Rab10 in engulfment and maturation of inert particles and M. bovis BCG. We showed that Rab10 association with phagosomes is transient and confocal microscopy revealed detectible levels of Rab10 on phagosomal membranes at very early time points, occurring even before Rab5 acquisition. Rab10 recruitment had strong functional consequence, as the knockdown of endogenous Rab10 by RNA interference or overexpression of Rab10 dominant-negative mutant delayed maturation of phagosomes of IgG-opsonized latex beads or heat killed-mycobacteria. These results can be explained, at least in part, by the involvement of Rab10 in recycling of some phagosomal components. More importantly, overexpression of the constitutively active mutant of Rab10 partially rescued live-Mycobacterium-containing phagosomes maturation. Indeed, we found that the membrane harboring Mycobacterium acquired Early Endosome Antigen-1, a marker excluded from phagosomes in control cells. Altogether these results indicate that Rab10, acting upstream of Rab5, plays a prominent role in phagolysosome formation and can modulate Mycobacterium-containing phagosomes maturation.

Introduction

Phagocytosis allows the internalization of large particles into the cells and plays an essential role in innate immunity. After internalization, the particle surrounded by a membrane and an actin shield loses the actin and starts to interact with the components of the endocytic pathway, a process referred to as phagosome maturation. This interaction is sequential and culminates with fusion to lysosomes, which is crucial to avoid infection (for review see (1-4)). In the vast majority of cases, the microbe inside the phagosome is killed and digested, but a number of important pathogens, including Mycobacterium tuberculosis, which kills around two million people each year, has acquired the ability to survive, and even replicate, by subverting the host signaling machinery, thus preventing phagosome maturation (5).

Rab proteins (Rab GTPase family) are known to localize to specific compartments in the endocytic pathway and after recruiting their effectors have a direct role in promoting membrane transport, vesicle tethering and fusion (6, 7). Several reports suggest that Rab proteins are required for the interaction of phagosomes with components of the endocytic pathway (3). Thus, a phagosome, normally maturing into a phagolysosome, undergoes a transition between the stages marked by early endocytic Rabs (e.g. Rab5) and late endocytic GTPases (e.g. Rab7) (8). Indeed Rabs 5 and 7 are the best characterized with respect to their localization to phagosomes membrane and maturation. Rab5 is known to control fusion of nascent phagosomes to sorting endosomes and is required for the acquisition of Rab7. Rab7 allows phagosomes to interact with late endosomes culminating with the formation of late phagosomes (9, 10). Other Rabs have been detected on phagosomal membranes, such as Rab11, a GTPase involved in endocytic recycling, which appears to be involved in pseudopod extension. It is also known that inactive Rab11 prevents particle engulfment (11). More recently, Smith et al. (12), published that the expression of inactive Rabs 23 and 35 inhibited the fusion of phagosomes containing a Salmonella mutant with lysosomes.

The function of Rab GTPases can be modulated by pathogens to allow their intracellular growth. Indeed, many studies have shown that selective inclusion or retention of Rab GTPases on vacuolar membranes could regulate the biogenesis of phagosomes inhabited by several bacteria, including Mycobacterium (13). In the case of mycobacterial phagosome maturation, block occurred between the stages controlled by Rab5 and Rab7, with no Rab7 acquisition by the phagosomes (5). Subsequently, it has been shown that mycobacterial phagosomes retain several Rab5 effectors, but prevent phosphatidylinositol 3-phosphate (PtdIns(3)P) formation and, consequently, the recruitment of PtdIns(3)P-binding proteins to phagosome maturation, thus contributing to the arrest of phagosomal maturation (5, 14, 15). More recently, Rab14 and Rab22 were identified as contributors to the arrest of mycobacterial phagosomes by promoting phagosomal fusion with early endocytic organelles and thus playing a role in the maintenance of Mycobacterium tuberculosis-phagosome in its immature early endosomal-like stage (13).

There are over 70 Rabs identified in mammalian cells and more than 20 on phagosomal membranes (12, 16, 17) but only a few of them, as mentioned above, have been studied in terms of phagocytosis and phagolysosome biogenesis. Proteomic studies have detected Rab10 on
Results

**Rab10 associates transiently with phagosomes and A431 cells stably expressing the FcγRIIA are a suitable model to study Fc-mediated phagocytosis**

Professional phagocytes can present a challenge to research. Though macrophages, monocytes and pre-differentiated neutrophile cell lines exist and are useful models, they are usually refractory to DNA transfection. In addition, it is difficult to study the signaling consequences of a single phagocytic receptor because professional phagocytes express an array of receptors, some of which recognize the same ligand (25, 26). By contrast, non-professional phagocyte cell lines such as CHO and COS cells are easier to maintain in culture, amenable to genetic manipulation and are suitable to study phagosome maturation (27). However, the information on Chinese hamster and African Green monkey genomes is still very limited. Therefore, we decided to generate an engineered human phagocyte cell line to study the involvement of Rab10 on Fc-mediated phagocytosis and on phagosomal maturation. We decided to use the human A431 cell line (ATCC CRL-1555, established from an epidermoid carcinoma) and stably expressed the FcγRIIA, an abundant and widely expressed phagocytic receptor, using a retroviral system. We chose opsonized inert particles (latex beads and sheep-red blood cells) as our model to generate phagosomes.

