# Rab5a GTPase regulates fusion between pathogen-containing phagosomes and cytoplasmic organelles in human neutrophils

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## Summary

**Biogenesis of phagolysosomes proceeds through a sequential series of interactions with endocytic organelles, a process known to be regulated by Rab and SNARE proteins. The molecular mechanisms underlying phagosome maturation in neutrophils are, however, not clearly understood. We investigated fusion between phagosomes containing the intracellular pathogen *Mycobacterium tuberculosis* versus the extracellular pathogen *Staphylococcus aureus* (designated MCP for mycobacteria-containing phagosome and SCP for *S. aureus*-containing phagosome) and cytoplasmic compartments in human neutrophils. Western blot analysis of phagosomes isolated after internalisation revealed that lactoferrin (a constituent of secondary granules) and LAMP-1 were incorporated into both SCP and MCP, whereas hck (marker of azurophil granules) interacted solely with SCP. The subcellular distribution of the proteins Rab5a and syntaxin-4 suggested a role in docking of granules and/or endosomes to the target membrane in the neutrophil. We observed that during phagocytosis, Rab5a in GTP-bound form interacted with syntaxin-4 on the membrane of MCP and were retained for up to 90 minutes, whereas the complex was recruited to the SCP within 5 minutes but was selectively depleted from these vacuoles after 30 minutes of phagocytosis. Downregulation of Rab5a by antisense oligonucleotides efficiently reduced the synthesis of Rab5a, the binding of syntaxin-4 to MCP and SCP and the capacity for fusion exhibited by the pathogen-containing phagosomes, but it had no effect on bacteria internalisation. These data indicate that the difference in granule fusion is correlated with a difference in the association of Rab5a and syntaxin-4 with the phagosomes. Intracellular pathogen-containing phagosomes retain Rab5a and syntaxin-4, whereas extracellular pathogen-containing phagosomes bind briefly to this complex. These results also identified Rab5a as a key regulator of phagolysosome maturation in human neutrophils.**

Key words: Neutrophil, *Mycobacterium tuberculosis*, Rab5, Phagocytosis, Fusion

## Introduction

As one of the most efficient phagocytic cells of the immune system, neutrophils restrict the initial, local replication of numerous extracellular and intracellular pathogens, thereby delaying their systemic spread. When microorganisms are engulfed by neutrophils, these cells degranulate, for example, the storage granules discharge their contents into the phagocytic vacuole; subsequently, the microorganisms are killed and digested. Phagolysosome biogenesis is essential for a neutrophil to kill invading microbes, and it occurs through the sequential fusion of newly formed phagosomes with other granules and/or endosomes (Ricevuti et al., 1993). Neutrophils are equipped with at least four distinct secretory compartments, referred to as: azurophil, specific, gelatinase granules and secretory vesicles. In addition, multivesicular bodies (MVB) and the multilaminar compartment (MLC) were recently identified; these display characteristic concentric internal membranes that bear an integral membrane protein of the lamp family, LAMP-1 (lysosome-associated membrane protein-1) and serve as late endosomal compartments in neutrophils (Cieutat et al., 1998; Bainton, 1999).

Fusion of these compartments with the phagosomes is controlled by various signals and proteins, including calcium, actin, annexins and Src kinases (Stendahl et al., 1994; Diakonova et al., 1997; Korade-Mirnics and Corey, 2000). Indeed more than 140 proteins have been found in isolated phagosomes from macrophages (Garin et al., 2001). In particular, small GTP-binding proteins of the Rab family regulate the docking and fusion of endocytic organelles. Rab5 is a member of this family, and it is expressed in three different isoforms (designated a, b and c) that appear to exhibit overlapping intracellular distributions (Bucci et al., 1995). Studies of macrophages have shown that Rab5a regulates events in the fusion of bacteria-containing vacuoles and early endosomes (Bucci et al., 1995; Sturgill-Koszycki et al., 1996; Alvarez-Dominguez and Stahl, 1999) and that the arrest of maturation can be linked to a block between Rab5- and Rab7-controlled steps (Via et al., 1997). In neutrophils, as well as in other eukaryotic cells, this protein participates in intracellular trafficking (Vita et al., 1996). At present, no information is available about the involvement of Rab5 in phagolysosome biogenesis in neutrophils. In addition to Rab GTPases, a

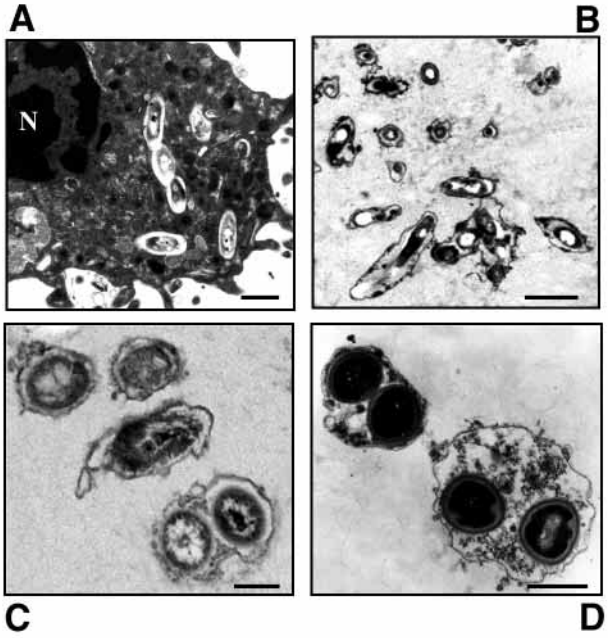



**Fig. 1.** Transmission electron microscopy (TEM) of neutrophils with ingested bacteria and of isolated phagosomes. Neutrophils were allowed to phagocytose mycobacteria of the H37Rv strain or *S. aureus*, and the bacteria-laden phagosomes (MCPv and SCP, respectively) were subsequently isolated. The micrographs show a neutrophil after internalisation of H37Rv for 60 minutes (A); isolated MCPv devoid of contamination with other cellular organelles (B); individual MCPv (C); and individual SCP (D). N, stands for nucleus and the scale bars represent 1 μm (A,B) and 0.5 μm (C,D).

