Diverting intracellular trafficking of *Salmonella* to the lysosome through activation of the late endocytic Rab7 by intracellular delivery of muramyl dipeptide

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Summary

Previously, we showed that live *Salmonella*-containing phagosomes (LSP) recruit early acting Rab5 and promote fusion with early endosomes, thus avoiding transport to the lysosomes. Therefore, live *Salmonella* survive in a specialized compartment. Here we show that scavenger-receptor-mediated intracellular delivery of muramyl dipeptide (MDP) to macrophages leads to efficient killing of *Salmonella* both in vitro and in vivo. To understand the intracellular trafficking modulation of *Salmonella* by delivery of MDP, we investigated the levels of endocytic Rab proteins, which are the major regulators of vesicular transport. Western blot analysis reveals reduced Rab5 and enhanced Rab7 content in the maleylated bovine serum albumin-MDP (MBSA-MDP)-treated cells. The reduced content of Rab5 in the treated cells and on phagosomes inhibits the fusion of *Salmonella*-containing phagosomes with early endosomes, and the enhanced Rab7 content in these cells facilitated targeting of LSP to lysosomes, which contain cathepsin D and vacuolar ATPase, for killing. In vitro reconstitution of lysosomal transport demonstrated that a reduced content of Rab5 and an enhanced level of Rab7 in MBSA-MDP-treated cells is primarily responsible for targeting *Salmonella* to lysosomes. Intracellular delivery of MDP thus offers a general strategy against macrophage-associated infections caused by intracellular pathogens that survive in the host cell by resisting transport to lysosomes.

Key words: Endocytosis, Phagocytosis, *Salmonella*, Rab GTPases, Fusion, Lysosomes

Introduction

Consigning intracellular pathogens to lysosomes after internalization through phagocytosis is a major tool host cells use to protect themselves. This defence mechanism is often countered by specific pathogens through various mechanisms (Garcia-del Portillo, 1999). Intracellular trafficking of phagosomes depends on the vesicular membrane composition as well as the intravesicular content and involves dynamic modulations of the phagosomal membrane (Desjardins et al., 1994; Garcia-del Portillo and Finlay, 1995), which are brought about by fusion with other endocytic vesicles and recruitment of various proteins from the cytosol. Small GTP-binding proteins of the Rab family regulate intercompartmental transport (Rothman, and Sollner, 1997; Schimmoller et al., 1998; Zerial and Mc Bride, 2001). Among the endocytic Rabs, Rab5 mediates homotypic fusion among early compartments (Gorvel et al., 1991; Barbieri et al., 1996), whereas Rab7 on the late endosomes regulates the transport between early to late compartments (Feng et al., 1995; Mukhopadhyay et al., 1997a; Mukhopadhyay et al., 1997b). Intracellular pathogens modulate the recruitment of these proteins on phagosomes to avoid or induce specific interactions of phagosomes with other vacuolar compartments for survival (Sturgill-Koszycki et al., 1996; Uchiya et al., 1999; Fratti et al., 2001). For instance, a block in vesicle fusion between the stages controlled by Rab5 and Rab7 arrests maturation of phagosomes containing live *Mycobacterium tuberculosis*, inhibiting their transport to lysosomes (Via et al., 1997). Recently, we showed that live *Salmonella*-containing phagosomes (LSP) recruit the early acting Rab5 and the fusion factors NSF and α-SNAP to promote fusion with early endosomes (Mukherjee et al., 2000; Mukherjee et al., 2001), thus avoiding transport to the lysosomes. Therefore, live *Salmonella* persist in a low-acidity compartment lacking active lysosomal enzymes and transferrin receptors but retaining Rab5 and Rab18 (Hashim et al., 2000). Rab GTPases are major regulators of intracellular trafficking (Rothman and Sollner, 1997; Schimmoller et al., 1998; Zerial and McBride, 2001), and overexpression of late endocytic Rab7 modulates intracellular trafficking of cathepsin D (Press et al., 1998) and *Salmonella* inside the host cells (Meresse et al., 1999). Thus, the alteration of endocytic Rab content by intervention in appropriate signal transduction events may offer a new strategy to modulate the targeting of invading organisms to a degradative compartment for efficient killing. Here we report that intracellular delivery of muramyl dipeptide (MDP) to macrophages decreases the cellular content of Rab5 and increases the Rab7 level, thereby promoting the transport of live *Salmonella* to the lysosomes, leading to efficient killing.

Materials and Methods

Materials

Unless otherwise stated, all reagents were obtained from Sigma. Tissue culture supplies were obtained from Life Technologies (Grand Island, NY). N-hydroxy succinimidobiotin (NHS-biotin), avidin-horseradish peroxidase (Avidin-HRP), avidin, bicinchoninic acid (BCA) reagents were purchased from Pierce Biochemicals (Rockford, IL). Muramyl dipeptide was purchased from Fluka (Switzerland). Lysotracker Red was purchased from Molecular Probes (Eugene, OR), and ECL reagents were procured from Amersham International. Other reagents used were of analytical grade.

Antibodies and recombinant proteins

Monoclonal antibody (mAb) 4F11, a mouse IgG_{2ak} mAb specific for the C-terminus of mouse Rab5, and an affinity-purified rabbit polyclonal antibody that recognizes the C-terminal domain of Rab7 were generously provided by A. Wandinger-Ness (University of New Mexico, Albuquerque, NM). Recombinant GDI and different constructs of Rab5 and Rab7 were kindly provided by Philip Stahl (Washington University School of Medicine, St Louis, MO). Anti-vacuolar ATPase antibody was a kind gift from Andrea Jahraus (EMBL, Heidelberg, Germany). V. Bal (National Institute of Immunology, New Delhi) kindly provided the virulent strain of *Salmonella typhimurium*. GFP-*Salmonella* was obtained as a gift from A. Aballay (Massachusetts General Hospital, Boston). Anti-cathepsin D and all the second antibodies labelled with HRP were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Mouse anti-actin and anti-Rab6 antibodies were purchased from Calbiochem (La Jolla, CA), and the anti-transferrin receptor antibody was obtained from Zymed Laboratories.