As shown in Figure 1A and B, and as previously published for some non-human cell lines (28), the expression of Fc receptors on the surface of non-phagocytic cells confers upon them the ability to internalize IgG-coated particles. We then tracked the association of GFP-Rab10 with the model phagosomes in A431 cells by doing pulse-chase experiments (Fig. 1 A-C). In parallel, we transfected RAW cells (Fig. 1D-F), professional phagocytes, with GFP-Rab10 and monitored Rab10 association with phagosomes. The dynamics of Rab10 in phagosomes of both cell lines was analyzed by fluorescence confocal microscopy. In both professional and engineered phagocytes, Rab10 associated transiently with phagosomes (Fig. 1C and F). The maximal association occurred after the pulse, of 15 min for RAW and 25 min for A431 cells (= 0 min chase), and Rab10 remained associated with latex-beads phagosomes for a few minutes, in RAW cells (Fig. 1C and F). We counted a positive association of Rab10 with the model phagosome as a distinct ring around the particles (enlargements in Fig. 1A and D). At each time point investigated, we determined Rab10 association for at least 100 internalized particles, ensuring we only counted cells where expression of Rab10 was relatively low. Additionally, to ensure that we counted only internalized beads, we immunostained for human IgG on ice before chase in order to exclude the phagosomes that were formed after the pulse. Rab10 has been previously identified on purified latex beads phagosomes (18) and on phagosomes of a Salmonella mutant (12). The results we obtained correlated well with previous data describing the association of this Rab with phagosomes, both in professional and non-professional phagocytes.

We then decided to follow maturation of phagosomes formed in our engineered phagocyte model and address the functional relevance of Rab10 acquisition by the phagosome.

**Both the inactive Rab10 expression and the absence of Rab10 delay phagolysosome formation**

The normal nucleotide cycle of Rab can often be inhibited by the expression of activated or inactive mutant variants. In general, the constitutively active Rab mutants cannot be turned OFF due to their inability to hydrolyze...
GTP, while expression of dominant-negative mutants sequesters nucleotide exchange factors thus inactivating endogenous Rabs (6). To explore the possible role of Rab10 in phagocytosis and/or phagosomal maturation, we introduced Rab10T23N (inactive form) and Rab10Q68L (activated form) (21) by transfecting them into cells. Expression of the GFP tagged inactive Rab10T23N, activated Rab10Q68L and wild-type Rab10 caused no obvious effect on the internalization of opsonized IgG-particles both in professional and non-professional phagocytes (results not shown). We then analyzed the trafficking of phagosomes. Again phagosomal maturation and the functional role of Rab10 were studied in both professional and engineered phagocytes. The effect of Rab10 mutants on phagosomal maturation was assessed by measuring the acquisition of the early endosomal marker EEA1, and the late endosomal protein Lamp-2. At each time, cells were fixed and processed for immunofluorescence microscopy. For EEA1 assessment, A431 and RAW cells were challenged with IgG-opsonized particles for 25 and 15 min, respectively, and then chased for 15 and 30 min (Fig. 2). In A431 cells, at zero min chase there was a decrease in EEA1 acquisition (Fig. 2C) in cells expressing Rab10T23N (23.2 ± 7.9 %) compared to the control cells (50.5 ± 6.0 %, cells not expressing the inactive Rab10, Fig. 2C, black columns). Conversely, the activated Rab10Q68L (Fig. 2B) and the wild-type Rab10 (Fig. 2A) did not affect the acquisition of this early phagosomal marker. Though, the acquisition of EEA1 was not attenuated in RAW cells expressing the inactive Rab10 (Fig. 2E).

To examine whether the effect of the inactive Rab10 was specific for fusion with early endosomes, we next tested Rab10 effects on phagosome-late endosome/lysosome fusion (Fig. 3). A431 cells were challenged with IgG-opsonized particles for 25 min and RAW cells for 15 min and then chased for the times indicated on the graphs axis. Acquisition of Lamp-2, both in A431 and RAW cells, was delayed in cells expressing inactive Rab10 (Fig. 3 C-F and H). For example, at 45 min chase time, only 50.9 ± 5.9 % of the phagosomes were positive for Lamp-2 in contrast with 86.2 ± 8.5 % in control A431 cells. At 2 h chase, this difference was attenuated. Similar values were obtained in RAW cells, where at 45 min chase 54.3 ± 4.6 % of the phagosomes were positive for Lamp-2 versus 87.1 ± 5 % in control cells. In the same way as for EEA1 acquisition, the expression of wild-type and activated Rab10 did not affect Lamp-2 acquisition (Fig. 3 A, B and G). Though Rab10 is required, both in RAW and A431 cells for Lamp-2 acquisition, this requirement was not observed for EEA1 in RAW cells (Fig. 2). RAW are professional phagocytes and phagosomal maturation is faster than in A431 cells.

Furthermore, EEA-1 association with phagosomal membranes is transient. Thus, taking into account these two parameters (kinetics and association time) it is more difficult to assess effects on EEA-1 acquisition in RAW cells than in A431 cells. This can explain the discrepancy observed on the effect of the inactive Rab10 overexpression on EEA-1 acquisition.

The effect of inactive Rab10 was a strong indication that Rab10 function is required for phagosome maturation. To fully demonstrate this, we used RNA interference (RNAi) to deplete the endogenous protein in A431 cells (Fig. 4). Retrovirus-mediated expression of short hairpin RNAs followed by elimination of non-transduced cells by antibiotic selection reduced Rab10 mRNA levels by 92.0 ± 1.0 % [mean ± standard error of the mean (SD), n=3]. We could not measure Rab10 protein levels directly since we did not have a suitable antibody. Yet, expression of transfected GFP-Rab10 was almost completely suppressed in Rab10 knockdown cells (Rab10 KD cells) (Fig. 4 A-C), suggesting that the endogenous Rab10 had largely been removed. As shown in Figure 4 H-K and L (empty columns), the percentage of positive Lamp-2 phagosomes in Rab10-depleted cells was lowered when compared with that of control cells (Fig. 4 D-G and black columns in L). At 45 min chase, in cells infected with control empty virus, the acquisition of Lamp-2 was 81.3 ± 2.1 % compared to 52.4 ± 5.0 % in Rab10 KD cells. The effect of Rab10-depletion on the association of Lamp-2 with engulfed particles was also verified by immunoblotting of purified phagosomes obtained from A431 cells (Fig. 4 M-O). Phagosomes isolated from Rab10 KD cells exhibited less Lamp-2 on their membranes. The density of the Lamp-2 band dropped to 37.4 % and 44.9 % in phagosomes from Rab10 depleted cells, respectively, after a 30 and 45 min chase period when compared with those from control cells (considered 100%). However, at 3 h chase a negligible difference was observed between control and KD cells, suggesting once more that in absence of Rab10 phagosomal maturation is delayed and not blocked.