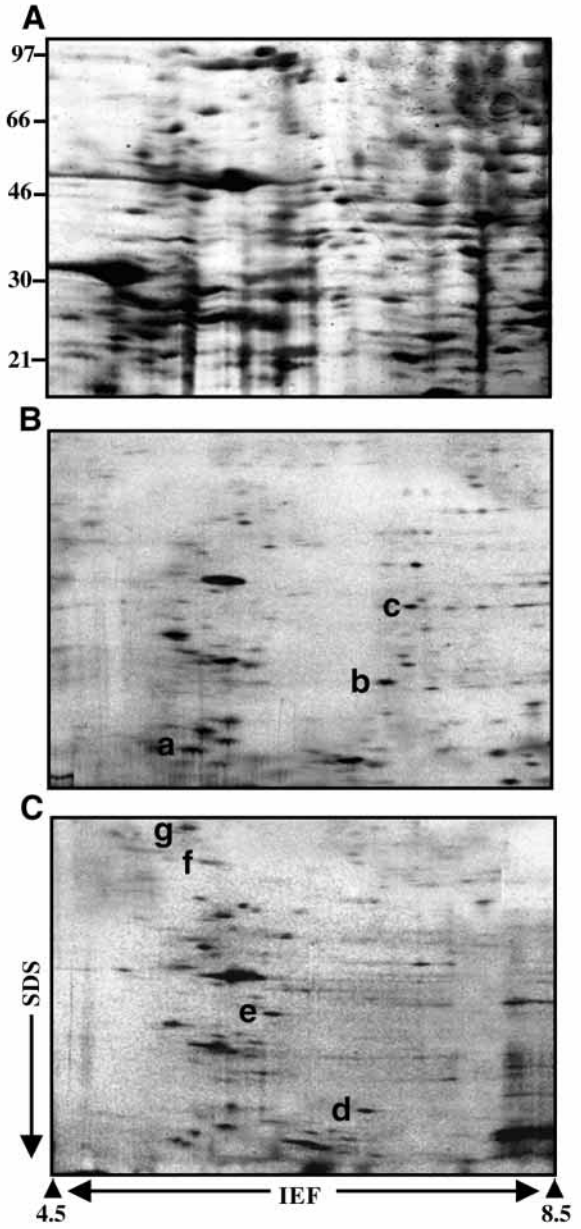



**Fig. 2.** Comparison of the protein composition of lysates of intact neutrophils (A), isolated MCPv (B) and isolated MCPa (C). Neutrophils ($4\times10^8$ cells) were allowed to phagocytose H37Rv or H37Ra for 60 minutes and were then homogenized and the phagosomes were isolated on 12% sucrose. Equivalent amounts of proteins from the whole lysates of neutrophils and isolated phagosomes were separated according to their isoelectric points on immobilized pH gradient 3-10 gels and then by standard SDS-PAGE. The letters a-c in (B) (MCPv gel) and d-g in (C) (MCPa gel) indicate as yet unidentified proteins (the kDa and pI values of these proteins are given in the text). The molecular weigh markers are indicated to the left in (A). The images shown are representative of silver stained gels from three separate experiments.

second family of vesicle-targeting molecules, the SNARE proteins, seems to convey another element of specificity to Rab-dependent membrane fusion (Brennwald, 2000). The differential subcellular distribution (plasma membrane versus granules) of SNAREs together with their in vitro interaction suggests a role for SNAREs in docking granules with the neutrophil plasma membrane during exocytosis (Ligeti and Mócsai, 1999). Experiments on these cells have shown that the t-SNARE, syntaxin-4, accumulates in or near the lamellipodium, which is the region of the cell that is involved in phagocytosis (Brumell et al., 1995). Moreover, microorganisms have also developed various methods to avoid being ingested and killed. For example, the intracellular bacteria *Mycobacterium tuberculosis* can actually survive and multiply within macrophages (Sturgill-Koszycki et al., 1996). Extracellular bacteria can interfere with the phagocytic capacity of the host cell and some of them, such as *Staphylococcus aureus*, produce toxins that destroy phagocytes (Thelestam, 1983). Despite this, these microorganisms are usually efficiently phagocytosed by and killed within neutrophils.

Despite the obvious importance of the functions of neutrophils and the complexity of intracellular trafficking, the molecular mechanisms underlying phagosome biogenesis are poorly understood. To explore these mechanisms, we used human neutrophils and the following bacteria: a virulent and attenuated strain of *M. tuberculosis* (H37Rv, and H37Ra respectively), to represent an intracellular parasite that infects a variety of cell types (Hernandez-Pando et al., 2000) including neutrophils (Perskvist et al., 2000), and *S. aureus*, to represent an extracellular pathogen. We characterized the molecular mechanisms governing fusion between phagosomes containing *M. tuberculosis* (H37Rv or H37Ra) or *S. aureus* and various granules and endosomal compartments. Using

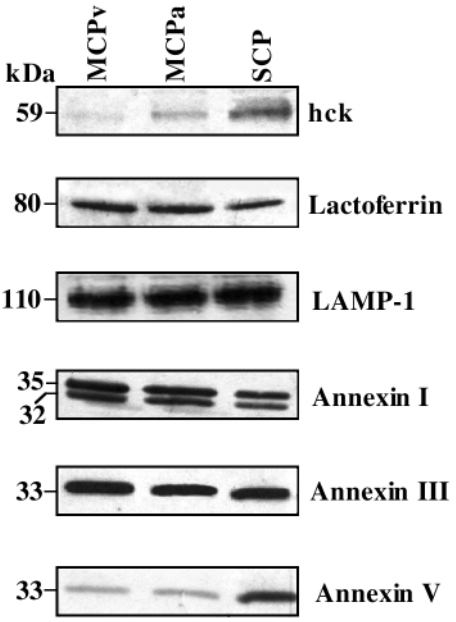



**Fig. 3.** Association of endosomal/granule markers and cytosolic proteins with the MCPv, MCPa and SCP. Neutrophils ($4\times10^8$ cells) were allowed to ingest H37Rv, H37Ra or *S. aureus* for 60 minutes. Thereafter, the phagosomes were isolated and equal amounts of the protein associated with the phagosomes were assayed for hck, lactoferrin and LAMP-1 and for annexin I, III and V by western blotting with appropriate antibodies. The illustrated immunoblots are representative of five independent experiments.

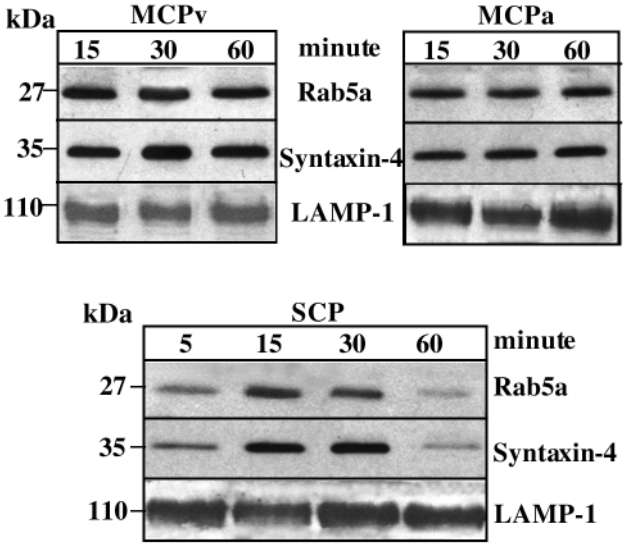



**Fig. 4.** Recruitment of Rab5a and syntaxin-4 to the MCPv, MCPa and SCP. Neutrophils ($4\times10^8$ cells) were allowed to ingest H37Rv, H37Ra or *S. aureus* for the indicated periods of time. Thereafter, the phagosomes were isolated and equivalent amounts of proteins were separated by SDS-PAGE and immunoblotted with anti-Rab5a or anti-syntaxin-4 antibodies. LAMP-1 was used as an internal control for the amounts of protein loaded. The blots shown are one representative of five separate experiments.

isolated bacteria-laden phagosomes, we found that Rab5a is an essential molecule that controls fusion between phagosomes containing bacteria and intracellular compartments, thereby regulating the ability of neutrophils to restrict the spreading of the pathogens. Whereas phagosomes containing *S. aureus* display the complex of Rab5a and syntaxin-4 briefly, phagosomes containing either strains of *M. tuberculosis* bind to the complex for an extended period of time, potentially leading to delayed phagolysosome maturation.

