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SopE-Mediated Recruitment of Host Rab5 on Phagosomes Inhibits *Salmonella* Transport to Lysosomes

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Summary

Phagocytosis is a process by which invading organisms are taken up by macrophages and targeted to the lysosomes, where they are degraded. However, many pathogens modulate this central process of macrophage-mediated killing by inhibiting their transport to the lysosomes through a variety of pathogen-derived mechanisms. Given the importance of Rab proteins in the regulation of intracellular transport pathways, we investigated the role of different host endocytic Rabs on the maturation of *Salmonella*-containing phagosomes in macrophages. Initially, we have developed a ligand mixing assay to measure the transport of the *Salmonella*-containing phagosomes to lysosomes. Using this assay we have shown that *Salmonella* decline their transport to the lysosomes. In order to determine whether inhibition of *Salmonella* transport to lysosomes is due to their sustained fusion with early endosomes, we have developed an in vitro fusion assay between *Salmonella*-containing phagosomes and early endosomes. Here, we have discussed how these methodologies are helpful to determine the mechanism of evasion of *Salmonella* transport to the lysosomes.

Key Words: *Salmonella*; phagosome; lysosomes; transport; endosome; fusion; Rab5; SopE.

1. Preface

Targeting of invading microorganisms to lysosomes after internalization by phagocytes is a major tool host cells use to protect themselves. This defense mechanism is often countered by specific pathogens through various mechanisms ***(1)***. For example, it has been shown that some pathogens, although

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targeted to the lysosomes, inhibit the function of lysosomal enzymes, e.g., *Leishmania* ***(2)***; pathogens like *Listeria* and *Trypanosoma* lyse the phagosomal membrane and reside in the cytosol of the infected host cells ***(3,4)*** or they simply inhibit their transport to the lysosomes and survive in a specialized endocytic compartment, e.g., *Mycobacterium* and *Salmonella* ***(5–7)***. These results suggest that pathogens somehow alter the host trafficking mechanisms and thereby they evade lysosomal transport. Current knowledge about the regulation of intracellular trafficking of the internalized cargo suggests that the transport of cargo from donor compartment to the acceptor compartment is tightly regulated by specific Rab GTPases along with their interacting proteins ***(8)***. These proteins are localized on a particular compartment and mediate transport between two specific vesicles ***(9–12)***. Thus, interference with these regulatory proteins by exogenous effector molecules from pathogens might perturb the normal cellular transport process.

Our laboratory has been trying to understand the mechanisms *Salmonella* uses to evade the degradative pathway of the host cell. We hypothesized that if *Salmonella*-containing phagosomes somehow interact constitutively with early endosomal compartment, this might inhibit their transport to the lysosomes in macrophages. Thus, we have developed some novel in vitro reconstitution assays to study the interactions between *Salmonella*-containing phagosomes and various endocytic compartments namely, early endosomes and lysosomes. The present chapter deals with these methods and describes how these methods are useful to dissect out the intracellular route of *Salmonella* in macrophages. Using these methods, we have demonstrated that a *Salmonella* effector protein, SopE, recruits the host Rab5 on live *Salmonella*-containing phagosomes and promotes their fusion with early endosomes. These events constitute the basis by which *Salmonella* survive in the host cells by inhibiting their transport to the lysosomes.

2. Materials

Unless otherwise stated, all reagents are obtained from Sigma Chemical Co. (St. Louis, MO) and bacterial culture media are purchased from Difco, France. Tissue culture supplies were obtained from the Grand Island Biological Co. (Grand Island, NY). *N*-Hydroxy succicinimidobiotin (NHS-biotin), avidin-horseradish peroxidase (AHRP), avidin, and bicinchoninic acid (BCA) reagents were purchased from Pierce Biochemicals (Rockford, IL).

Cells are routinely counted using a hemocytometer (Neubauer chamber, depth 0.1 mm, 1/400 mm^2; ROHEM, India). Teflon cell scrapers (Falcon, 25 cm handle and 1.8 cm blade) were purchased from Becton-Dickinson (Franklin Lakes, NJ).