Preparation of MBSA-MDP conjugates

MDP was conjugated to BSA by using water-soluble carbodiimide in the presence of N-hydroxysulfosuccinimide as described previously (Srividya et al., 2000). Briefly, 10 mg of the MDP was incubated in the presence of 3.6 mg of EDC and 3.2 mg of sulfo-NHS in 0.1 ml of water for 10 minutes at room temperature to generate an active ester of MDP. Subsequently, activated MDP was reacted with 10 mg of BSA in 0.2 M sodium-carbonate buffer, pH 9.5, for 1 hour at room temperature. BSA-MDP conjugate was purified through Sephadex G25 column chromatography, and the purified conjugate was maleylated with maleic anhydride at a pH of 8.0. A conjugate with 15:1 molar ratio of MDP to protein was used in these studies.

MBSA-MDP-mediated killing of *Salmonella* in J774E macrophages

The virulent *Salmonella typhimurium* strain was a clinical isolate from Lady Harding Medical College, New Delhi. Bacteria were grown overnight in Luria broth (LB) at 37°C with constant shaking (300 rpm). The bacteria were harvested in the stationary phase and were washed twice in phosphate-buffered saline (PBS) and used for infection. To determine the effect of MDP in the free or conjugated form on the microbicidal properties of macrophages, J774E macrophages (1×10^6 cells/well) were cultured in RPMI-1640 medium containing 10% FCS in the presence of MBSA-MDP or free MDP for 12 hours. Cells were washed twice with PBS, and 1×10^7 *Salmonella typhimurium* were added to each well and centrifuged at a low speed (500 ***g***, 5 minutes at 4°C) to synchronize the infection. Subsequently, cells were incubated for 20 minutes at 37°C for infection, and then the cells were washed five times with PBS to remove uninternalized bacteria. The infected macrophages were incubated in the respective drug-containing medium at 37°C. After incubation for 12 hours, the macrophages were lysed in solubilization buffer (SB, PBS containing 0.5% Triton X-100), and an aliquot of the cell lysates was plated on *Salmonella-Shigella* agar plates to determine the number of viable *Salmonella* present in the lysate in terms of colony-forming units.

Treatment of *Salmonella*-infected mice with MBSA-MDP

To determine the efficacy of MBSA-MDP for treatment of *Salmonella* infection in vivo, C57Bl-6 mice (6 week's old, weighing about 20 g each) were injected intraperitoneally with 1×10^3 *Salmonella* (in 100 μl of PBS) as described (Pashine et al., 1999) on day 0. Subsequently, *Salmonella*-infected animals received intraperitoneal injections of free MDP or MBSA-MDP (1 μg/ mouse of MDP equivalent; 50 μg/kg body weight) or ciprofloxacin (1.4 mg/mouse; 70 mg/kg body weight) daily for four consecutive days. Finally, mice were sacrificed on day 10, and the spleen was dissected out from each animal. A portion of the spleen (50 mg) from each animal was teased apart in SB, and aliquots of the cell lysates were plated on *Salmonella-Shigella* agar plates to determine the number of viable *Salmonella* present in the spleen in terms of colony-forming units.

Detection of Rab proteins in MBSA-MDP-treated cells

To determine whether MBSA-MDP treatment induced the expression of endocytic Rabs, J774E cell monolayers were incubated with free or conjugated MDP (MDP equivalent, 1 μg/ml) for 12 hours at 37°C in RPMI-1640 medium with 10% FCS. Cells were washed and 80 μg of the cellular proteins were analysed by 12% SDS-PAGE. The proteins were transferred onto nitrocellulose membranes and checked for the presence of actin, Rab5, Rab7 and Rab6 using the respective antibodies. Proteins were visualized by appropriate HRP-labelled second antibodies and ECL.

Purification of phagosomes

Salmonella-containing phagosomes were purified from untreated cells as well as from MDP- and MBSA-MDP-treated cells as described previously (Mukherjee et al., 2000; Hashim et al., 2000). Briefly, J774E macrophages were pretreated with MDP in the free or conjugated form (MDP equivalent, 1 μg/ml) for 12 hours at 37°C. Subsequently, macrophages were allowed to internalize *Salmonella* (1 macrophage: 10 *Salmonella*) for 5 minutes to restrict their entry primarily to the early compartment. Cells were washed five times with PBS and resuspended (2×10^8 cells/ml) in homogenization buffer (HB: 250 mM sucrose, 0.5 mM EGTA and 20 mM Hepes-KOH, pH 7.2 containing protease inhibitors) and homogenized in a ball-bearing homogenizer. Homogenates were centrifuged at a low speed (400 ***g*** for 5 minutes) at 4°C to remove nuclei and unbroken cells. The post-nuclear supernatants were centrifuged at 12,000 ***g*** for 6 minutes at 4°C to pellet the phagosomal fraction. Finally, the pellets were resuspended in 100 μl of HB and loaded on a 12% sucrose cushion. Samples were centrifuged at 1,700 ***g*** for 45 minutes at 4°C, and the purified phagosomes were recovered from the bottom of the tube. The phagosomes, thus purified, were free of plasma membrane, endosomes, lysosome, Golgi and endoplasmic reticulum contamination (Hashim et al., 2000). To determine the expression of endocytic Rabs on purified phagosomes, 40 μg of the respective phagosomes were subjected to 12% SDS-PAGE. The proteins on the gels were transferred onto nitrocellulose membranes and checked for the presence of transferrin receptor, Rab5 and Rab7 as described above.

Preparation of endosome

Early endosomes containing avidin-HRP were prepared as described previously (Mukherjee et al., 2000). Briefly, J774E macrophages were incubated with avidin-HRP (1 mg/ml) in internalization medium (MEM containing 10 mM HEPES and 5 mM glucose, pH 7.4) at 4°C for 1 hour to allow cell-surface binding. Internalization was carried

out by the addition of prewarmed medium and incubated for 5 minutes at 37°C to label the early endosomal compartment, and uptake was stopped by the addition of ice-cold medium. Cells were washed with ice-cold medium and homogenized in HB at 4°C, and post nuclear supernatants (PNS) were prepared and quickly frozen in liquid nitrogen. To prepare the enriched endosomal fraction, thawed PNS was diluted with HB (1:3) and centrifuged at 37,000 ***g*** for 1 minute at 4°C. The supernatant was again centrifuged at 50,000 ***g*** for 5 minutes at 4°C. The resultant pellet enriched in early endosomal vesicles was used for the in vitro fusion assay.