Taken together, the results presented in Figures 2-4 showed that phagosomes in engineered phagocytes proceed to mature in a manner that is indistinguishable from that of professional phagocytes. More importantly, we demonstrated that depletion of functional Rab10, either through RNA depletion or by overexpression of inactive Rab10, delayed phagosome maturation.

**Rab10 is acquired before Rab5**

Since Rab10 plays a prominent role in phagolysosome formation and in A431 cells affects the phagosomal recruitment of the Rab5-effector, EEA-1, we then asked whether
this small G protein acts upstream or in parallel with Rab5. To answer this question, we co-transfected RAW cells with Rab10 and 5, challenged these cells with opsonized particles and subjected them to live-confocal microscopy. Live confocal microscopy applied to RAW cells expressing both GFP-Rab10 and mRFP-Rab5, a GTPase known to enhance early endosomes fusion (29), revealed that Rab10 was recruited earlier than Rab5 during phagosome formation (Fig. 5). Soon after addition of opsonized particles, pseudopods labeled with GFP-Rab10 extended from the macrophage surface and made contact with the IgG-opsonized particles (Fig. 5). This is best illustrated in enlargement of the inset 0 min, green color of Figure 5. A few minutes after the recording, Rab10 was no longer associated with the phagosomal membrane (Fig. 5 and enlargements of the insets 0.5 and 1 min). Some co-localization was also observed between Rab10 and the Rab5-effector, EEA-1 on phagosomal membranes (results not shown).

To better define the stage of the phagocytic sequence of Rab10 acquisition, we examined whether Rab10 was co-localizing with actin. As can be observed in Figure 6 A-F pseudopods and nascent phagosomes decorated with actin (rhodamine-phalloidin staining) also had GFP-Rab10, confirming that this small G protein is localized to the phagocytic cup. Since the effect of the inactive Rab10 on acquisition of Rab5-effector, EEA-1, can be due to inhibition of the recruitment and/or activation of Rab5 we decided to monitor the phagosomal association of Rab5 and two Rab5-GEFs, Rabex-5 and RIN-1, with phagosomal membranes in the presence and absence of Rab10 (Fig. 6H). The overexpression of both wild-type and activated Rab10 did not seem to affect Rab5 recruitment but the expression of inactive Rab10 decreased Rab5 acquisition (Fig. 6G). However, this effect cannot be attributed to absence of Rabex-5 or RIN-1 since at 0 min chase the phagosomal association of Rabex-5 and RIN-1 was not significantly reduced in cells depleted of Rab10. Thus, Rab10 does not seem to be involved in the recruitment of these two Rab5-GEFs, although it affected Rab5 acquisition to the phagosomal membranes. The effects of Rab10T23N on EEA-1 and Rab5 acquisition suggest that Rab10 functions upstream of Rab5, though it is not known the role of Rab10 on Rab5 acquisition but it is possible that the former supplies signals for acquisition of the latter.

**Overexpression of activated Rab10 changes the properties of Mycobacterium containing phagosome**

Some pathogens regulate host trafficking pathways by the selective inclusion or retention of Rab GTPases on membranes of the vacuoles that they occupy in host cells during infection. Furthermore, Rab10 is acquired very early and the phenotype of phagosomes in Rab10-depleted cells bears some resemblance to that of mycobacterial phagosomes. Both are deficient in Lamp proteins and appearing to be affected at an early stage of maturation. We therefore tested whether Rab10 is recruited to phagosomes containing intracellular surviving mycobacteria. As shown in Figure 7A and C, Rab10 associated poorly with mycobacterial phagosomes. Indeed Rab10 was found more frequently in phagosomes containing *Mycobacterium smegmatis*, a species completely killed within macrophages by 48 h, (results not shown) or with phagosomes containing inert particles (Fig. 1) than in phagosomes with *Mycobacterium bovis* BCG. This raises the possibility that impairment of Rab10 recruitment is part of the mechanism used by pathogenic mycobacteria to prevent phagolysosome formation. Interestingly, we observed that activated Rab10 had greater association with live *Mycobacterium*-containing phagosomes (Fig. 7A, light grey columns and B). Therefore, we decided to address whether the overexpression of wild-type and the Rab10 mutants could change the fate of the *Mycobacterium*-containing phagosome. As can be observed in the graphs D and E of the Figure 7, expression of activated Rab10 partially overcame phagosome maturation block imposed by live mycobacteria. Indeed, we found that the membrane harboring live *Mycobacterium* acquired Phosphatidylinositol 3-phosphate [PI(3)P], as judged by the association of the 2-FYVE construct that binds specifically to this inositol, and EEA-1, markers excluded from the phagosomes in control cells (cells not transfected, black columns) (Fig. 7 D-E). However, no effect of the activated Rab10 overexpression on Lamp-2 acquisition was observed within the period investigated (Fig. 7G). Concerning Rab10 acquisition by the live BCG phagosomes, the overexpression of the dominant-negative Rab10 did not seem to have major effects on the kinetics of association nor on the percentage of Rab5-positive phagosomes but overexpression of activated Rab10 increased the percentage of Rab5-positive phagosomes (Fig. 7F). Thus, Rab10 overexpression changed the properties of the live *Mycobacterium*-containing phagosomes, though it was not sufficient for fusion with lysosomes.
All the experiments to assess the involvement of Rab10 in maturation described above were mainly performed with IgG-opsonized particles that are engulfed via the Fc-receptors. Mycobacterium recognition by phagocytes is likely to involve more than one phagocytic receptor. Thus, we decided to assess whether Rab10 had any functional relevance in maturation of phagosomes containing heat-killed *Mycobacterium* that, like IgG-opsonized- red blood cells (RBC) or latex beads, follows the canonical maturation process, meaning that the heat-killed *Mycobacterium* phagosome fuses with lysosomes. As shown in Figure 7H, overexpression of the dominant negative Rab10 mutant also delayed fusion of heat-killed *Mycobacterium* phagosomes with late endocytic compartments. Taken together our results suggest that Rab10 is a central and general regulator of the transition of the nascent phagosome to early phagosome, affecting the transition of this organelle into a degradative compartment.