2.1. Transport of Salmonella-Containing Phagosomes to Lysosomes

1. The virulent wild-type *S. typhimurium* strain (a clinical isolate from Lady Harding Medical College, New Delhi, India) and the auxotrophic mutant, aro A, of *S. typhimurium* (SL3235 from Dr. K. Sanderson of *Salmonella* Genetic Stock Centre, Calgary, Canada) were both obtained from Dr. Vineeta Bal of National Institute of Immunology, New Delhi, India.
2. Luria broth (LB): 10 g of pancreatic digest of casein, 5 g of yeast extract, and 10 g of sodium chloride is dissolved in 1 L of distilled water and pH is adjusted to 7.0.
3. *Salmonella* and *Shigella* agar (SS Agar): 5 g of bacto beef extract, 5 g of bacto proteose peptone, 10 g of bacto lactose, 8.5 g of bacto bile salt No.3, 8.5 g of sodium citrate, 8.5 g of sodium thiosulfate, 10 g of ferric citrate, 13.5 g of bacto agar, 0.33 mg of brilliant green, and 0.025 g of neutral red dissolved in 1 L of distilled water and pH is adjusted to 7.0. The medium is boiled for 1 min and 25 mL is poured into each Petri dish (94/16 mm).
4. Phosphate-buffered saline (PBS): 10 m*M* sodium phosphate buffer, pH 7.0 containing 150 m*M* NaCl.
5. J774E, a well-characterized mannose receptor positive mouse macrophage cell line kindly provided by Dr. Philip Stahl (Washington University School of Medicine, St. Louis, MO).
6. Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 10% (v/v) fetal calf serum (FCS, Biological Industries, Israel) and gentamycin (Gibco BRL, NY) at a concentration of 50 μg/mL.
7. Hanks balanced salt solution (Gibco BRL, NY) pH 7.4 containing 10 m*M* hydroxyethyl piperazine sulfonate (HEPES), 10 m*M* TES, 10 mg/mL bovine serum albumin (HBSA).
8. Solubilization buffer (SB): PBS containing 0.5% Triton X-100.
9. Substrate buffer: 0.05 *N* sodium acetate buffer, pH 5.0 containing O-phenylenediamine (0.75 mg/mL) and 0.006% H_2O_2.

2.2. Fusion of Salmonella-Containing Phagosomes with Early Endosomes

1. Internalization medium (IM): minimum essential medium (Gibco BRL, NY) supplemented with 10 m*M* HEPES and 5 m*M* glucose. pH is adjusted to 7.4 and filtered through a 0.2 μm filter.
2. Cells are homogenized by forcing the cell suspension via attached syringes through a stainless steel ball-bearing homogenizer ***(13)*** that has the advantage of permitting rapid and reproducible breakage of the cells.
3. Homogenization buffer (HB): 250 m*M* sucrose, 0.5 m*M* EGTA, 20 m*M* HEPES. pH is adjusted to 7.2 with KOH.
4. Protease Inhibitor cocktail (Roche, Germany): One tablet dissolved in 2 mL Milli-Q water to prepare a 25X stock solution. Store at –20°C. Use at a final concentration of 1X.

5. G-25 Sepharose (Amersham Biosciences, UK) powder is dispersed in several volumes of Milli-Q water and stored at 4°C to enable swelling of the matrix.
6. Fusion buffer (FB): 250 m*M* sucrose, 0.5 m*M* EGTA, 20 m*M* HEPES-KOH, pH 7.2, 1 m*M* dithiothreitol, 1.5 m*M* $MgCl_2$, 100 m*M* KCl, including an ATP-regenerating system, 1 m*M* ATP, 8 m*M* creatine phosphate, 31 units/mL creatine phosphokinase, and 0.25 mg/mL avidin as the scavenger.