## Materials and Methods

### Regents and antibodies

A rabbit polyclonal antibody (Ab) against human LAMP-1 was generously provided by Sven Carlsson (University of Umeå, Umeå, Sweden), rabbit polyclonal anti-human lactoferrin was a gift from Inge Olsson (University of Lund, Lund, Sweden) and rabbit polyclonal Abs against annexins I, III and IV were kindly provided by Joel Ernst (San Francisco General Hospital, San Francisco, CA). Anti-Rab5a (S-19) Ab, rabbit anti-hck (N-30) and anti-actin (C-2) mAb were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse mAb anti-human syntaxin-4 was from Transduction Laboratory (Lexington, KY) Alexa488-conjugated anti-rabbit and Alexa594-conjugated anti-mouse Abs were obtained from Molecular Probes (Eugene, OR). Electrophoresis and ECL reagents, dexteran, Ficol-paque, [$\alpha$-$^{32}$P] GTP and [$^{35}$S]-methionine were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated in the text. All reagents were of the highest purity available.

### Bacterial preparations

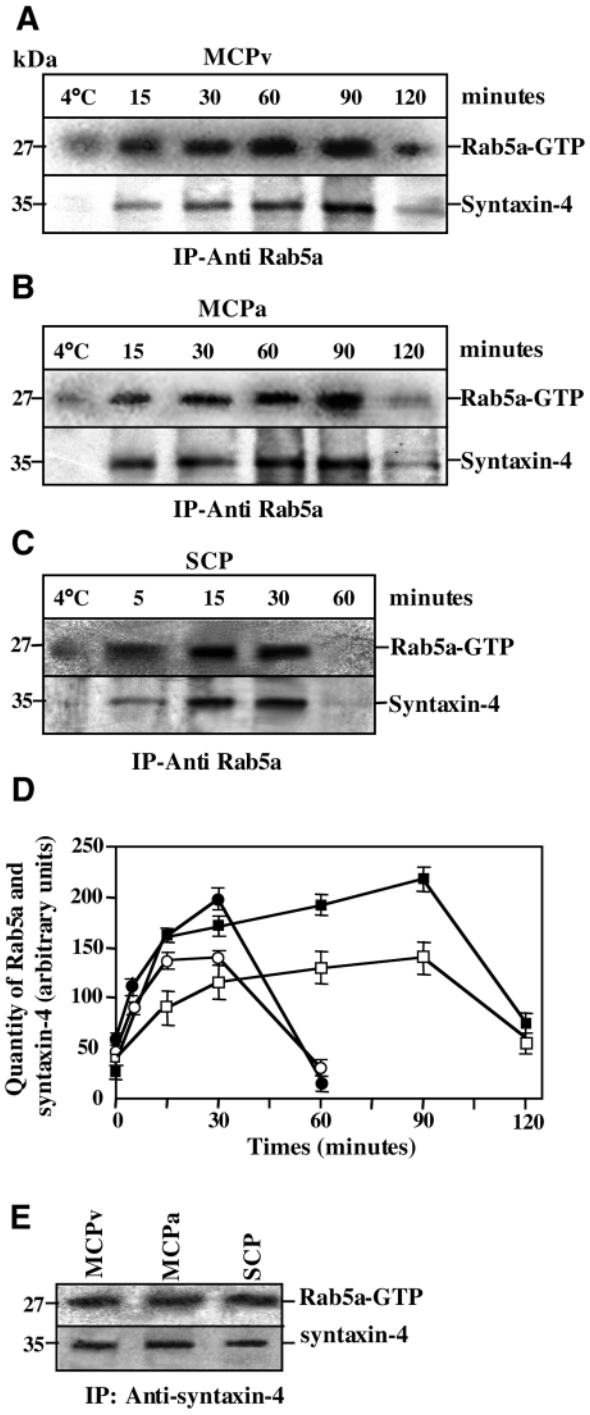
The virulent *M. tuberculosis* strain H37Rv was obtained from the Swedish Institute of Infection Laboratory (Stockholm, Sweden), and the attenuated strain H37Ra (ATCC 25177) was obtained from the American Type Culture Collection (Manassas, VA). Early passages of mycobacteria were prepared and maintained in Middlebrook broth (Difco Laboratories, Detroit, MI) supplemented with OADC (oleic acid, albumin, dextrose and NaCl; Difco). Initial colonies of H37Rv were expanded and frozen in aliquots (Perskvist et al., 2000). Because continuous passage of mycobacteria in liquid culture is associated with loss of virulence, freshly thawed aliquots were not passaged more than once before use in the experiments. Single-cell mycobacterial suspensions were prepared using a syringe equipped with a 27 G 3/4 0.4×19 needles. After several passages through the needle, the bacteria were filtered in a sterile Pasteur pipette equipped with cotton wool (Perskvist et al., 2000). Microscopic examination confirmed that a significant portion (>95%) were present as single bacteria. The *S. aureus* were of the catalase-positive WOOD 46 strain that is devoid of protein A. These bacteria were stored at –70°C and prior to experiments, were cultured at 37°C for 18 hours in Luria broth (LB) with constant shaking (300 rpm) and then transferred to fresh medium and cultured for another 3 hours (Wilsson et al., 1996). C3b/bi-opsonized bacteria were obtained by exposure to 20% normal human serum, as previously described (Perskvist et al., 2000). The viability of the bacteria was assessed at each step by comparing bacterial counts determined by microscopy and assays of colony forming units (CFU). All bacterial were replenished after one passage. The integrity of the cell wall of the bacilli was confirmed by electron microscopy.

### Neutrophils preparation and phagocytosis

Neutrophils were isolated by subjecting buffy coat from healthy blood donors to dextran sedimentation and Ficoll-Paque gradient centrifugation (Böyum, 1968). A brief hypotonic lysis was performed

to remove contaminating red blood cells, resulting in >90% neutrophil purity. The neutrophils were subsequently resuspended ($1\times10^7$ cells/ml) in Krebs-Ringer glucose (KRG) buffer containing 1 mM $Ca^{2+}$ and $Mg^{2+}$. The cells were routinely pretreated with 5 mM diisopropyl fluorophosphate (DFP) for 15 minutes on ice to minimize proteolysis.