**Rab10 affects recycling from phagosomal membranes**

We next examined by which mechanism(s) Rab10 contributes to the phagosomal maturation delay. Since maturing phagosomes undergo both fusion with other organelles and concomitant fission and because Rab10 was found to be involved in recycling, we tested whether it also contributes to recycling of phagosomal components back to the plasma membrane. In this context the effect of Rab10 depletion on the detachment of a plasma membrane marker GFP-GL-GPI (glycosyl phosphatidyl inositol-anchored green fluorescent protein) and on transferrin receptor recycling on phagosomes was assessed. The results of these studies are shown in Figure 8. The plasma membrane protein, GFP-GL-GPI that demarcated the forming and early phagosomes (results not shown) was eliminated with time from maturing phagosomes both in control (Fig. 8A, black columns) and Rab10-depleted (Fig. 8A, empty columns) cells. However, the percentage of GFP-GL-GPI-positive phagosomes was higher in Rab10-depleted than in control cells. This effect was more pronounced at 30 min chase where in control cells only 22.58 ± 2.4 % of the phagosomes were positive for GFP-GL-GPI versus 40.15 ± 2.0 % for Rab10 KD cells. Similar results were obtained in cells expressing the inactive Rab10 mutant (results not shown).

Rab10 depletion showed some tendency to slow transferrin receptor recycling from phagosomal membranes (Fig. 8B) but it was less pronounced than in GFP-GL-GPI recycling (Fig. 8A).

Jointly, these observations indicate that the loss of components of the phagosomal membranes is affected in the absence of Rab10, which can explain, at least in part, the delay in phagolysosome formation.

**Discussion**

Intracellular bacterial pathogens have evolved highly specialized mechanisms to enter and survive within their eukaryotic hosts resulting in devastating diseases such as tuberculosis, food poisoning, etc. In order to do this, bacterial pathogens need to avoid host cell degradation and obtain nutrients and biosynthetic precursors, as well as evade detection by the host immune system. To create an intracellular niche that is favorable for replication, some intracellular pathogens inhibit the maturation of the phagosome or exit the endocytic pathway by modifying the identity of their phagosome and the exploitation of the host cell trafficking pathways. If we take into account that in eukaryotic cells, organelle identity is determined, in part, by the composition of active Rab GTPases on their membranes, the retention or exclusion of Rab proteins from the phagosomal membranes can explain, at least in part, their escape from the degradative endosomal or lysosomal pathways. Indeed, evidence indicates that a number of intracellular bacterial pathogens, including *Mycobacterium tuberculosis*, *Salmonella enterica* serovar Typhimurium, *Legionella pneumophila* and *Chlamydia pneumoniae* exploit Rab GTPases to regulate the biogenesis of the phagosomes and to ensure their intracellular survival (5, 10, 30, 31).

In this context, the aim of this study was to better understand the requirement of Rab10 for phagosomal maturation in professional and engineered phagocytes with two different types of phagocytic particles. We focused on the small G protein, Rab10 for two reasons: i) it has been identified in phagosomes by proteomic and other approaches (12, 17, 18); ii) and it has been implicated in membrane trafficking (19-21). Our results indicate that Rab10 is required for phagosome maturation, both in professional and non-professional phagocytes. More importantly, the overexpression of its activated mutant changes, at least in part, the properties of *Mycobacterium* containing phagosome.

The functional involvement of Rab10 on phagocytosis and phagosomal maturation was tested by overexpression of Rab10 mutants and by RNAi. In cells expressing the inactive Rab10T23N or in Rab10 KD cells phagolysosome formation was delayed, indicating a role of Rab10 on phagolysosome biogenesis. In earlier proteomic studies Rab10 was detected along with Rab5 in purified latex beads phagosomal preparations (18). Here, confocal live microscopy showed that Rab10 is
acquired even before Rab5, suggesting that the kinetic aspects of bulk biochemical studies (18) and video microscopy cannot be directly compared. However, it is difficult to determine where exactly Rab10 may act. We observed that in coverslip-grown A431 cells GFP-tagged wild-type Rab10 localizes mostly at the Golgi and partially with early endosomal compartments (Supplementary Figure). The constitutively active mutant (Rab10G68L) localized mainly in the early endosomal vesicles as shown by pronounced co-localization with Rab5 (Supplementary Figure), while the inactive mutant of Rab10 (Rab10T23N) localized to the Golgi and distributed in the cytosol. These results are consistent with previous studies of Rab10 localization, which showed that GFP-Rab10 is distributed between the Golgi and endosomes in polarized intestinal cells in Caenorhabditis elegans (19). Rab proteins usually localize to the sites at which they regulate membrane trafficking events. Based on the general principle that Rab proteins interact with their effector proteins only after GDP-to-GTP exchange, it is likely that the GTP-bound Rab mutants accumulate on their target membrane domains in association with their effectors, whereas the inactive GDP-bound state indicate where membrane recruitment takes place (e.g. (32)). Thus, in A431 cells it is likely that Rab10 is involved in transport of carrier membranes between these two compartments, Golgi and endosomes.