In vitro fusion assay

In vitro fusion of phagosomes containing the biotinylated *Salmonella* with early endosomes containing avidin-HRP was carried out using the procedure described previously (Mukherjee et al., 2000). Briefly, phagosomes isolated from untreated or treated cells were mixed with early endosomes in fusion buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM HEPES-KOH, pH 7.2, 1 mM dithiothreitol, 1.5 mM $MgCl_2$, 100 mM KCl, including an ATP regenerating system, 1 mM ATP, 8 mM creatine phosphate, 31 units/ml creatine phosphokinase and 0.25 mg/ml avidin as scavenger) supplemented with gel-filtered cytosol prepared from untreated or MBSA-MDP treated cells. Fusion was carried out for 10 minutes at 37°C, and the reaction was stopped by chilling on ice. The HRP-avidin-biotin bacterial complex was recovered by centrifugation (10,000 ***g*** for 5 minutes) after solubilization of the membrane in solubilization buffer (SB, PBS containing 0.5% Triton X-100 with 0.25 mg/ml avidin as scavenger). The enzymatic activity of avidin-HRP associated with the biotinylated bacteria was measured as a fusion unit. The maximum fusion between endosomes and phagosomes isolated from untreated control cells was observed at 0.5 mg/ml of normal cytosol concentration, which was expressed as 1 unit of relative fusion. HRP activity corresponding to 1 unit is mentioned in the figure legends.

Assay for transport to lysosomes

To determine the transport of *Salmonella* to the lysosomes in MBSA-MDP-treated cells, J774E cells (1×10^6 cells) were treated with MDP in the free or conjugated (MDP equivalent, 1 μg/ml) form for 12 hours at 37°C as described in a previous section. Transport of *Salmonella* from early to late lysosomes was detected using an assay described previously (Hashim et al., 2000). Briefly, J774E cells were incubated in the presence of avidin-HRP (200 μg/ml) at 4°C to allow binding. Subsequently, avidin-HRP was chased for appropriate time (90 minutes) at 37°C to label the lysosomes. After washing, cells were allowed to bind to biotinylated live or dead *Salmonella* (1×10^7 cells) at 4°C for 1 hour. Cells were resuspended in prewarmed medium and uptake was carried out for 5 minutes at 37°C to restrict internalization predominantly to the early compartment. Cells were washed three times to remove unbound bacteria by centrifugation at low speed (300 ***g*** for 6 minutes). Uninternalized surface-bound biotinylated bacteria were quenched by adding free avidin (0.25 mg/ml). Cells were washed twice and chased for the indicated time at 37°C. The reaction was stopped by chilling on ice, and the cells were solubilized in solubilization buffer (SB, PBS containing 0.5% Triton X-100 with 0.25 mg/ml avidin as scavenger). The HRP-avidin-biotin bacterial complexes in the lysates were recovered by centrifugation (10,000 ***g*** for 5 minutes). The enzymatic activity of avidin-HRP associated with the biotinylated bacteria was measured in relative transport units.

Reconstitution of phagosome-lysosome transport in permeabilized cells

To directly demonstrate the role of altered Rab5 and Rab7 content of MBSA-MDP-treated cells in lysosomal targeting of *Salmonella*, reconstitution of phagosome-lysosomes transport assay was carried out in permeabilized cells using a similar assay to one described previously (Funato et al., 1997). Briefly, lysosomes of J774E cells (1×10^6 cells) were loaded with avidin-HRP, and subsequently cells were allowed to bind to biotinylated live or dead *Salmonella* (1×10^7 cells) at 4°C for 1 hour followed by 5 minutes uptake at 37°C to restrict internalization predominantly to the early compartment as mentioned in a previous section (Hashim et al., 2000). Cells were washed three times to remove unbound bacteria. In order to permeabilize the cells using the freeze-thaw method (Klenchin et al., 1998), cells were resuspended in ice-cold permeabilzation buffer (10 mM K-phosphate, 120 mM KCl, 0.5 mM EGTA, pH 7.2 containing 50 μg/ml avidin) and quickly frozen in liquid nitrogen. Subsequently, cells were kept at –80°C for 12 hours. The cell suspension was thawed by warming the tubes at room temperature. Under these conditions more than 80% of the cells were permeabilized, as measured by the release of lactate dehydrogenase, a cytosolic marker. However, less than 5% of the HRP was released from the avidin-HRP-loaded lysosomes. After permeabilization, cells were incubated for 30 minutes at 4°C to deplete cytosol and gently washed twice with HB. Subsequently, endogenous Rab proteins from the cells were depleted using Rab-GDI along with GDP, as described earlier (Funato et al., 1997). Loading of reconstituted cytosol was carried out by incubating the cells at 4°C for 30 minutes in 40 μl of fusion buffer containing an ATP regenerating system in the presence of indicated cytosol (4 mg/ml) reconstituted with respective Rab protein (300 ng). Rab5 or Rab7 was immunodepleted from the respective macrophage cytosol as described previously (Mukherjee et al., 2000), and Rab proteins were preincubated with cytosol in the fusion buffer at room temperature for 30 minutes for in vitro prenylation (Lombardi et al., 1993). Finally, cells were incubated for 60 minutes at 37°C to allow transport to the lysosomes. The reaction was stopped by chilling on ice, and the cells were solubilized in solubilization buffer (SB, PBS containing 0.5% Triton X-100 with 0.25 mg/ml avidin as scavenger). The HRP-avidin-biotin bacterial complexes in the lysates were recovered by centrifugation (10,000 ***g*** for 5 minutes). The enzymatic activity of avidin-HRP associated with the biotinylated bacteria was measured in relative transport units.

Determination of *Salmonella* transport to lysosome in MBSA-MDP-treated cells by confocal microscopy

To confirm the transport of *Salmonella* to the lysosomes in MBSA-MDP-treated cells, J774E cells (1×10^6) were plated on sterile glass coverslip placed in six-well tissue culture plate and treated with MBSA-MDP (MDP equivalent, 1 μg/ml) for 12 hours at 37°C as described in the previous section. Subsequently, treated or untreated J774E cells (1×10^6 cells) were incubated with GFP-*Salmonella typhimurium* (1×10^7 cells) for 10 minutes at 37°C to restrict their entry to the early compartment. Cells were washed three times to remove unbound bacteria and chased for another 90 minutes at 37°C. During the last 30 minutes of the chase, Lysotracker Red (100 nM) was added to GFP-*Salmonella*-infected macrophages to label the lysosomes. Cells were washed three times with cold PBS, and confocal microscopy was carried out using a Zeiss, LSM 510 confocal microscope using an oil immersion objective.