To obtain phagosomes, neutrophils were allowed to ingest opsonised bacteria for the amount of time indicated in the figure legends. The cells ($4\times10^8$ in 40 ml of KRG) were exposed to H37Rv or H37Ra (at a ratio of one neutrophil to 20 mycobacteria) or *S. aureus* (one neutrophil to 10 bacteria) at 37°C. Adding ice-cold KRG stopped phagocytosis; thereafter the neutrophils were washed and the phagosomes were isolated. The suitable neutrophil-to-bacteria ratio was determined by incubating the cells for 15, 30 and 60 minutes with FITC-conjugated (Majeed et al., 1997) and opsonised bacteria and utilizing the trypan blue exclusion test (Jaconi et al., 1990). The results showed that the percentages of neutrophils ingesting H37Rv were 23%±5, 55%±7 and 70%±9 (means±s.e.m. *n*=5), and the corresponding values for *S. aureus*-ingesting neutrophils were 32%±3, 60%±8 and 85%±11. These results gave roughly equivalent quantities of bacteria ingested by neutrophils, thus these proportions were used in subsequent experiments. It is plausible that the difference in the nature of the phagosomes between mycobacteria and *S. aureus* relates to differences in the rate and amount of phagocytosis between these two bacterial species within the time frame of the assay. To eliminate this probability, in some experiments, we utilised synchronised phagocytosis. Briefly the bacteria were added to the neutrophils and incubated for 10 minutes at 37°C for mycobacteria and 5 minutes for *S. aureus.* The non-adherent bacteria were removed by centrifugation at 4°C, and internalisation of the bound bacteria was stimulated by rapidly warming the cells to 37°C. After 0 to 60 minutes, cells were processed for isolation of the phagosomes.




## Preparation of phagosomes containing bacteria

The *M. tuberculosis* strains H37Rv or H37Ra and *S. aureus*-containing phagosomes (respectively designated MCPv, MCPa and SCP) were isolated using a procedure modified from two previously described methods (Desjardins et al., 1994; Chakraborty et al., 1994). The neutrophil pellets were resuspended in 2 ml of homogenisation buffer (250 mM sucrose, and 3 mM imidazole, pH 7.4), containing 1 mM $Na_3VO_4$ and 10 μg/ml aprotinin, leupeptin and pepstatin. Passing the suspension through a tuberculin syringe with a 23-gauge needle disrupted the cells. The disruption was monitored in a light microscope and stopped before there was any major damage to the nuclei. The intact neutrophils and nuclei were sedimented by centrifugation at 300 ***g*** for 10 minutes; the pellet was discarded, and the postnuclear supernatant was loaded onto 12% sucrose in 3 mM imidazole (w/w) and centrifuged at 800 ***g*** for 45 minutes. This yielded a pellet containing bacteria-laden phagosomes, which was gently resuspended in 4 ml of homogenisation buffer without inhibitors. This suspension was run (by gravity) through a 47 mm, 3 μm pore Nucleopore filter followed by an additional 3 ml volume of homogenisation buffer, and the flow-through was loaded onto 12% sucrose and centrifuged at 800 ***g*** for 45 minutes to collect the phagosomes in the bottom of the tubes. All procedures were performed at 4°C.

**Fig. 5.** Interaction between Rab5a GTP and syntaxin-4 on neutrophil phagosomes during phagocytosis. Neutrophils ingested H37Rv, H37Ra or *S. aureus* for the indicated periods of time, after which the proteins associated with the isolated phagosomes (750 μg of protein for each phagosome fraction) were solubilized and Rab5a or syntaxin-4 was immunoprecipitated as described in the Materials and Methods. The immunoprecipitates (IP) were separated by SDS-PAGE transferred and blotted on the membranes. The GTP-binding state of Rab5a was detected on MCPv, MCPa and SCP by an [α-$^{32}$P] GTP overlay assay and visualized by autoradiography. The same membrane was stripped and analysed for the presence of syntaxin-4. Interaction of GTP-bound Rab5a and syntaxin-4 on the MCPv (A) and MCPa (B) during 120 minutes and SCP (C) during 60 minutes of phagocytosis are shown. The immunoblots are representative of three separate experiments. (D) The kinetics of association of Rab5a-GTP on the MCPv (■) and on SCP (●) and that of the synatxin-4 on the MCPv (□) and SCP (○) are illustrated. Data are expressed as mean±s.e.m of three experiments. (E) Syntaxin-4 immunoprecipitated from the MCPv, MCPa and SCP fractions isolated after 30 minutes phagocytosis and Rab5a-GTP co-immunoprecipitated with anti-syntaxin-4. The blot is representative of three independent experiments.

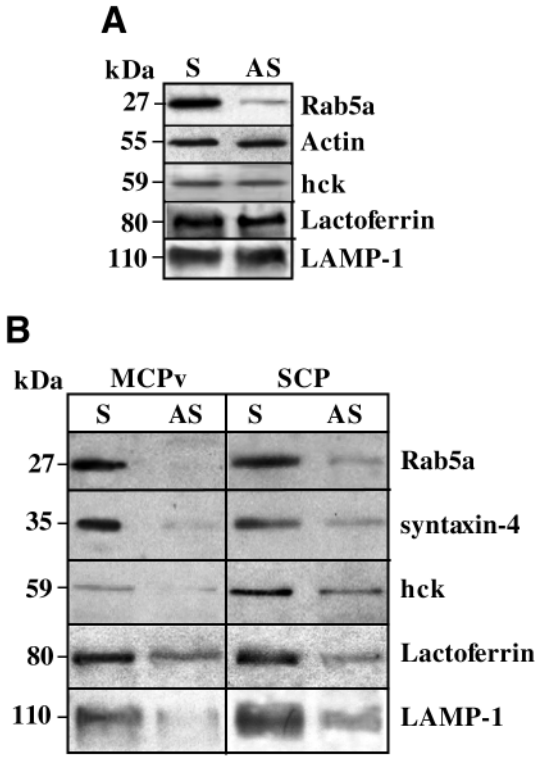



**Fig. 6.** Downregulation of Rab5a impaired the capacity of the phagosomes to fuse with other organelles. Neutrophils were treated with Rab5a-antisense (AS) or -sense (S) oligonucleotides as described in Materials and Methods. (A) The cells were subsequently lysed and equal amounts of the proteins were separated by SDS-PAGE and immunoblotted with the antibodies raised against Rab5a, actin, hck (azurophil granules), lactoferrin (secondary granules) and LAMP-1 (late endosomes) proteins. (B) Antisense- or sense-treated neutrophils ($1\times10^8$ cells) were allowed to ingest H37Rv at the ratio of one cell to 20 H37Rv or to 10 *S. aureus* for 30 minutes. Thereafter, the phagosomes were isolated and equivalent amounts of the phagosomal proteins were separated by SDS-PAGE and immunoblotted for the expression of Rab5a, syntaxin-4, hck, lactoferrin and LAMP-1 using appropriate antibodies. The blots shown are representative of three experiments.

The viability of the bacteria in the phagosomes was determined by selective lysis of the phagosomal membranes using KRG containing 0.5% Triton X-100 followed by comparison of bacterial counts and CFU results. The integrity of the phagosomal membrane was confirmed by exposing the phagosomes containing FITC-labelled bacteria to trypan blue. This test showed that >90% of the FITC bacteria were not quenched by this dye. Staining of these phagosomes by LAMP-1 verified that the bacteria were retained inside phagosomes. The amount of phagosomal protein loaded for SDS-PAGE was first adjusted using colorimetric measurement of the protein concentration and DC protein reagents (Bio-Rad) and thereafter ascertained as the level of LAMP-1 expression. We found that LAMP-1 is a consistent marker that is expressed on the isolated vacuoles containing mycobacteria or *S. aureus*.