How exactly Rab10 acts, remains to be answered. It is thought that Rab10 acts in a similar recycling pathway as Rab8a since both Rabs share a common GAP activating protein (GAP), As160 (33) and can interact with Myosin V (34), a motor protein implicated in membrane recycling. However, the exact role(s) of Rab10 is/are not well established. Recent work from three groups has placed Rab10 within the endocytic recycling system in C. elegans (19) intestinal cells, MDCK cells and in human gastric parietal cells (35). One recent study suggests that Rab10 controls exocytic transport in polarized MDCK cells, but the authors do not rule out that it may also be needed, directly or indirectly, for endocytic trafficking (21). Indeed, this phenotype was obtained only by expression of the activated Rab10, which is likely to sequester and thus inactivate a number of effector proteins, whereas the corresponding inactive mutant or Rab10-depletion by RNAi had subtle or no effects (21). Furthermore, Sec4p, the yeast homologue of Rab10, is involved in the exocytic pathway (36). However, it is possible that the multiple functions of Sec4p in yeast are carried out by separate Rabs in higher organisms, so that Rab8, Rab10 and possibly Rab13 could represent split versions of Sec4p. Many parallels are likely to exist between endocytosis and phagocytosis. Based on our own data Rab10 seems to promote phagosome maturation by mediating recycling from phagosomal components, which is necessary for phagolysosome biogenesis. In fact, Rab4 and Rab11, known to be involved in the recycling pathway, are present on phagosomal membranes, suggesting that recycling processes might also be important for phagolysosome biogenesis (37, 38). Alternatively, Rab10 may affect interactions between phagosomes and early endosomal organelles due to its involvement in the biosynthetic pathway. In its absence, Golgi to endosome transport might be affected, disrupting the supply of components to the endocytic machinery, indirectly affecting maturation.

Several lines of evidence indicate that the mycobacterial phagosome maintains an early endosomal niche in infected macrophages. Firstly, the phagosome associates with early endosomal Rabs or transferrin receptor but not with late endosomal GTPase, Rab7, or late endosomal tetraspanin, CD63, (5, 14). Secondly, mycobacterial phagosomes are known to have diminished generation of PI3P, leading to exclusion of PI3P-binding effectors, such as Hrs and EEA-1, thus preventing membrane tethering necessary for onward progression to late endosomes (14, 15). Thirdly, it has been well established that mycobacterial phagosomes do not acquire the vacuolar proton pump necessary for acidification of phagosomes (39). Hitherto, the studies of early endocytic Rabs in bacterial pathogenesis have been restricted to only a few Rabs, mainly Rab5 (5, 14). Furthermore, recent work has identified more two host GTPases, Rab14 and Rab22a, involved in the blockade of normal phagolysosome biogenesis during mycobacterial infection (13, 40). Here, we identified Rab10, a protein that plays an essential role in early phagosome-to-phagolysosome transition, as part of the host machinery that is also targeted by Mycobacterium. Indeed, we found that the requirement of Rab10 is considerably lower in the case of live BCG than in that of opsonized latex particles or heat-killed BCG. These findings suggest that the inability to recruit Rab10 may contribute to the arrest of phagosomal maturation induced by pathogenic mycobacteria. More importantly, this arrest can be partially rescued by overexpressing the activated Rab10. Thus, here we identified Rab10 as a potential attractive target that should be taken into account for the development of therapeutics that can restore “normal” phagosome maturation. With over 70 Rabs identified in the human genome, much remains to be learned regarding the role of these regulatory GTPases in general intracellular trafficking, and in phagosomal and endosomal organelles, as well as in microbial pathogenesis. Our data indicate that the process
of phagosome maturation is far more complex than a single Rab5 to Rab7 transition. Based on the inhibitory effect of the inactive Rab10 on EEA-1 phagosomal acquisition we suggest that Rab10 is a central regulator of the transition of a nascent phagosome to an early phagosome, affecting the transition of this organelle into a degradative compartment. Furthermore, we were able to monitor the trafficking of a pathogenic bacterium and characterize the divergence of its vacuolar compartment from the normal degradative pathway. Future studies are required to define the molecular mechanisms by which Rab10 controls phagosome maturation and how intracellular pathogens, like *Mycobacterium*, modulate their function. Understanding the precise function of Rab10 and how it is connected with other parts of the transport machinery, such as cargo adaptors, molecular motors, vesicle fusion proteins and other Rab GTPases, will require the identification of all Rab10-effector proteins.

**Material and Methods**

**Antibodies**

Monoclonal were anti-human (H4B4), anti-mouse (ABL-93) Lamp-2 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and anti-mouse (M2) Flag (Sigma-Aldrich, USA). Polyclonal were goat anti-EEA-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-GFP (D. Drechsel, MPI-CBG). Secondary antibodies were from Molecular Probes or from Jackson ImmunoResearch. Rhodamin-phalloidin was from Molecular Probes and DAPI from Fluka.

**Plasmids and generation of A431 cells stably expressing the FcγRIIA**

GFP-GL-GPI, Canine pGFP-Rab10-wt (wild-type), pGFP-Rab10T23N (inactive) and pGFP-Rab10Q68L (active) plasmid constructs, were kindly provided by Kai Simons (Max-Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany) and were described elsewhere (21). Tagged-Rab5 and 2-FYVE-GFP were kindly provided by Kai Simons (Max-Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany). Flag-mRIN-1 and Flag-mRabex-5 plasmid constructs were kindly provided by Dr. Sayaka Yoshiki (Kyoto University, Japan). Human FcγRIIA tagged with myc was subcloned into the retroviral vector pBABE-puro. In order to generate A431 cells stably expressing FcγRIIA, retrovirus production, cells infection and selection were done as described before (41).