Results and Discussion

SCR-mediated targeting using maleylated bovine serum albumin (MBSA) as a carrier has been exploited extensively for intracellular delivery of pharmacologically active molecules for modulating macrophage function (Mukhopdhyay et al., 1989; Majumdar et al., 1991). To determine the effect of MBSA-MDP on *Salmonella*-infected macrophages, MBSA was conjugated with MDP using a water-soluble carbodiimide (Srividya et al., 2000). Subsequently, J774E macrophages were

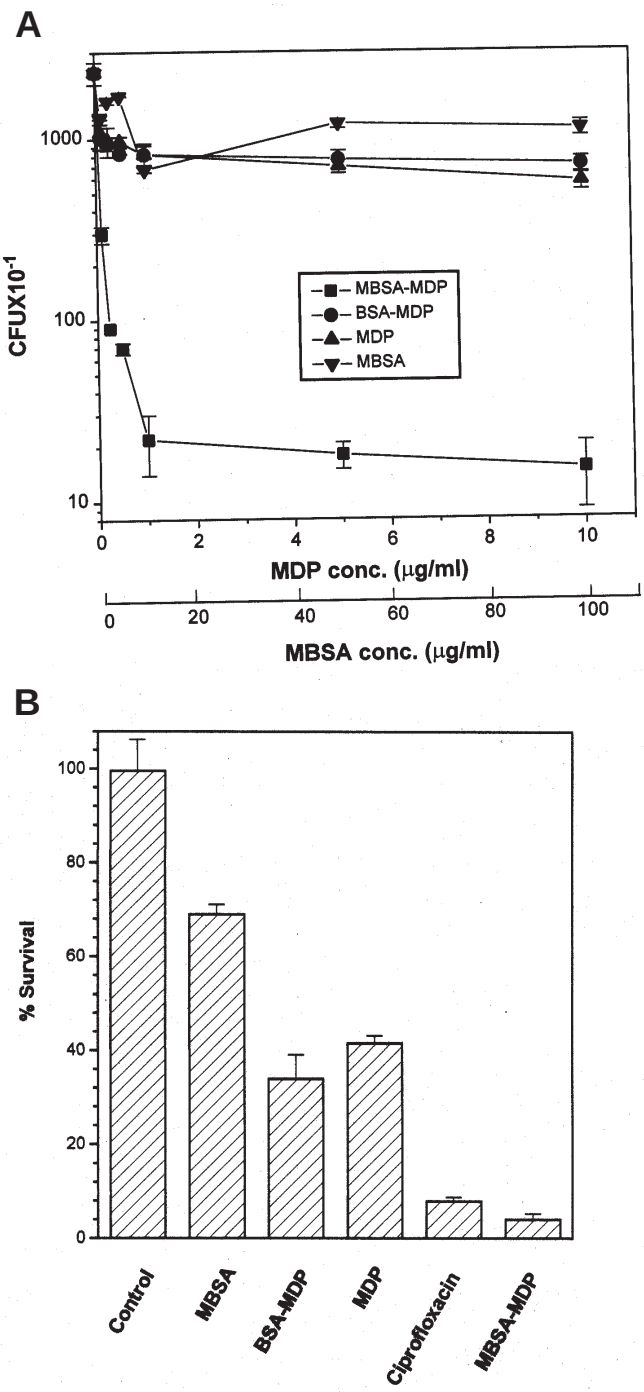

Fig. 1. MBSA-MDP-mediated killing of *Salmonella* in vitro and in vivo. (A) J774E macrophages (1×10^6 cells/well) were treated with the indicated concentrations of MBSA-MDP or free MDP for 12 hours. Treated macrophages were infected with *Salmonella typhimurium* (1×10^7) as described in the Materials and Methods, and the infected cells were incubated in the respective drug-containing medium. After incubation for 12 hours at 37°C, the macrophages were lysed in solubilization buffer, and an aliquot of the cell lysates was used to determine the level of viable bacteria they contain (colony-forming units). Results are expressed as an average of three determinations±s.d. (B) To determine the efficacy of MBSA-MDP for treatment of *Salmonella* infection in vivo, C57Bl-6 mice were infected with *Salmonella* as described in the Materials and Methods on day 0. Subsequently, *Salmonella*-infected animals received intraperitoneal injections of free MDP or MBSA-MDP (1 μg/mouse of MDP equivalent; 50 μg/kg body weight) or ciprofloxacin (1.4 mg/mouse; 70 mg/kg body weight) daily for four consecutive days. Finally, the splenic load of *Salmonella* was determined by measuring the colony forming units on day 10 as described in the Materials and Methods. Results are expressed as percentage survival of three independent experiments±s.e. The number of colonies in untreated control mice (2.21×10^4 CFU±s.e.) was taken as 100%.

cultured in the presence of various concentrations of MBSA-MDP or free MDP for 12 hours, then washed and infected with *Salmonella typhimurium* at a ratio of 1:10 (macrophage:*Salmonella*). The infected macrophages were washed to remove the unbound bacteria and incubated in the respective drug-containing medium for 12 hours. Subsequently, the cells were lysed, and the number of viable *Salmonella* was determined by their colony-forming ability. Data in Fig. 1A show that about 91% of the intracellular *Salmonella* were eliminated by 0.1 μg/ml of MBSA-MDP (MDP equivalent) treatment, whereas a 100-fold higher concentration of free MDP (10 μg/ml) eliminated only 70% of the bacteria, indicating that MBSA-MDP is nearly 100 times as effective as free MDP. Similar results were obtained when macrophages infected with *Salmonella* were treated with MBSA-MDP, indicating that the enhanced killing is caused by intracellular delivery of MDP (data not shown). MBSA-MDP or free MDP treatment did not affect the viability of macrophages, as determined by MTT assay; they also did not induce the release of lactate dehydrogenase, a cytosolic marker (data not shown).

To determine the efficacy of MBSA-MDP for treatment of *Salmonella* infection in vivo, C57Bl-6 mice were infected with 10^3 *Salmonella* per mouse as described previously (Pashine et al., 1999). The animals received intraperitoneal injections of free or MBSA-MDP (1 μg/ mouse of MDP equivalent; 50 μg/kg body weight) or ciprofloxacin (1.4 mg/ mouse; 70 mg/kg body weight) daily for four consecutive days. On day 10, the mice were sacrificed to determine the content of viable *Salmonella* in the spleen (Pashine et al., 1999). Data in Fig. 1B show that administration of MBSA-MDP (50 μg/kg body weight) eliminated about 96% of the bacteria from the infected spleen, whereas free MDP at the same dose eliminated only 60% of the *Salmonella*. In comparison, the therapeutic dose (70 mg/kg body weight) of ciprofloxacin, which is about 1500-fold higher than the MDP concentration in the conjugate, removed about 90% of the *Salmonella* from the mice. These results are consistent with the previous observations that macrophages activated by MDP augment the host defense against various infections (Koff et al., 1985; Sarkar et al., 1997), but the mechanism of killing of intracellular pathogens by MDP-mediated macrophage activation is not known.