2.3. Recruitment of Host Rab5 on Salmonella-Containing Phagosome

1. SDS sample buffer: 60 m*M* Tris-HCl, pH 6.8 containing 2% sodium dodecyl sulfate (w/v), 10% glycerol (v/v), 3% β-mercaptoethanol (v/v), 0.001% bromophenol blue (w/v).
2. Transfer buffer (TB): Tris-glycine buffer, 25 m*M* Tris, 200 m*M* glycine containing 20% methanol.
3. Nitrocellulose membrane for protein transfers: 0.45 μm (BioRad, CA).
4. Photographic film for capturing ECL signals purchased from Konika X-ray Film, Goa, India.
5. Antibodies for Western blotting: A mouse IgG monoclonal antibody, 4F11, specific to the C-terminus of mouse Rab5 ***(14)*** and affinity purified rabbit anti-Rab7 polyclonal antibody were kind gifts from Dr. A. Wandinger-Ness (University of New Mexico, Albuquerque, NM). Mouse anti-transferrin receptor antibody was purchased from Zymed, San Francisco. HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).
6. Antibodies for electron microscopy: A rabbit polyclonal anti-Rab5 antibody was a gift from Dr. J. Gruenberg (EMBL, Heidelberg, Germany) and goat anti-rabbit IgG conjugated with 20 nm colloidal gold was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA).
7. Grids for electron microscopy were purchased from Electron Microscopy Sciences, Washington.
8. Enhanced ChemiLuminiscence (ECL) reagent was purchased from Amersham Biosciences, UK, and used according to manufacturer's instructions.

2.4. SopE, a Type III Secretary Protein of Salmonella, Binds Host Rab5

1. The plasmid construct for expression of GST-Rab5 was received as a kind gift from Dr. Philip Stahl (Washington University School of Medicine, St. Louis, MO). GST-Rab5 is expressed and purified from *E. coli* using glutathione-agarose beads (Sigma Chemical Co.) according to manufacturer's instructions.
2. Anti-*Salmonella* antibodies (anti-SopE, anti-SopB, and anti-SipC) were kindly provided by Dr. E. E. Galyov from Institute of Animal Health (Berkshire, UK).

3. Methods

Several intracellular pathogens modulate the regulatory molecules of the host endocytic pathway to evade targeting to lysosomes. In order to determine whether *Salmonella* inhibits their transport to the lysosomes to survive in macrophages, we have initially developed an in vitro ligand mixing assay to measure the kinetics of transport of live or dead *Salmonella* to lysosomes. Using this assay, we have shown that live *Salmonella* avoid their transport to the lysosomes. Inhibition of transport of *Salmonella*-containing phagosomes to the lysosomes is due to the enhanced fusion of live *Salmonella*-containing phagosomes (LSP) with early endosomes as determined by the in vitro reconstitution of fusion between biotinylated *Salmonella*-containing phagosomes and avidin-HRP (AHRP) loaded early endosomes. Moreover, this fusion step is inhibited by GTPγS, suggesting the possible role of Rab-GTPases in the regulation of this fusion event ***(15)***. As Rab5 is shown to regulate the homotypic fusion between early compartments ***(9,10)***, we look for the presence of Rab5 on *Salmonella*-containing phagosomes by Western blotting and electron microscopy using specific antibodies. Our results show that *Salmonella*-containing phagosomes recruit host Rab5. Finally, using immobilized Rab5 in pull-out assays, we identify SopE, a *Salmonella* effector protein, which specifically binds with Rab5. Thus, we establish that the recruitment of host Rab5 on LSP via the bacterial effector molecule, SopE, is the underlying mechanism by which *Salmonella* evade their transport to the lysosomes.

3.1. Transport of Salmonella-Containing Phagosomes to Lysosomes

To understand the mechanism by which *Salmonella* survive in macrophages, we first measured the kinetics of transport of live and dead bacteria to lysosomes in J774E macrophages.