**Cells and transfection**

Mouse macrophages RAW 264.7 and A431 cells (American Type Culture Collection) were grown in Dulbecco’s Modified Eagle Medium (DMEM) high glucose, supplemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Life Technologies). The cells were transiently transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer’s recommendations.

**Phagocytosis and Bacterial Infection**

Fresh sheep-red blood cells (RBC) were opsonized with rabbit anti-sheep RBC antibody (1:50). Latex beads were opsonized with 1 mg of human IgG/mL. Opsonization was for either 2 h at room temperature or overnight at 4°C. The onset of phagocytosis was synchronized by allowing the particles to bind to cells on ice for 7 min, and ingestion was then initiated by incubation at 37°C. Excess particles were washed away with phosphate-buffered saline (PBS) and, when indicated, the cells were incubated in culture medium at 37°C for the specified additional chase period. To identify adherent particles that were not internalized, the cells were incubated at 4°C with Cy5/Cy3/Cy2-labeled donkey anti-rabbit IgG (1:1000) or Cy5/Cy3/Cy2-labeled donkey anti-human IgG (1:1000) for 5 min. For live cell imaging, RAW macrophages grown on circular coverslips were transferred to a live cell imaging chamber and bathed in Hepes-buffered RPMI 1640 (Sigma). In these experiments phagocytosis was not synchronized. *Mycobacterium bovis* BCG harbouring a pMN437 plasmid (a kind gift from Michael Niederweis, Department of Microbiology, University of Alabama at Birmingham, Birmingham) was grown until exponential phase on Middlebrook’s 7H9 broth medium (Difco) supplemented with 10 % Middlebrook OADC (Oleic acid, albumin, dextrose, catalase) (Difco) (v/v) and 0.05 % Tween 80 (v/v) at 37°C/5% CO2. Media were supplemented with 50 µg/mL hygromycin (Roche) for selection of recombinant mycobacteria. Bacteria were prepared for macrophage infection as described previously (24). A bacterial suspension with an OD 600 nm of 0.3 was added to RAW macrophages, centrifuged at 300 g for 3 min at 37°C and incubated at 37°C in a 5 % CO2 atmosphere for 2 or 12 h. Extracellular mycobacteria were removed as described previously (24) and the protocol described for latex beads was followed.

**Immunofluorescence and Confocal microscopy**

Cells were grown on 24 well plates and fixed with Methanol or 4 % PFA for 30 min, followed by quenching of the aldehyde groups with glycine or ammonium chloride and permeabilization with
Triton-X100. Cells were then incubated with the primary antibodies (EEA-1 or Lamp-2) for 1 h at room temperature, washed and finally incubated with the secondary antibodies conjugated with a fluorophore for another 1 h. The antibody dilutions used were EEA-1 (1:100), Lamp-2 (1:100) and Flag (1:400). Flag antibody was incubated for 2 h at room temperature, washed and finally incubated with the secondary antibody anti-mouse conjugated with a Cy3-fluorophore for another 1 h. DAPI at a final concentration of 30 nM was incubated for 20 min at room temperature. Live cell imaging was performed in CO2-independent medium at 37°C. Both live and fixed samples were analyzed by using the LSM 510 META point-scan confocal laser microscope (Zeiss) with a 63x oil immersion objective.

Transferrin Uptake and Recycling from phagosomal membranes
Control and Rab10-depleted A431 cells stably expressing the FcγRIIA were plated on glass coverslips, washed with cell culture medium, and incubated for 1 h at 37°C in serum-free DMEM. Then 0.05 µg/ml rhodamin-conjugated transferrin (Molecular Probes) and opsonized latex-beads were added to the cells followed by incubation at 37°C for 25 min. After this incubation time (pulse) the cells were extensively washed and the extracellular beads stained with secondary antibodies. Finally the cells were chased for various time intervals, fixed with 4 % PFA and imaged.

RNAi
The Rab10 target sequence has been described previously (21). Retrovirus-mediated RNAi and quantitative RT-PCR were done as before (41).

Phagosomes isolation and Western blot
Phagosomes were isolated by the method of Desjardins et al. (42) from A431 cells grown on 14-cm-diameter petri dishes to 60 to 70 % confluence. The protein concentration of the phagosomal preparation was determined with the bicinchoninic acid assay (Pierce), using albumin as the standard. Isolated phagosomes or whole-cell extracts were solubilized in Laemmli's sample buffer, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes. The membranes were blocked for 50 min with 5 % milk in PBS and 0.1 % Tween 20. Antibodies to GFP were used at 1:10000 dilution, and Lamp-2 antibody was used at a 1:1000. Immunoreactive bands were visualized by ECF reagent (GE healthcare, Amersham, Piscataway, N.J.).

Statistical analysis
Results were expressed as the means ± standard deviations (S.D.). Statistical significance was assessed by the Student t-test (two-tailed). A p value of < 0.05 was considered to be statistically significant.
Legends

Figure 1: Rab10 associates transiently with phagosomes. A431-Fc/RIIA and RAW cells were transfeceted with GFP-Rab10 and allowed to ingest IgG-opsonized latex beads for 25 and 15 min, respectively. A, Distribution of GFP-Rab10 in A431 cells. B, Distribution of GFP-Rab10 in RAW cells. B and E are the corresponding differential interference contrast (DIC) images. Arrows indicate co-localization between latex vacuoles and Rab10. The insets are enlargements of the areas outlined with the squares. Scale bars are 10 µm. Acquisition of Rab10 by phagosomes in A431 (C) and RAW 264.7 (F) cells. IgG-opsonized beads were added to the cells and allowed to phagocytose for 25 and 15 min, respectively in C and F, (chase time = 0 min), at which time point external beads were stained using antibodies and excluded from the calculations. The phagosomes were allowed to mature for the times indicated in the graphs abscissa. Data shows the percentages of Rab10-positive phagosomes and are means ± SD of three independent experiments (100 phagosomes for each condition). Samples were analyzed by fluorescence confocal microscopy.