Recently, we reported that intracellular delivery of MDP to macrophages through scavenger receptor (SCR)-mediated endocytosis triggers the secretion of different cytokines (Srividya et al., 2000). Several independent studies have shown that activation of macrophages through different cytokines alone or in concert can contribute to the antimycobacterial potential of these cells, resulting in the control of infection in vitro (Rook et al., 1986; Chan et al., 1992; Flynn et al., 1995; Schaible et al., 1998; Via et al., 1998). However, the

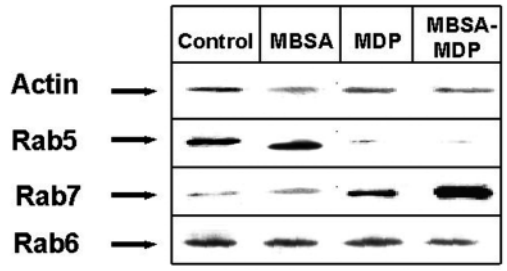

Fig. 2. Detection of the content of Rab5 and Rab7 in MBSA-MDP-treated cells. J774E cell monolayers were incubated with free or conjugated MDP (MDP equivalent, 1 μg/ml) for 12 hours at 37°C in RPMI-1640 medium. An equivalent number of cells (80 μg of protein each) was solubilized in SDS buffer, boiled and subjected to SDS-PAGE. Western blot analyses were carried out for the detection of actin, Rab5, Rab7 and Rab6 in untreated, MBSA-MDP- or MDP-treated cells. Proteins were visualized using an appropriate HRP-labelled second antibody using ECL. Results from western blots are representative of three independent preparations.

mechanism of killing of *Mycobacterium* by cytokine-activated macrophages is not known. In view of the reports that IFNγ alters the expression of Rab5 (Alvarez-Dominguez et al., 1998), and Rabs are the major regulators of intracellular transport, we investigated whether the intracellular delivery of MDP, which induces the secretion of different cytokines, modulates the level of endocytic Rabs. Western blot analysis of the cell lysate with specific antibodies after 12 hours of treatment with MDP or MBSA-MDP revealed that MBSA-MDP treatment resulted in a significant increase in the level of Rab7 and a decrease in the content of Rab5 in the macrophages compared with free MDP treatment (Fig. 2). By contrast, content of the Golgi-associated Rab6 (Mallard et al., 2002) remained unaltered with MBSA-MDP treatment, suggesting that the effects seen with MBSA-MDP might be restricted to endocytic Rabs like Rab5 and Rab7. However, treatment with MBSA alone did not alter the level of endocytic Rab proteins in the cells, suggesting that the observed enhanced effect of MDP in MBSA-MDP is caused by SCR-mediated uptake of more MDP than fluid phase uptake of free MDP (Fig. 2). As Rab7 is the targeting signal to the late lysosomal compartment (Mukhopadhyay et al., 1997a), enhanced expression of Rab7 in MBSA-MDP-treated cells probably explains the enhanced killing of *Salmonella* in macrophages by MBSA-MDP treatment.

To investigate the consequences of the altered cellular levels of Rab5 and Rab7 in the maturation of *Salmonella*-containing phagosomes, we purified early (5 minutes) LSP from untreated cells (Mukherjee et al., 2000) as well as from MBSA, MDP and MBSA-MDP-treated cells and measured their levels of Rab5 and Rab7 by western blot analysis. Data in Fig. 3A show that LSP from untreated control cells display significant levels of Rab5 and little Rab7, whereas those from MBSA-MDP-treated cells primarily display Rab7 and almost no Rab5. LSP isolated from free MDP-treated cells, however, showed substantially less Rab7 than those from MBSA-MDP-treated cells. The higher content of Rab5 on LSP compared with the total Rab5 content in MDP-treated cells is due to SopE, a secretory protein of *Salmonella*, which mediates recruitment of Rab5 on LSP (Mukherjee et al., 2000; Mukherjee et al., 2001). Since the contents of viable bacteria were similar in early LSP isolated from the control cells and cells treated with MBSA, MDP or MBSA-MDP (data not shown), enhanced recruitment of Rab7 by LSP in MBSA-MDP-treated cells is not caused by the differential viability of the bacteria. Possibly, higher expression of Rab7 in MBSA-MDP-treated cells than in untreated or MDP-treated cells correlates with the SCR-mediated enhanced uptake of MDP by macrophages (Mukhopadhyay et al., 1989; Majumdar et al., 1991). It is tempting to speculate that the enhanced secretion of various cytokines by MBSA-MDP- treated cells (Srividya et al., 2000) regulates the expression of the Rab proteins. This is supported by a recent finding that macrophages treated with IFN-γ induced Rab5 expression (Alvarez-Dominguez et al., 1998). However, macrophages treated with MBSA-MDP do not secrete IFN-γ (data not shown), which is in agreement with the fact that IFN-γ is exclusively secreted by NK cells and certain subpopulations of T lymphocytes (Billiau et al., 1996). Furthermore, reduced Rab5 content in MBSA-MDP-treated macrophages did not alter the uptake of *Salmonella* (data not shown), suggesting that the increased Rab7 content of MBSA-MDP-treated cells presumably compensates for the endocytic function of Rab5, which is consistent with the report that injection of Rab7 alone into frog oocytes stimulated HRP uptake (Mukhopadhyay et al., 1997a).