The level of contamination of the phagosome preparations with other cellular components was analysed as described elsewhere (Russell et al., 1996). In short, we prepared four tubes of neutrophils (in each there were $1\times10^8$ cells in 10 ml of KRG), two of which were metabolically labelled with 15 μCi/ml [$^{35}$S] methionine for 30 minutes on ice. Bacteria were added to one labelled and one unlabelled tube. After 30 minutes of phagocytosis, the labelled neutrophils that contained bacteria were combined with unlabelled cells without ingested bacteria, and the unlabelled bacteria-containing neutrophils were combined with the labelled bacteria-free cells. After the isolation procedures were completed, equal aliquots of the two samples were measured for radioactivity. To calculate the percentage of contamination the cpm from the bacteria in unlabelled cells was divided by a value representing the combined cpm values from bacteria in labelled and unlabelled cells multiplied by 100. In the protocols given, the levels of contaminations varied between 5 and 7% for SCP and between 10 and 12% for MCP. The purity of the MCPv, MCPa and SCP was further analysed by electron microscopy and two-dimensional gel electrophoresis.

### Transmission electron microscopy

Both intact neutrophils with ingested bacteria and isolated phagosomes were prepared for transmission electron microscopy. Specimens were fixed by adding 2% glutaraldehyde in 100 mM sucrose-sodium cacodylate-HCl buffer (pH 7.2), and post-fixed in 2% osmiumtetroxide and then centrifuged in 2% agar (2500 ***g***, 2 minutes). Pieces of the resulting agar pellet were stained on block with 2% uranyl acetate in 50% ethanol and subsequently dehydrated and embedded in Epon-812. Ultrathin sections were cut with a diamond knife (DIATOME, Bienne, Switzerland), stained with lead citrate and examined and photographed in a JEOL 1200 EX electron microscope (Tokyo, Japan).

### Protein gel electrophoresis and immunoblotting

Proteins were separated according to their isoelectric point and molecular weight using high-resolution two-dimensional gel electrophoresis based on the method of O'Farrel (O'Farrel, 1975). For each sample in a given experiment, the same amount of proteins was solubilised in urea sample buffer (9 M urea, 0.8% [w/v] pharmalyte (pH 3-10), 1% [w/v] DTT, 2% [w/v] CHAPS and 0.01% [w/v] bromophenole blue). Separation in the first dimension was done with an Immobiline DryStrip Kit (Pharmacia Biotech. Uppsala, Sweden) using a non-linear pH gradient from 3 to 10. After focusing, the strips were positioned over a vertical 8-18% Exel SDS-PAGE slab gel (Pharmacia Biotech.) and subjected to electrophoresis according to the instructions of the manufacturer. The gels were then silver stained for protein pattern analysis.

SDS-PAGE was performed using a 12% separating gel as described by Laemmli (Laemmli, 1970). Isolated phagosomes were lysed for 30 minutes at 4°C with 100 μl of RIPA buffer (pH 7.5) containing 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl and the same protease inhibitors as in the homogenisation buffer. Equal amounts of protein were separated by SDS-PAGE and then electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with 5% BSA in PBS, and the phagosomal proteins on the blots were detected with either 1 μg/ml mAbs (1:500 dilution) or 0.5 μg/ml polyclonal Abs (1:1000) in 3% BSA in PBS for 60 minutes at room temperature (RT) followed by HRP-conjugated anti-mouse or anti-rabbit Ab (1:3000 dilution) and then visualized with a commercial ECL kit. To confirm that the lanes received equal amounts of proteins, the blots were stripped and reprobed with anti-LAMP-1 Ab. The intensity of the bands was measured by densitometric assay using a Howtek scanner and Quantity One Software (Advanced American Biotechnology, Fullerton CA)

### Coimmunoprecipitation and GTP-binding overlay assay

MCPv, MCPa and SCP (750 μg protein each) were lysed in 100 μl of 20 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 1 mM DTT, 5 mM Mg $Cl_2$ and 0.6% CHAPS and then incubated for 30 minutes at 4°C and subsequently centrifuged at 35,000 ***g*** for 60 minutes

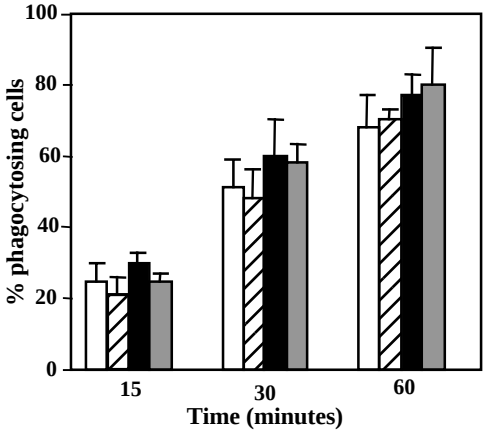



**Fig. 7.** The effect of down-regulation of Rab5a on the phagocytic capacity of neutrophils. Neutrophils either treated with Rab5a antisense and then allowed to ingest (□) H37Rv or (■) *S. aureus*, or with Rab5a sense and then phagocytosed (stripes) H37Rv or (grey) *S. aureus* as described in Materials and Methods. The percentage of the phagocytosed cells with ≥1 bacteria was counted by trypan blue exclusion. Data represent mean±s.e.m. of four experiments.

(Mukherjee et al., 2000). The supernatants were precleared once with protein-A-agarose and then incubated at 4°C with 2 μg of anti-Rab5a Ab for 60 minutes and thereafter with 30 μl of 50% (v/v) protein A-agarose for an additional 60 minutes. The concentration of the protein eluted from beads was measured by colorimetric assay as above and the same amounts of proteins were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. Essentially the same procedure was used to precipitate syntaxin-4, except that the phagosome fractions were lysed in RIPA buffer without inhibitors and centrifuged at 15,000 ***g*** for 15 minutes. After lyses, the protein was immunoprecipitated with 2.5 μg of anti-syntaxin-4 mAb. An overlay assay utilizing 1 μCi/ml of [α-$^{32}$P] GTP (3.000 Ci/mM, DuPont NEN) in 50 mM phosphate buffer (pH 7.5) containing 5 mM $MgCl_2$, 1 mM EGTA and 0.3% Tween 20 and 1 μM ATP as competing substrate (Deretic et al., 1995) was used to determine the GTP-binding state of the Rab5a, which was then visualized by autoradiography and GTP binding was quantified in a PhosphorImager. The same membranes were washed thoroughly with 0.5% triton X-100 in PBS and then stripped and immunoblotted with Abs as specified in the figure legends. Duplicate gels were silver stained to establish whether other proteins coimmunoprecipitated with anti-Rab5a Ab. To confirm the molecular mass of GTP-bound Rab5a, the same membrane was washed and then immunoblotted with anti-Rab5a Ab. The specificity of the [α-$^{32}$P] GTP-binding was examined by addition of 10 μM GTPγs, a nonhydrolysable analogue of GTP, to the various phagosome fractions