Figure 2: Inactive Rab10T23N attenuates EEA-1 acquisition in engineered phagocytes. Cells were transected with wild-type (A and D), activated (B) and inactive Rab10 (C and E). The transected A431 (A-C) and RAW cells (D and E) with the indicated Rab10 variants were pulsed with IgG-opsonized latex beads for 25 or 15 min, respectively. The external beads were stained and the cells chased for the times indicated in the graphs. Then the cells were fixed, stained for EEA-1 and examined by confocal microscopy. Black columns represent control cells and empty columns, the cells expressing Rab10 variants. Results are means (± SD) of three to four independent experiments.*, p< 0.05 comparing differences between non-transfected and transfected cells.

Figure 3: Inactive Rab10T23N attenuates phagolysosome formation. Cells were transected with wild-type (A and G), activated (B) or inactive Rab10 (C-F and H). The transected A431 (A-F) and RAW cells (G and H) with the indicated Rab10 variants were pulsed with IgG-opsonized latex beads for 25 and 15 min, respectively. After the indicated chase time point the cells were fixed, stained for Lamp-2 and examined by scanning confocal microscopy. Black columns represent control cells and empty columns, the cells expressing Rab10 variants. Results are means (± SD) of three to four independent experiments. Note that the percentage of 100 % was set as the value for the latest chase time point in the Lamp-2 acquisition experiments. **, p< 0.01 and ***, p< 0.001 comparing differences between non-transfected and transfected cells. D, shows Lamp-2 staining at 45 min chase. E, shows cells expressing the inactive GFP-Rab10. F, shows the respective DIC image. *, indicates a Rab10T23N transfected cell. Solid and open arrows point to Lamp-2-positive or -negative phagosomes, respectively. Scale bars is 10 µm.

Figure 4: Depletion of Rab10 by RNAi delays phagolysosome formation. Effects of Rab10 depletion by RNAi on phagosomal maturation. Knockdown cells were generated by retrovirus-mediated RNAi and A431 cells transfected with empty retrovirus respectively as control. Rab10 knockdown (Rab10 KD) cells suppress expression of GFP-Rab10 (A-C). Control and Rab10 KD cells were transected with GFP-Rab10 using Lipofectamine 2000. Equivalent amounts of cell lysate were separated by SDS–PAGE and subjected to immunoblotting with antibodies to GFP and Lamp-2 (loading control). The table below shows the downregulation efficiency in terms of mRNA, assessed by RT-PCR, and in terms of protein quantified by Western blot, using Quantity One® Software (BioRad). (B, C) fluorescence images showing the expression of GFP-Rab10 in control and Rab10 KD cells, respectively. Green represents GFP-Rab10 and blue, nuclei visualized with DAPI. Rab10 knockdown affects Lamp-2 acquisition (D-O). Lamp-2 acquisition was assayed in control and Rab10 KD cells by immunofluorescence, as described in the legend of Figure 3. A typical experiment is illustrated in panels D to K. D-G represent control cells and H-K, Rab10 KD cells. (E, I) Show the distribution of Lamp-2. (G, K) Show the particles internalized after the pulse, stained with secondary antibodies conjugated with Cy2 (green color). Panels D and H show the respective merge images. Panels F and J show the corresponding DIC images. Solid and open arrows point to Lamp-2-positive and -negative phagosomes after a chase of 45 min, respectively (E and I). Scale bars are 10 µm. (L) Quantification of the effect of Rab10 depletion on Lamp-2 acquisition. Black columns represent control and empty columns, Rab10 KD cells. The presence of Lamp-2 on phagosomes (L) was assessed after a 25 min pulse of phagocytosis followed by 45 or 120 min chase. Data are means ± SD of three separate experiments (200 cells were counted in each). *, p< 0.05 comparing differences between control and Rab10-depleted cells. The effect of Rab10 depletion on Lamp-2 acquisition was also assessed by Western blot of isolated phagosomes from cells exposed to opsonized particles for 35 min and then chased for 30, 45 and 180 min (M-O), as indicated. 12 µg of protein were loaded in each lane.
Figure 5: Distribution of Rab10 and Rab5 during phagocytosis.
Recruitment of Rab10 and Rab5 to phagosomes. RAW cells were co-transfected with GFP-Rab10 and mRFP-Rab5. Data are means ± SD of three separate experiments (200 cells were counted in each); *, p < 0.05; **, p < 0.01.

Figure 6: Rab10 is recruited earlier than Rab5 to phagosomal membranes.
RAW (A-C) and A431 (D-F) cells expressing GFP-Rab10 (A, D) were allowed to initiate phagocytosis of RBCs (A) or latex beads (B), followed by fixation and staining for F-actin with rhodamine-phalloidin (B and E); C and F are the corresponding DIC images. Arrows point to phagocytic cups/phagosomes positive for actin and Rab10. Scale bars are 10 μm. Quantification of the effect of Rab10 depletion on Rab5 acquisition by phagosomes (G). A431 cells were co-transfected with the indicated Rab10 constructs and Rab5 for 24 h. Quantification of the effect of Rab10-depletion on RIN-1 and Rabex-5 acquisition by phagosomes (H). Control cells, Black columns. Rab10 KD cells, empty columns. In G and H the cells were challenged with IgG-opsonized particles for 25 min. Then the cells were fixed and examined by confocal microscopy. Data are means ± SD of three separate experiments (200 cells were counted in each); *, p < 0.05; **, p < 0.01.