3.1.1. Culture of Salmonella

1. Wild-type *Salmonella* from a frozen glycerol stock (*see* **Note 1**) are inoculated in 3 mL LB and grown overnight at 37°C with constant shaking at 300g. A small aliquot of this culture is spread on SS Agar containing Petri plate (90 mm, disposable, 25 mL medium/plate) and incubated for 12 h at 37°C to obtain isolated *Salmonella* colonies (*see* **Note 2**).
2. For experimental purposes, a single *Salmonella* colony is inoculated into 10 mL of LB in a 50 mL Falcon tube (30 × 115 mm) and incubated at 37°C with constant shaking (300g) for about 12 h to grow the culture to a cell density corresponding to an OD_{600} of about 0.9–1. Thereafter, 0.1 mL of bacterial suspension is diluted into 10 mL of fresh LB and cells are grown under similar conditions for about 2 h to an OD_{600} of about 0.5. These log phase cells are harvested by centrifugation (*see* **Note 3**), washed twice with PBS and used for further studies.

3.1.2. Preparation of Dead Salmonella

1. *Salmonella* are killed by incubating 1×10^{10} *Salmonella* suspended in 1 mL of PBS at 65°C for 45 min. To achieve 100% killing, cells are subsequently fixed with 1% glutaraldehyde (v/v) for 30 min at 4°C. Cells are washed with PBS.
2. The viability of treated bacteria is checked by plating an aliquot (25 µL) of the cell suspension on SS agar plates. No colony appears under these conditions indicating complete loss of viability of the treated bacteria.

3.1.3. Biotinylation of Live or Dead Salmonella

1. *Salmonella* grown in LB as described above are biotinylated for use as a phagocytic probe in subsequent transport and fusion assays. Bacteria (1×10^{10} cells) are washed twice with PBS and incubated with 0.5 mg/mL of *N*-hydroxysuccinimidobiotin in 10 m*M* PBS, pH 8.0, containing 0.1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$, on a rotary shaker for 1 h at 4°C. Cells are washed twice with 10 m*M* PBS containing 50 m*M* NH_4Cl to quench excess unreacted biotin and finally resuspended in PBS. The viability of bacteria before and after biotinylation is checked (*see* **Note 4**).
2. To prepare dead biotinylated *Salmonella*, an aliquot of live biotinylated bacteria is killed as described above. Aliquots of live and dead biotinylated bacteria containing identical numbers of cells bind the same amount of AHRP, suggesting a comparable extent of biotinylation in both preparations. Biotinylated live and dead *Salmonella* are used as phagocytic probes to study transport to lysosomes in macrophages.

3.1.4. Culture of J774E Macrophages

1. Macrophages are well characterized to internalize and degrade invading organisms by phagocytosis. To understand the trafficking of *Salmonella*-containing phagosomes in macrophages, we have used J774E clone, a well-characterized mannose receptor positive mouse macrophage cell line.
2. J774E macrophages are cultured at 37°C in 5% CO_2 95% air atmosphere in RPMI-1640 medium supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS) and gentamicin (50 µg/mL).
3. Cells are cultured in flasks (Greiner Bio-one 550 mL, 175 cm^2 tissue culture flasks) till they form a confluent monolayer. The cells are dislodged by gentle tapping and subcultured at 1×10^7 cells per flask into 25 mL of fresh medium (*see* **Note 5**).

3.1.5. Assay for Transport of Salmonella to Lysosomes

1. J774E macrophages (1×10^6cells) are harvested into10 mL of FCS free RPMI-1640 medium by gentle scraping with a cell scraper (*see* **Note 6**) and washed three times in chilled PBS by centrifugation at 4ºC (*see* **Note 7**).