Macrophages are phagocytic cells that usually target the invading microorganisms to the lysosomes where the lysosomal hydrolases in the acidic compartment degrade them. By contrast, morphological studies have shown that *Salmonella* bypass the M-6-P-receptor-positive compartment (Portillo and Finlay, 1995) and reside in a unique compartment that clearly diverges from the degradation pathway of the macrophages (Rathman et al., 1997; Buchmeier and Heffron, 1991; Alpuche-Aranda et al., 1992). But, the molecular mechanism for inhibition of *Salmonella* transport to lysosomes was not known. Recently, we have shown that live *Salmonella*-containing phagosomes (LSP) transport a bacterial protein, SopE, on the surface of phagosomes and thereby recruit the early acting Rab5 and fusion factors like α-SNAP and NSF to promote fusion with early endosomes (Mukherjee et al., 2000; Mukherjee et al., 2001). *Salmonella* persist in a specialized low-acidity compartment lacking active lysosomal enzymes and transferrin receptors but retaining Rab5 and Rab18 (Hashim et al., 2000). Thus, SopE-mediated recruitment of Rab5 on LSP, which promotes fusion with early endosomes, is the major mechanism by which *Salmonella* survive in macrophages. Therefore, the downregulation of Rab5 expression in MBSA-MDP-treated cells might prevent the interaction of *Salmonella*-containing phagosomes with early endosomes, whereas simultaneous upregulation of Rab7 could induce the transport of *Salmonella*-containing phagosomes to the lysosomes. The data presented in Fig. 3B show that the extent of in vitro fusion of early endosomes with LSP isolated from MBSA-MDP-treated cells in the presence of cytosol prepared from MBSA-MDP-treated cells is significantly lower than that observed with LSP isolated from untreated control cells in the presence of normal cytosol. This suggests that the reduced content of Rab5 in MBSA-MDP-treated cells inhibits fusion with early endosomes, thus subverting the primary survival mechanism of *Salmonella* (Mukherjee et al., 2000; Hashim et al., 2000; Mukherjee et al., 2001).

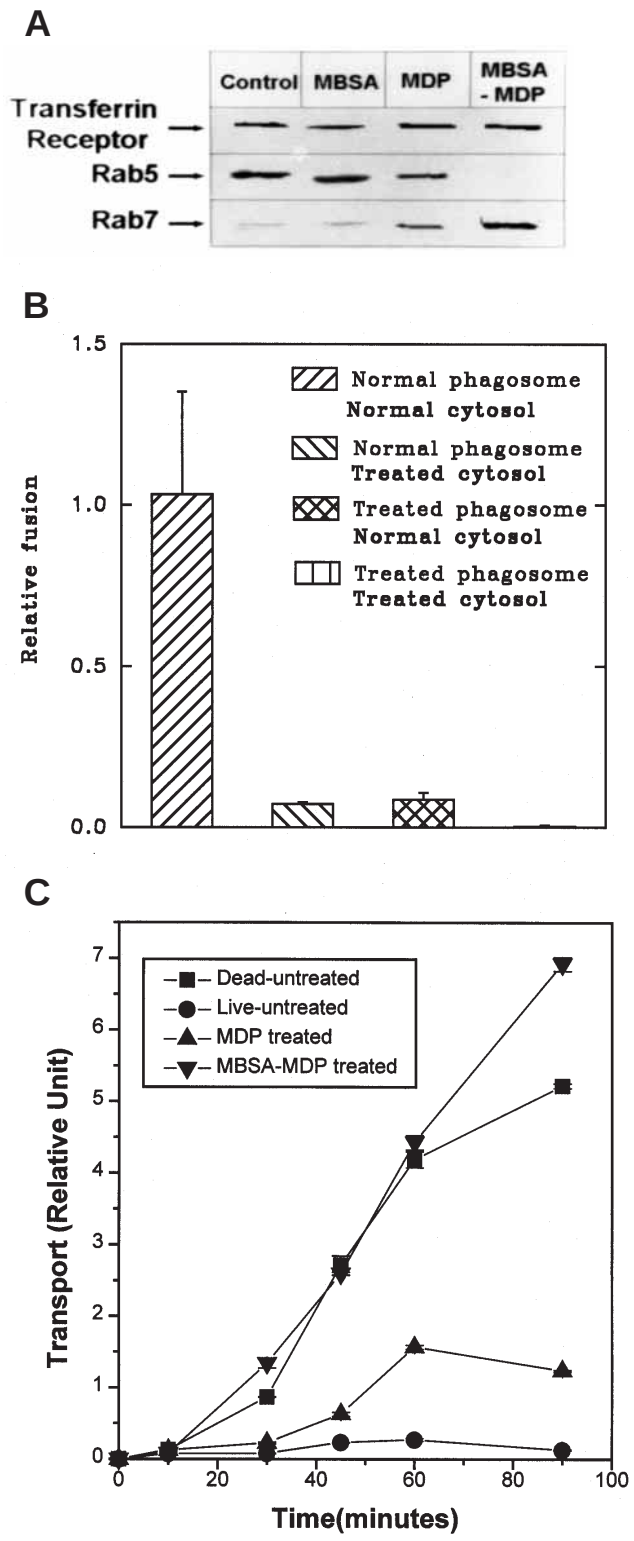

Fig. 3. Intracellular transport of *Salmonella* in MBSA-MDP-treated cells. (A) *Salmonella*-containing early phagosomes were purified from untreated cells as well as from MDP and MBSA-MDP-treated cells as described in the Materials and Methods. Proteins from the respective phagosomes (40 μg each) were separated by SDS-PAGE and transferred to nitrocellulose membranes. After incubation with appropriate dilutions of specific antibodies, the proteins on the membranes were visualized using an appropriate HRP-labelled second antibody using ECL. Results from western blots are representative of three independent preparations. (B) In vitro fusion of early endosomes with *Salmonella*-containing phagosomes isolated from untreated or MBSA-MDP-treated cells. Fusion between early endosomes with respective *Salmonella*-containing phagosomes isolated from untreated or MBSA-MDP treated cells was carried out either in the presence of normal cytosol or cytosol prepared from MBSA-MDP-treated cells as indicated. Fusion was measured as described in the Materials and Methods. Maximum fusion between endosomes and phagosomes isolated from untreated control cells was observed at 0.5 mg/ml of normal cytosol concentration, which was normalized to one unit. The results are expressed as relative fusion from three independent experiments±s.d. One unit corresponds to 10.2 ng of HRP activity/mg of protein. (C) Intracellular transport of live *Salmonella* to the lysosomes in MBSA-MDP-treated cells. To determine the transport of *Salmonella* to the lysosomes by MBSA-MDP-treated cells, J774E cells (1×10^6 cells) were treated with MDP in the free or conjugated form for 12 hours at 37°C as described earlier. Subsequently, J774E macrophages were preloaded with avidin-HRP and chased for 90 minutes to label the lysosomes. Cells were pulsed with live or dead biotinylated *Salmonella* at 37°C for a short period of time (5 minutes) to restrict their entry to the early compartment followed by a chase as described in the Materials and Methods. At indicated times the formation of the bacteria biotin-avidin-HRP complex was measured to determine the transport of the *Salmonella* to lysosomes. Each point represents the mean±s.d. from three independent experiments and is expressed as relative units of transport to lysosomes.