### Treatment of neutrophils with antisense oligonucleotides

We used the antisense oligonucleotide sequences Rab5a-antisense (AS), 5′-TGC GCC TCG ACT AGC CAT GT–3′ and Rab5a-sense (S), 5′-ACA TGG CTA GTC GAG GCG CA-3′ (20 mer), which were chosen in view of results published by Alvarez-Dominguez and Stahl (Alvarez-Dominguez and Stahl, 1999) and were purchased from Life Technology, Inc. A pair of bases was included before the ATG to maximize hybridisation and specificity. In all oligonucleotides, the internucleoside linkages were completely phosphorothioate-modified to activate RNAse H. 5′ and 3′ terminal bases were also modified to resist nuclease attack. Equal enhancement of delivery of the oligonucleotides was achieved with the cationic lipid DMRIE-C (1,2-dimyristoyloxypropyl-3-dimethyl-hyroxyethyl ammonium bromide/cholesterol, 1:1, M/M) (Life Technology, Inc.). We chose DMRIE-C because it lacks dioctanoyl phosphatidylethanolamine, which can be toxic to phagocytic cells (Korchak et al., 1998). Neutrophils ($7\times10^7$ cells) were preincubated in 10 ml of Opti-MEM I-reduced serum medium (Life Technology, Inc) in 160 ml tissue culture flasks for 30 minutes at 37°C in a humidified $CO_2$ incubator. Rab5a-AS or -S oligonucleotide (120 μg) was suspended in Opti-MEM and incubated with 360 μl of DMRIE-C at room temperature for 30 minutes and thereafter added to neutrophils for 4 hours. Following incubation, the cells were exposed to the opsonised H37Rv or *S. aureus* for 30 minutes at the same ratio as previously mentioned, and the induction and isolation of phagosomes were carried out as described above. This treatment did not affect the viability of the cells, as assessed by the trypan blue test.

To determine whether downregulation of Rab5a affected the phagocytic uptake of bacteria, neutrophils were treated with Rab5a-antisense or -sense oligonucleotide as described above. The cells ($10^6$) were allowed to ingest FITC-conjugated and opsonised bacteria at a ratio of one neutrophil to 20 H37Rv or to 10 *S. aureus* for 15, 30 and 60 minutes. The percentage of ingesting neutrophils was determined by the trypan blue exclusion test.

## Results

A prerequisite for biochemical analysis of phagolysosome fusion is that pure phagosomes can be isolated. Using a combination of two methods (Desjardins et al., 1994; Chakraborty et al., 1994), we were able to successfully separate both MCP and SCP. These phagosome preparations showed a low level of contamination with other intracellular organelles. Transmission electron microscopy revealed that isolated phagosomes accommodating bacteria were surrounded by a continuous and distinct membrane (Fig. 1C,D), similar to the membrane of H37Rv-containing phagosomes in intact cells (Fig. 1A). A vast majority of the isolated vacuoles were devoid of other cell organelles (Fig. 1B). Furthermore, we performed 2D gel electrophoresis to analyse the protein composition of isolated phagosomes. This analysis gave significantly simpler polypeptide profiles for the MCPv (Fig. 2B) and MCPa (Fig. 2C) than for whole cell lysates (Fig. 2A), which demonstrates the validity of our approach.

### MCPv, MCPa and SCP acquire different endosome and granule markers and cytosolic proteins

We used the following proteins to investigate the interaction between different neutrophil granules and newly formed phagosomes: hck, a src-kinase associated with the membrane of azurophil granules (N'Diaye et al., 1998); lactoferrin, which is found in secondary granules; and LAMP-1, a marker of late endosomal compartments. (Cieutat et al., 1998; Bainton et al., 1999). In initial experiments, neutrophils were allowed to phagocytose H37Rv or H37Ra or *S. aureus* for 60 minutes prior to isolation of phagosomes. The same amount of protein found associated with the phagosomes was subjected to SDS-PAGE and subsequent immunoblot analysis. Other investigators (N'Diaye et al., 1998) have used immuonoelectron microscopy to study neutrophils and observed that phagosomes containing mycobacteria fail to acquire myeloperoxidase (MPO) and hck. Our results showed that the amount of hck protein associated with the membrane of MCPv and MCPa was markedly reduced compared with that of the SCP, indicating a limitation of fusion between MCP and azurophil granules (Fig. 3). Azurophil granules contain

numerous lysosomal enzymes and bactericidal factors (Bainton, 1999). Thus the fusion incompetence of azurophil granules indicates a mechanism by which mycobacteria avoid the toxicity of these factors. Moreover, both lactoferrin and LAMP-1 were associated with the membranes of MCPv, MCPa and SCP, suggesting that the vacuoles remained accessible to the specific granules and late endosomal organelles (Fig. 3).

The differences we observed between the MCP and SCP regarding fusion with azurophil granules prompted us to examine which cytosolic proteins are involved in phagolysosome fusion. We analysed the ability of isolated phagosomes to bind to annexins, proteins that are implicated in fusion of intracellular membranes (Diakonova et al., 1997). Western blotting of isolated phagosomes showed that annexin I, III and V were present in SCP, whereas only annexins I and III were detected in lysates of MCP preparation (Fig. 3). Whole neutrophil lysates also contained all three kinds of the annexins (data not shown). The anti-annexin I Ab reproducibly recognized two distinct bands in western blots, which reflected post-translation modification by proteases (Movitz et al., 1999). The level of expression of LAMP-1 was also used as an internal control of loading of the phagosomal proteins.

### Recruitment of Rab5a and syntaxin-4 to MCPv, MCPa and SCP

Rab GTPases are known to be key regulators of vesicular transport in mammalian cells. There is evidence that Rab5a is present in the cytosol of human neutrophils and that upon cell activation with PMA Rab5a translocates to the membrane (Vita et al., 1996). Roberts et al. (Roberts et al., 2000) found that Rab5a associated transiently with newly formed phagosomes in macrophages. Thus we studied the kinetics of interaction of Rab5a with the MCPv, MCPa or SCP to determine whether this protein is involved in the maturation of these phagosomes. The results showed that MCPv and MCPa acquired Rab5a within 15 minutes and the amount of the protein increased slightly to a level that was maintained up to 60 minutes, whereas SCP recruited Rab5a within 5 minutes but the protein was selectively depleted after 30 minutes (Fig. 4). Densitometric quantification of Rab5a at 15, 30 and 60 minutes indicated 245±10, 251±9 and 268±12 arbitrary units of Rab5a in the MCPv, and at 5, 15, 30 and 60 minutes the corresponding values for SCP were 52±11, 230±10, 213±13 and 25±9 arbitrary units. These data demonstrated that SCP transiently recruited Rab5a during fusion with endosomes and granules, whereas MCP retained Rab5a throughout a longer period of time.