2. Washed J774E cells are resuspended in 0.5 mL chilled HBSA containing 200 μg/mL AHRP and incubated for 60 min at 4°C to promote binding of the ligand.
3. Bound AHRP is internalized by warming the cells to 37°C for 10 min. Cells are washed three times with HBSA and recovered by centrifugation (*see* **Note 7**).
4. Subsequently, cells are suspended in 0.5 mL prewarmed HBSA and incubated for 80 min at 37°C to promote transport of AHRP to lysosomes. AHRP is chased in the presence of 1 mg/mL mannan to prevent efflux of the internalized probe ***(16)***.
5. The AHRP-loaded J774E cells are washed three times in HBSA, resuspended in 0.5 mL HBSA and incubated with 1×10^7 (MOI 1:10) live or dead biotinylated *Salmonella* for 1 h at 4°C. Subsequently, unbound *Salmonella* are separated from the cells by centrifugation (*see* **Note 7**). Cells are resuspended in 1 mL of pre-warmed HBSA and incubated at 37°C for 5 min to restrict entry of bacteria into the early compartment.
6. Cells are washed three times with HBSA and uninternalized, surface-bound biotinylated bacteria are quenched by the addition of free avidin (0.25 mg/mL) for 30 min at 4°C and washing twice in chilled HBSA.
7. To measure kinetics of transport of *Salmonella* to lysosomes, J774E cells loaded with AHRP and biotinylated *Salmonella* are incubated at 37°C for 15, 30, 45, 60, and 90 min. At each time point, cells are promptly chilled on ice to stop the transport (*see* **Note 8**).
8. Cells are solubilized in SB containing 0.25 mg/mL free avidin as scavenger (*see* **Note 9**) and the bacteria biotin–AHRP complexes generated after fusion are separated from residual unbound AHRP by centrifugation (*see* **Note 3**).
9. The resulting pellets are resuspended in 50 μL PBS and transferred to a 96-well microtiter plate. The transport of *Salmonella* to lysosomes is measured as a read out of the enzymatic activity of HRP associated with the biotinylated bacteria.
10. The HRP activity associated with the fusion complexes is measured following the addition of 100 μL substrate buffer to each well and incubating the plate at room temperature until a pale yellow color develops (*see* **Note 10**). The reaction is stopped by addition of 100 μL 1 *N* H_2SO_4 and absorbance is read at 492 nm in an ELISA reader (Anthos HtII). Specific transport is deduced after subtracting the background HRP activity associated with biotinylated bacteria when the transport reaction is carried out at 4°C.
11. The results for transport of live and dead *Salmonella* to lysosomes in J774E cells are illustrated in **Fig. 1**. Dead *Salmonella* are efficiently transported to AHRP-loaded lysosomes within 45 min, and maximum fusion is observed at 90 min. In contrast, live *Salmonella* do not fuse with lysosomes even after 90 min, demonstrating that *Salmonella* evades transport to lysosomes. Our data also suggest that inhibition of *Salmonella* transport to the lysosomes possibly depends on some of the effector molecule/s derived from live bacteria, since dead *Salmonella* are not protected from lysosomal targeting.

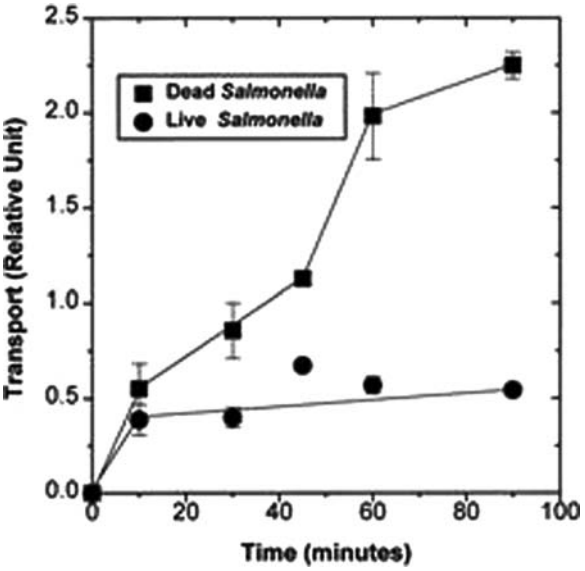

Fig. 1. Intracellular transport of live or dead *Salmonella* to the lysosomes. J774E macrophages are preloaded with AHRP and chased for 80 min to label the lysosomes. Subsequently, cells are pulsed with live or dead biotinylated *Salmonella* at 37°C for a short period of time (5 min) to restrict their entry to the early compartment and incubated for the indicated times at 37°C. At the indicated times, the cells are lysed by SB containing avidin as scavenger. HRP activity associated with bacteria-biotin-AHRP complex is measured to determine the transport of the *Salmonella* to lysosomes. Each point represents the mean ± S.D. from three independent experiments. (From **ref. *17***.)