Figure 7: Rab10 overexpression modulates Mycobacterium-containing phagosomes maturation.
Acquisition of Rab10, EEA-1, PI(3)P, Rab5 and Lamp-2 by BCG-containing phagosomes. RAW macrophages (control) or RAW macrophages transfected with wild-type mRFP-Rab10, mRFP-Rab10Q78L or mRFP-Rab10T23N were infected with live (A-G) or fed with heat killed BCG (H) for 15 min. The recruitment of wild-type Rab10 (empty columns) and activated Rab10 (light grey columns) by phagosomes was assessed (A). In macrophages infected with live BCG we observed less recruitment of Rab10 to phagosomal membranes. A representative picture of the recruitment of activated Rab10 is shown (B) and the arrow points to a positive phagosome. A representative picture of absence of recruitment of wild-type Rab10 is shown (C) and the arrow points to a negative phagosome. In red, mRFP-Rab10Q78L (B) or mRFP-wildtype Rab10 (C) and in green, live BCG. Scale bars are 10 μm. Insets show a magnification of the boxed areas showing a Rab10-positive or negative phagosomes. Quantification of the acquisition of EEA-1 (D), PI(3)P (E), Rab5 (F) and Lamp-2 (G) by phagosomes containing live BCG. Both control and transfected macrophages were followed and compared. In E and F, RAW macrophages were co-transfected with a construct consisting of two tandem PI(3)P-binding FYVE domains of EEA-1 fused with GFP (2FYVE-GFP) or with Rab5-GFP and the indicated Rab10 constructs. A delay in Lamp-2 acquisition by phagosomes containing heat killed BCG was observed in macrophages transfected with mRFP-Rab10T23N (H). Black columns represent control; light grey columns, Rab10Q78L; empty columns, wild-type Rab10 and dark grey, Rab10T23N. Data are means ± SD of three separate experiments (200 cells were counted in each); *, p < 0.05; **, p < 0.01.

Figure 8: Rab10 is involved in recycling.
Control and Rab10-depleted A431 cells stably expressing the FcγRIIA were plated on glass coverslips and then challenged with opsonized IgG-particles. Effect of Rab10-depletion on the disappearance of GFP-GL-GPI from the phagosomes (A). The cells were transfected with GFP-GL-GP 24 h before the phagocytosis assays. Effect of Rab10-depletion on transferrin receptor recycling from the phagosomes (B). The experiment was performed as described in the Material and Methods Section. Control cells, black columns, Rab10-depleted cells, empty columns. The cells were exposed to latex beads for 25 min and then chased for the times indicated on the graphs. Then the cells were fixed and examined by confocal microscopy. Data are means ± SD of three separate experiments (200 cells were counted in each); ***, p < 0.001 comparing differences between control and Rab10-depleted A431 cells.
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Supplementary Data: Subcellular Localization of Rab10 in A431 cells

An important step in understanding the function of a membrane trafficking protein is to identify its subcellular localization. Towards this objective, we compared the distribution of Rab10 with well-characterized markers of Golgi, endosomal, and lysosomal compartments by immunofluorescence microscopy in A431 cells. Thus, in the supplementary figure we report the subcellular distribution of Rab10.

In A431 cells, overexpressed Rab10 was mainly localized to Golgi (A), and early endosomes (B and C). Indeed, GFP-Rab10 in the perinuclear region localized to great extent with the Golgi marker phosphatidylinositol-4-phosphate, visualized by overexpressing the PH domain of FAPP1 (A). Rab10 also partially overlapped with EEA-1 and Rab5, markers of early endosomes (B and C).

Of interest, the activated GFP-Rab10Q68L co-localized strongly with Rab5 (D). In contrast, GFP-Rab10T23N was mainly present in perinuclear tubular structures that overlapped with those positive for phosphatidylinositol-4-phosphate, again visualized by overexpressing the PH domain of FAPP1 (E).

The subcellular localization of this small G protein indicates that Rab10 probably regulates the transport of carrier membranes between Golgi and early endosomal compartments. Of note, in A431 cells there is no overlap of Rab10 with the late endosomal/lysosomal marker, Lamp-2 (F), nor with GFP-GL-GPI (G), indicating that Rab10 is not associated to late endosomal/lysosomal compartments nor to the plasma membrane.

Legend:

Supplementary Figure: Rab10 localizes primarily to the Golgi and partially in early endosomes.

GFP-Rab10 was introduced into A431 cells grown on coverslips by transient transfection and analyzed by immunofluorescence confocal microscopy. Weakly expressing cells were chosen for imaging to avoid mislocalization due to high levels of expression.

(A–G) After methanol or PFA fixation and Triton-X100 permeabilization, cells were stained as described in Material and Methods section. Wild-type mRFP-Rab10 and GFP-FAPP1-PH (A), wild-type GFP-Rab10 and mRFP-Rab5 (C), GFP-Rab10Q68L and mRFP-Rab5 (D), mRFP-Rab10T23N and GFP-FAPP1-PH (E), or GFP-GL-GPI and mRFP-Rab10WT (G) were co-expressed transiently in A431 cells. (B) Wild-type Rab10 and endogenous EEA-1 or (F) wild-type Rab10 and Lamp-2. Scale bars are 10 μm.

References


Figure -1
Figure -2

A  Wild-type Rab10

B  Activated Rab10Q68L

C  Inactive Rab10T23N

D  Wild-type Rab10

E  Inactive Rab10T23N

■ Control  □ Rab10 variant
Figure -3

A. Wild-type Rab10
B. Activated Rab10G33L
C. Inactive Rab10T23N

D, E, F. Additional images showing different conditions or treatments.

G. Wild-type Rab10
H. Inactive Rab10T23N

Legend:
- Control
- Rab10 variant
Figure -5
Figure -6

A B C
D E F

G

H

Rab 5 positive phagosomes (%)

wild type Rab10 Rab10G68L Rab10T23N

0 20 40 60 80

Control Rab10 KD

CEFs positive phagosomes (%)
Figure -7 cont.
Supplementary figure
Supplementary figure cont.