As previously mentioned, proteins of the t-SNARE family ensure that vesicles are delivered to their correct target membrane, and Rab GTPases appear to act upstream of SNARE molecules during membrane docking/fusion (Cao et al., 1998). Syntaxin-4 is a t-SNARE protein in neutrophils, thus we investigated the possibility that it is also involved in fusion of endosomes and/or granules with neutrophils phagosomes. The same kinetics of association were observed for syntaxin-4 and Rab5a (Fig. 4). Densitometric measurements confirmed this result. In our assay, an insufficient amount of MCP was recovered after less than 15 minutes of phagocytosis, hence the kinetic analyses were done from 15 to 60 minutes of exposure to mycobacteria. The expression levels of LAMP-1 were determined to verify that the same amount of each protein was loaded. Synchronised phagocytosis was initially performed using FITC-conjugated and opsonized bacteria. The results showed that 75%±4 or 83%±6 (mean±s.e.m., $n$=3) of neutrophils bound to mycobacteria or *S. aureus*, whereas the percentage of neutrophils ingesting these bacteria was 3%±1.3 or 5%±2, respectively. Thus these short periods of time allowed for adequate numbers of bacteria to attach to the cell surface before the warming period, with a low percentage of ingested neutrophils. Consequently, the synchronised phagocytosis assay was used to verify the kinetics of Rab5a and syntaxin-4 recruitment to phagosome-containing mycobacteria or *S. aureus*. Similar data were obtained compared to the unsynchronised phagocytosis assay.

### Interaction between GTP-bound Rab5a and syntaxin-4 on the membrane of MCP and SCP

The assembly of Rab5a and syntaxin-4 on the membrane of phagosomes showed that the fusion events in neutrophils occur through a highly controlled mechanism. Similar to other low molecular weight GTPases, Rab5a is active in its GTP-bound form (Barbieri et al., 1994; Alvarez-Dominguez et al., 1996); thus, we investigated whether Rab5a in its GTP-bound form can interact with syntaxin-4 on the phagosomal membrane of the bacteria during the fusion process. We extended the phagocytic time of the mycobacteria to examine the later time points of association of Rab5a and syntaxin-4 on the phagosomes and allowed neutrophils to ingest H37Rv or H37Ra for 120 minutes or *S. aureus* for 60 minutes. Lysates of the phagosomes were precipitated with anti-Rab5a Ab, subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The GTP-binding state of Rab5a was detected using [$\alpha$-$^{32}$P] GTP, and the same membrane was then blotted to detect possible coprecipitation of syntaxin-4 with Rab5a. We found a sustained association between GTP-bound Rab5a and syntaxin-4 on the MCPv (Fig. 5A) and MCPa (Fig. 5B) up to 90 minutes after phagocytosis, and then it gradually declined. SCP retained the Rab5a-synatxin-4 complex for up to 30 minutes; thereafter the SCP was completely depleted from the complex (Fig. 5C). The kinetics of association of Rab5a-GTP and syntaxin-4 revealed that although these proteins are absent from SCP, functional Rab5a-GTP and syntaxin-4 persist on MCPv (Fig. 5D). Next we confirmed that Rab5a in its GTP-bound state coimmunoprecipitated with anti-syntaxin-4 in the phagosomal fractions obtained after 30 minutes of phagocytosis (Fig. 5E). There was no such interaction in lysates of non-ingesting neutrophils at 4°C (Fig. 5A,B,C), thus confirming that the association of syntaxin-4 with GTP-bound Rab5a occurred in neutrophils activated by phagocytosis. The results from silver staining showed that Rab5a also coprecipitated with other regulatory proteins than syntaxin-4. The nature of these proteins remains to be determined.

### Downregulation of Rab5a reduced the capacity of MCP and SCP to fuse with intracellular organelles

To elucidate the functional mechanism(s) of the interaction between Rab5a and syntaxin-4 on the phagosomal membrane, we examined the effects of downregulation of Rab5a on the

properties of fusion between pathogen-laden phagosomes and intracellular endosomes and granules. Initial experiments demonstrated that treatment of the neutrophils with antisense Rab5a markedly decreased the expression of Rab5a (Fig. 6A). Actin was used to confirm that equal amounts of the proteins were loaded. Expression of both Rab5a and syntaxin-4 declined on the isolated phagosomes (Fig. 6B). In light of the reduction of Rab5a and syntaxin-4, we considered the possibility that intracellular membrane trafficking had been altered. We thus evaluated the translocation of the endosomes and granule markers in antisense-treated neutrophils. We found that downregulation of Rab5a impaired the ability of the MCPv and SCP to incorporate hck, lactoferrin and LAMP-1 into their membranes (Fig. 6B). Finally, downregulation of Rab5a had no effect on the phagocytic capacity of neutrophils (Fig. 7). Together, these findings demonstrated that Rab5a did not play a role in bacterial uptake but was clearly involved in degranulation and intracellular trafficking in neutrophils. To rule out the non-specific effect of the Rab5a-antisense treatment, the whole cell lysate from treated or untreated neutrophils was subjected to SDS-PAGE and immunoblotted with antibodies to lactoferrin, hck and LAMP-1. The results showed that the levels of these proteins were not affected by the antisense treatment (Fig. 6A).

### MCPv and MCPa but not SCP displayed similar fusion properties

In this study we analysed the maturation of MCPv, MCPa and SCP in neutrophils. The results clearly confirmed that MCPv and MCPa exhibited similar fusion patterns to granules and/or endosomal organelles, which differed from that of SCP. Accordingly, we used 2D gel electrophoresis to compare the protein compositions of these phagosomes and found that there are indeed differences between MCPv and MCPa. For example, the following sequences were reproducibly detected in MCPv: 23 kDa, pI 5-5.5; 29 kDa, pI 6.3-6.5; 44 kDa pI 6.5-6.8 (for a, b, and c, respectively, in Fig. 2B), and the following proteins were found only in MCPa: 24 kDa, pI 6-6.5; 40 kDa, pI 5.5-5.8; 80 kDa, pI 5.3-5.6; and 98 kDa, pI 5-5.5 (d, e, f, and g, respectively, in Fig. 2C). The different protein composition may reflect different intraphagosomal milieus. Further studies are needed to identify both these and other unknown proteins. However, we conclude that, despite the differing polypeptide compositions of MCPv and MCPa, these phagosomes exhibited similar intracellular fusion capacities to the endosomal/granule compartments.

## Discussion

Mounting a successful defence against internalised pathogens in neutrophils requires the maturation of the phagosomes through a complex remodelling process that involves regulated interaction with a series of granules and endosomal compartments. To be able to selectively control fusion between different granule populations and the newly formed phagosomes, neutrophils must possess differentiating mechanisms to deliver granules to the target membranes. To elucidate phagosome biogenesis in neutrophils, we compared the molecular mechanisms underlying docking and fusion of phagosomes containing an intracellular pathogen (*M. tuberculosis* H37Rv or H37Ra) or an extracellular pathogen (*S. aureus*) with endosomal and granule compartments. We found that MCPv and MCPa did not acquire hck, indicating a reduced fusion with azurophil granules, whereas, they did fuse with specific granules and late endosomes. In contrast, SCP fused with all of the indicated organelles. In mature neutrophils, release of granules seems to occur largely in reverse order of the synthesis of these compartments, although this may depend on the nature of the inducing stimulus (Joiner et al., 1989). The densest azurophil granules are synthesized first but are the last to fuse with target membranes (Sengelov et al., 1995). The azurophil granules store a number of bioactive agents, such as cytotoxic polypeptides and digestive enzymes (Bainton, 1999). Thus exclusion of these factors owing to the fusion incompetence of the mycobacterial phagosome with this granule may affect the fate of the bacteria, whereas the exposition of *S. aureus* to the toxicity of these enzymes is unavoidable. The fact that mycobacteria are killed during neutrophil phagocytosis (Majeed et al., 1997; Brown et al., 1987) indicates that oxygen-dependent mechanisms that involve fusion with specific granules and secretory vesicles may be sufficient to kill ingested mycobacteria.