3.2. Fusion of Salmonella-Containing Phagosomes with Early Endosomes

To investigate whether *Salmonella* evade transport to lysosomes by enhanced fusion with the early endosomes, fusion between early endosomes loaded with AHRP and purified phagosomes containing live or dead biotinylated *Salmonella* is measured using an in vitro fusion assay.

3.2.1. Preparation of Avidin-HRP Loaded Early Endosomes

1. J774E macrophages (1×10^6cells) are harvested into 10 mL of FCS free RPMI-1640 medium by gentle scraping with a cell scraper (*see* **Note 6**) and washed three times in chilled internalization medium (IM) by centrifugation (*see* **Note 7**).
2. The resulting cell pellet is gently tapped to disperse cells and incubated in 300 µL of precooled IM containing AHRP (1 mg/mL) for 1 h at 4°C to allow surface binding of the ligand.

3. Subsequently, the cells are harvested by centrifugation (*see* **Note 7**), resuspended in 300 µL prewarmed IM, and incubated at 37°C for 5 min to label the early endosomes. Uptake is promptly stopped by the addition of excess chilled IM and incubating the cell suspension on ice (*see* **Note 11**).
4. Following internalization of AHRP, cells are washed three times with chilled PBS and harvested by centrifugation (*see* **Note 7**). The resulting cell pellet is resuspended in three volumes of chilled homogenization buffer (HB) containing protease inhibitor cocktail (*see* **Note 12**).
5. The cell suspension is homogenized on ice by using a ball bearing homogenizer by giving 10 strokes of the piston (*see* **Note 13**).
6. The homogenized material is clarified by centrifugation (400*g* for 5 min) in a refrigerated Eppendorf centrifuge using F34-6-38 rotor at 4ºC to remove unbroken cells and nuclei. The resulting postnuclear supernatant (PNS) is quickly frozen in liquid nitrogen as 1 mL aliquots and stored at –70°C.
7. To prepare early endosomes, the PNS aliquot is thawed and diluted (1:3) in chilled HB and centrifuged at 60,000*g* for 1 min at 4°C in a BeckmanTL-100 ultracentrifuge, rotor TLA100.3 (*see* **Note 14**). The resulting supernatant is again centrifuged at 1,00,000*g* for 5 min at 4°C in the same ultracentrifuge. The final pellet enriched in early endosomal vesicles is resuspended in 100 µl HB and used for the in vitro fusion assays.

3.2.2. Preparation of Phagosomes Containing Live or Dead Biotinylated Salmonella

1. Biotinylated live and dead *Salmonella* to be used as phagocytic probe are prepared as described in **Subheading 3.1.1.** and washed twice in chilled FCS-free RPMI-1640 medium by centrifugation (*see* **Note 3**).
2. J774E macrophages (1×10^8cells) are harvested into 10 mL of FCS free RPMI-1640 medium by gentle scraping with a cell scraper (*see* **Note 6**) and washed three times in chilled IM by centrifugation (*see* **Note 7**).
3. Washed macrophages are incubated with live or dead bacteria (MOI of 1:10) in 200 µL chilled FCS free RPMI-1640 for 1 h at 4°C to facilitate binding.
4. Subsequently, the cells are washed by low-speed centrifugation (1150*g*, 5 min, 4°C, in an Eppendorf centrifuge using rotor F-45-30-11) and resuspended in 500 µL prewarmed IM. Internalization of bound bacteria is carried out at 37°C for 5 min to restrict their entry into early compartment. Further transport of the bacteria is stopped by the addition of excess chilled medium (*see* **Note 11**). Uninternalized bacteria are removed by washing cells three times in chilled medium (*see* **Note 7**).
5. The resulting cell pellet is suspended in 1 mL chilled HB containing protease inhibitors and homogenized in a ball bearing homogenizer giving 10 strokes of the piston. The homogenate is centrifuged at 515*g*, 10 min, 4°C in an Eppendorf centrifuge using F-45-30-11 rotor. The post nuclear supernatant (PNS) is quickly frozen in liquid nitrogen and stored at –70°C as 0.5 mL aliquots.