To determine whether the enhanced content of Rab7 in macrophages induced by MBSA-MDP leads to enhanced lysosomal targeting of live *Salmonella*, we studied the transport of biotinylated live or dead bacteria to the lysosomes preloaded with avidin-HRP as described previously (Hashim et al., 2000). The results presented in Fig. 3C show that in MBSA-MDP-treated macrophages live biotinylated *Salmonella* colocalized with avidin-HRP-loaded lysosomes at a rate similar to that of dead biotinylated bacteria in untreated macrophages, whereas transport of live biotinylated *Salmonella* to the lysosomes is inhibited in untreated macrophages (Hashim et al., 2000). In free MDP-treated cells, by contrast, only ~20% of the live biotinylated *Salmonella* were transported to the lysosomes during the same interval (90 minutes).

Intracellular delivery of MDP may modulate several signal transduction molecules. To directly demonstrate that the altered Rab5 and Rab7 content of MBSA-MDP-treated cells is sufficient to drive the killing of *Salmonella* by lysosomal targeting, reconstitution of the phagosome-lysosome transport assay was carried out in permeabilized cells (Klenchin et al., 1998) in the presence of different combinations of in vitro prenylated Rab5 and Rab7 proteins. The results presented in Fig. 4 show that dead biotinylated *Salmonella* are transported to avidin-HRP-loaded lysosomes, whereas transport of live *Salmonella* is significantly inhibited in the presence of cytosol prepared from normal macrophages. Interestingly, when a similar assay was carried out in the presence of Rab5-immunodepleted cytosol, transport of the live bacteria to the lysosomes was partially induced in comparison with that in normal cytosol. These results suggest that the reduced content of Rab5 in the depleted cytosol might inhibit the fusion of LSP with the endosome, and endogenous Rab7 is probably

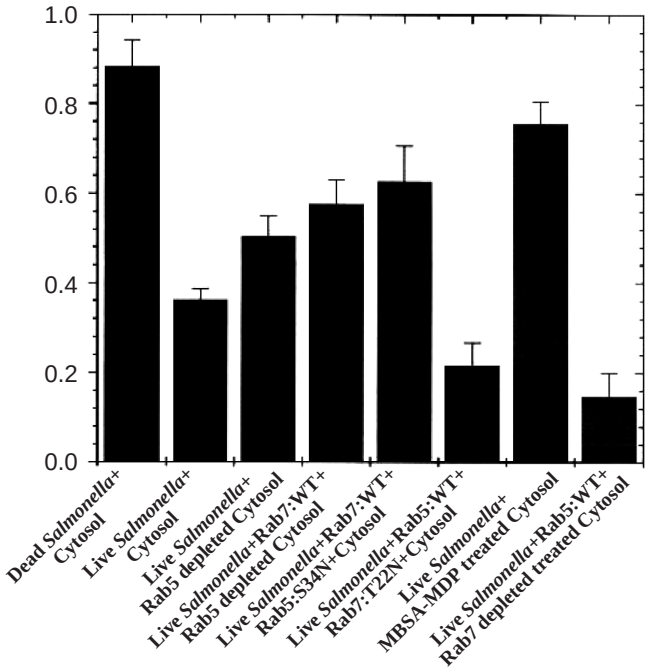

Fig. 4. Reduced Rab5 and enhanced Rab7 content of MBSA-MDP-treated cells is sufficient to drive lysosomal targeting. To determine the role of Rab5 and Rab7 content of MBSA-MDP-treated cells in lysosomal targeting of *Salmonella*, reconstitution of the phagosome-lysosomes transport assay was carried out in permeabilized cells in the presence of cytosol prepared from MBSA-MDP-treated cells or untreated cells containing in vitro prenylated Rab proteins as described in the Materials and Methods. Cells were incubated for 60 minutes at 37°C to allow transport. Subsequently, formation of the bacteria biotin-avidin-HRP complex was measured to determine the transport of the *Salmonella* to lysosomes. Each point represents the mean±s.d. from three independent experiments, and these are expressed as relative units of transport to lysosomes.

responsible for the partial transport of *Salmonella* to the lysosomes. However, the transport of *Salmonella* to the lysosome in Rab5-depleted cytosol was relatively low in comparison with MBSA-MDP-treated cytosol mainly because of the enhanced content of Rab7 in treated cells. This is further supported by the fact that addition of Rab7:WT in Rab5-depleted cytosol induced more transport of *Salmonella* to the lysosome. Moreover, transport of the live *Salmonella* to the lysosomes was significantly induced, almost to the extent observed in the presence of cytosol prepared from MBSA-MDP-treated cells, when reconstitution of transport was carried out in the presence of cytosol containing Rab5-S34N, a negative mutant of Rab5 locked in GDP form (Li and Stahl, 1993) along with Rab7:WT protein (Fig. 4). These results indicate that the reduced content of Rab5 and enhanced level of Rab7 in MBSA-MDP-treated cells are probably responsible for transport of *Salmonella* to the lysosomes. By contrast, transport of live *Salmonella* to the lysosomes was significantly blocked when reconstitution was carried out in the presence of cytosol containing Rab5:WT and Rab7:T22N, a negative mutant of Rab7 locked in the GDP form (Mukhopdhyay et al., 1997a). In addition, transport of *Salmonella* to the lysosome was significantly blocked in comparison with MBSA-MDP-treated cytosol when the transport assay was carried out in the presence of Rab7-immunodepleted cytosol supplemented with Rab5:WT protein (Fig. 4). These results unequivocally prove