According to previous studies of neutrophils performed in our laboratory, elevation of intracellular calcium does not occur during phagocytosis of mycobacteria (Majeed et al., 1997), although it does occur during ingestion of *S. aureus* (Wilsson et al., 1996). The absence of fusion of MCP with azurophil granules may in fact be due to a transient lack of intracellular calcium, because it is known that these granules require higher concentrations of intracellular calcium than the others to fuse with the membranes (Nüsse et al., 1998). It is also tempting to speculate that the decreased ability of MCPv and MCPa to fuse with azurophil granules may be related to the lack of translocation of certain calcium-regulated cytosolic proteins, for example, annexin V, to the membrane of these phagosomes and that such a translocation does occur in SCP. Extensive studies of vesicle trafficking along the endocytic pathway have indicated that various annexins regulate vesicle fusion in a calcium-dependent manner (Diakonova et al., 1997; Collins et al., 1997), hence the differential assembly of annexins in proximity to phagosomal membranes may influence the binding capacity of various intracellular organelles. It should be noted that our finding of annexin I on the MCPv and MCPa are not in line with our previously published results (Majeed et al., 1997). A possible explanation for this discrepancy is that the immunofluorescence staining performed in the cited study was not sensitive enough to detect the limited amount of annexin I present on mycobacterial phagosomes. Inasmuch as all three types of phagosomes we studied exhibited characteristics of the late endocytic pathway, the presence of LAMP-1 on these structures suggests that the MCPv and MCPa bypass the normal endocytic route, implying that the mycobacteria interfere with maturation of theirs phagosomes. By comparison, in human macrophages LAMP-1 translocates to the mycobacteria-containing phagosome (Zimmerli et al., 1996), they exclude the vacuolar ATPase in bone marrow-dived mouse macrophages and consequently do not become acidified (Sturgill-Koszycki et al., 1994). In neutrophils, maturation of bacterial vacuoles into phagolysosomes occurs through fusion with azurophil granules, thus the differential fusion competence of the

phagosomes with azurophil granules is related to the maturation process.

We found that, in neutrophils, Rab5a in GTP-bound form was expressed on MCPv and MCPa for a longer period of time during phagosome-granule fusion while targeted briefly to the membrane of the SCP. These results are in agreement with the findings of Clemens and co-workers (Clemens et al., 2000). Like the *M. tuberculosis* strains in our experiments, the *M. tuberculosis* strain Erdman used by Clemens et al. (Clemens et al., 2000) blocks phagosomal maturational in macrophages through persistent expression of Rab5a on the vacuole. The fact that Rab5a did not persist on the neutrophil phagosome and dissociated after 120 minutes may reflect the rapid and effective phagolysosome fusion of neutrophils. Alvarez-Dominguez and Stahl (Alvarez-Dominguez and Stahl, 1999) studying human macrophages reported that overexpression of Rab5a accelerates maturation of the phagosomes and enhances intracellular killing of *Listeria monocytogenes*, whereas the downregulation of Rab5a selectively impairs maturation of the phagosomes. We have found that in neutrophils downregulation of Rab5a by its antisense oligonucleotide reduced expression of Rab5a and syntaxin-4 on the phagosome of the ingested bacteria and consequently reduced the fusion capacity of the phagosomes with the intracellular compartments. The results underscore the importance of Rab5a in mediating phagosome-endosomal/granule interaction in neutrophils. This is in accordance with the results showing that Rab5a is a key molecule regulating phagolysosome biogenesis in macrophages (Duclos et al., 2000). In our study, the recruitment and maintenance of GTP-bound Rab5a in association with syntaxin-4 on the mycobacterial phagosomes correlated with exclusion of fusion with azurophil granule and thereby impairment of the phagosome maturation. In contrast, a brief expression of Rab5a GTP and syntaxin-4 on the SCP related to the complete phagolysosome maturation. In all likelihood the Rab proteins also cooperate with other regulatory molecules to create a platform for membrane organization. A direct interaction between the Rab GTPase Ypt1p and the SNARE Sed5p has been detected in yeast (Lupashin and Waters, 1997). Furthermore, Pep120, which is a yeast homologue of syntaxin-6, interacts with the Rab5 homologue Vps21p (Peterson et al., 1999). Hence it is conceivable that Rab5a interacts with syntaxin-4 in neutrophils. We noted that the translocation of syntaxin-4 has kinetics identical to the Rab5a and that it interacts with GTP-bound Rab5a. Thus the combination of these proteins involves the fusion events in neutrophils. Owing to the complexity of the conglomerate of effector proteins at the site of fusion, we expect that other effecter molecules are also integrated into the Rab5a/syntaxin-4 complex. In that context, Simonsen et al. (Simonsen et al., 1999) recently observed that Rab5 and syntaxin-6 bound competitively to the membrane-proximal C-terminus of EEA1, a Rab5 effector protein. This effector protein was recently shown to be targeted for mycobacterial phagosome maturation arrest (Fratti et al., 2001).

Bacterial factors can affect the state of Rab proteins in phagosomes. For example, within a phagosome, *Neisseria gonorrhoeae* continuously synthesizes porin, which is translocated into the phagosomal membrane and influences the association of Rab5a (Mosleh et al., 1998). Also it has been shown that SopE, a protein secreted by *Salmonella*, interacts with Rab5 in GTP bound forms (Hardt et al., 1998). This result suggests that mycobacteria may secrete a similar protein that affects the recruitment of Rab5, which in turn activates SNARE proteins and triggers vesicle fusion. After such recruitment, GTPase-activating protein (GAP) could increase the GTPase activity of the Rab protein, converting it into its GDP form, thereby initiating GDI-mediated release of the Rab to the cytosol, (Pfeffer, 1994). Thus the task of a secreted mycobacterial protein would be to inhibit GAP activity and thereby maintain Rab on the phagosomal membrane. In fact, Mukherjee and co-workers (Mukherjee et al., 2001) recently showed that SopE mediates recruitment of non-pernylated Rab5-GTP on *Salmonella*-containing phagosomes and acts as Rab5 specific exchange factor converting Rab GDP to GTP without the prerequisite of prenylation.

Neutrophils are quintessential phagocytes that contribute to the restriction of the extracellular and intracellular pathogens. Phagolysosome biogenesis involves the fusion of the phagosome-containing pathogens with various neutrophil granule and/or endosomal compartments. We conclude that whereas phagolysosome fusion in neutrophils occurs in a Rab5a-dependent pathway, the fusion of *M. tuberculosis* phagosomes with neutrophil organelles is different from that of the *S. aureus* phagosomes.

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