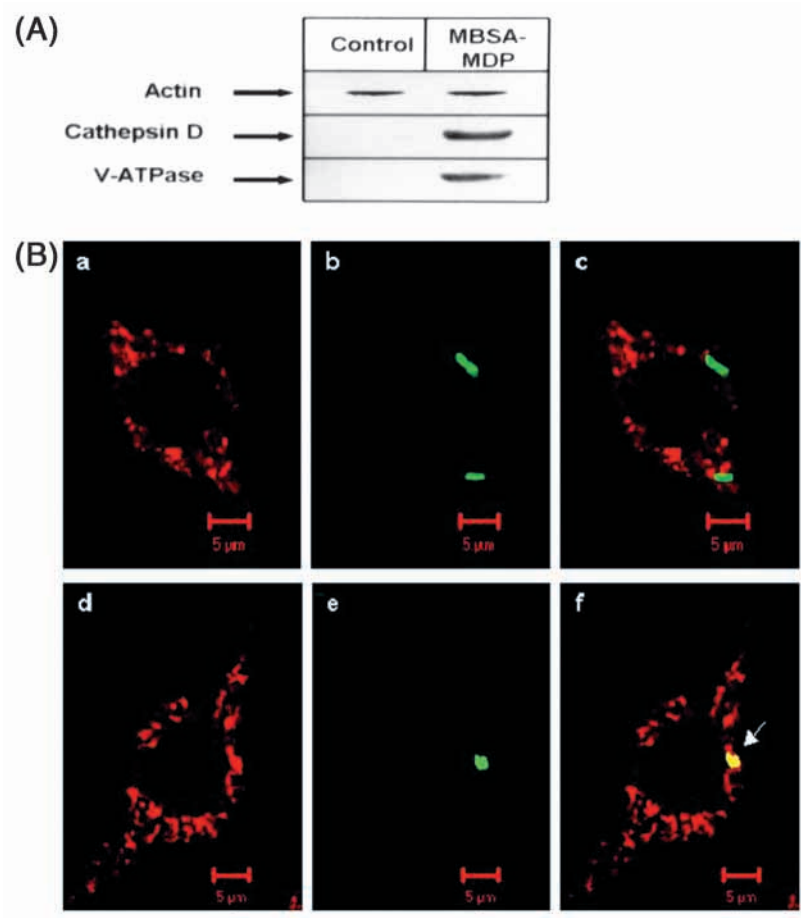

Fig. 5. MBSA-MDP induced the transport of *Salmonella* to the lysosomes. (A) To determine the nature of mature LSP, LSP were purified from untreated cells as well as from MBSA-MDP-treated cells as described in the Materials and Methods after 90 minutes of internalization in J774E cells. LSP were washed and the content of actin, cathepsin D and V-ATPase on respective LSP (40 μg each) was determined by western blot analysis using specific antibodies. Results from western blots are representative of three independent preparations. (B) Confocal images showing the GFP-*Salmonella* colocalization with Lysotracker-Red-labelled lysosomes in untreated (a-c) and MBSA-MDP-treated J774E cells (d-f). Untreated or MBSA-MDP-treated J774E cells were infected with GFP-*Salmonella typhimurium* and chased for 90 minutes to determine their colocalization with Lysotracker-Red-labelled lysosomes as described in the Materials and Methods. Lysotracker-Red-labelled lysosomes appear in the red channel (a,d); green channel shows the GFP-*Salmonella* (b,e); and yellow indicates the colocalization of GFP-*Salmonella* with Lysotracker-labelled lysosomes in merged images (c,f).

that enhanced content of Rab7 and reduced level of Rab5 in MBSA-MDP-treated cells is responsible for the induced transport of *Salmonella* to the lysosomes. Although, intracellular delivery of MDP may activate different signalling cascades, our results clearly demonstrate that downregulation of Rab5 in parallel with upregulation of Rab7 triggered by intracellular delivery of MDP is sufficient to target LSP to the lysosomes.

LSP isolated from MBSA-MDP-treated macrophages 90 minutes after internalization showed enhanced levels of cathepsin D and vacuolar ATPase in comparison with LSP isolated from untreated cells, indicating that in MBSA-MDP-treated cells the bacteria are transported to a fully competent lysosomal compartment containing lysosomal enzymes and vacuolar ATPase (Fig. 5A). However, in HeLa cells overexpression of Rab7 alone was reported to target *Salmonella* to a compartment that is positive for LAMP-1 and Rab7 but lacks lysosomal enzymes like cathepsin D (Meresse et al., 1999). Whereas, Rab7 overexpression in HeLa cells failed to target *M. Tuberculosis* and *L. pneumophila* to a LAMP1-containing compartment (Clemens et al., 2000). Although Rab 7 is the signal to transport the cargo to the lysosomes, the overexpression of Rab7 alone is unable to transport these microorganisms to fully competent lysosomes. This may be because of the bacteria-driven mechanism, which arrests their transport to the lysosomes. Finally, the results presented in Fig. 5B clearly demonstrate that GFP-*Salmonella* colocalized with the Lysotracker-Red-labelled lysosomal compartment in MBSA-MDP-treated cells (Fig. 5Bf), whereas GFP-*Salmonella* in untreated cells resides in a compartment that is not labelled with Lysotracker Red (Fig. 5Bc). It is pertinent to mention that more than 80% of the GFP-*Salmonella* colocalized with the Lysotracker-Red-labelled lysosomal compartment in MBSA-MDP-treated cells, whereas less than 10% of the GFP-*Salmonella* colocalized with the Lysotracker-Red-labelled compartment in untreated cells. Lysotracker Red was shown to accumulate in the compartment that labelled with Rab7 and LAMP1 antibodies, which is a characteristic feature of the late lysosomal compartment (Schaible et al., 1998; Magez et al., 1997; Wubbolts et al., 1996). Therefore, our data suggest that *Salmonella* is transported to fully competent lysosomes in MBSA-MDP-treated macrophages for efficient killing.

In conclusion, this is the first demonstration that intracellular delivery of MDP leads to simultaneous reduction of Rab5 and enhancement of Rab7. Reduction in Rab5 levels inhibits the fusion of LSP with early endosomes, subverting the mechanism by which *Salmonella* resist transport to the lysosomes, whereas Rab7, being involved in the transport of vesicles towards the late/lysosomal compartments, enhanced the content of Rab7 in the cells and presumably targets the *Salmonella* to the lysosomes for eventual destruction. Currently, we are trying to understand the mechanism by which MBSA-MDP-mediated enhanced secretion of different cytokines regulates the expression of Rab proteins. This novel approach of modulating the cellular contents of endocytic Rabs to ensure lysosomal targeting and destruction might be generally useful in combating intracellular pathogens, such as *Salmonella typhi*, *M. tuberculosis*, *L. pneumophilia* and *Toxoplasma gondii* that normally survive in the host cell by resisting transport to lysosomes.

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