6. To obtain the enriched phagosomal fraction, the PNS is quickly thawed, diluted (1:3) with chilled HB, and centrifuged at 500*g*, 10 min, 4°C using A-4-62 rotor in an Eppendorf centrifuge. The supernatant is carefully transferred to a fresh tube and centrifuged at 12,000*g*, 6 min, 4°C in an Eppendorf centrifuge using F-45-30-11 rotor.
7. The pellet enriched in *Salmonella*-containing phagosomes is suspended (*see* **Note 15**) in 1 mL HB and centrifuged at 500*g* for 2 min at 4°C in an Eppendorf centrifuge using rotor (A-4-62). The supernatant is collected carefully and further centrifuged at 12,000*g* for 6 min using rotor F-45-30-11.
8. The final pellet is resuspended in 100 µL of HB containing protease inhibitors and centrifuged at 500*g* for 2 min at 4°C in an Eppendorf centrifuge using rotor (A-4-62) to remove any residual particles.
9. To purify phagosomes, the resulting supernatant is loaded onto 1 mL 12% sucrose cushion (*see* **Note 16)** and centrifuged at 1700*g* for 45 min in an Eppendorf centrifuge using rotor F-45-30-11 (*see* **Note 17**).
10. The resulting pellet is resuspended in 0.5 mL HB and centrifuged at 12,000*g* for 6 min in an Eppendorf centrifuge using rotor F-45-30-11.
11. Highly purified live *Salmonella*-containing phagosomes (LSP) or dead *Salmonella*-containing phagosomes (DSP) (*see* **Note 18**) are recovered in the pellet from the bottom of the tube and subsequently resuspended (*see* **Note 15**) in 100 µL HB and used in endosome-phagosome fusion assays.

3.2.3. Preparation of Cytosol from J774E Macrophages

1. J774E macrophages (1×10^8cells) are harvested into 10 mL of FCS free RPMI-1640 medium by gentle scraping with a cell scraper (*see* **Note 6**) and washed three times in chilled PBS by centrifugation (*see* **Note 7**).
2. The resulting cell pellet is suspended in three volumes of chilled HB containing protease inhibitors and the suspension is homogenized on ice using a ball bearing homogenizer giving 20 strokes of the piston.
3. The homogenate is clarified by centrifugation (515*g* for 10 min) in an Eppendorf centrifuge using F34-6-38 rotor at 4ºC to remove unbroken cells and nuclei. The supernatant is carefully collected in a fresh tube and centrifuged at 1,40,000g at 4ºC for 30 min in a Beckman ultracentrifuge TL-100 using rotor TLA 100.3.
4. The resulting supernatant is carefully transferred to a fresh tube and centrifuged again under similar conditions in a Beckman ultracentrifuge as mentioned above.
5. The obtained supernatant containing cytosol is concentrated using Millipore Centricon (YM-10) (*see* **Note 19**). The concentrated cytosol is distributed as 200 µL aliquots, quickly frozen in liquid nitrogen and stored at –70°C.
6. Prior to use in fusion assay, the cytosol is gel filtered using spin columns. The gel filtration column is packed with preswollen G-25 sepharose up to the 1 mL mark in a 1 mL syringe which has been plugged with glass wool (*see* **Note 20**). The gel matrix is washed with five volumes of chilled HB by applying vacuum

and centrifuged at 515*g*, 5 min at 4°C using F34-6-38 rotor in an Eppendorf centrifuge to elute residual HB.

7. A 100 µL aliquot of frozen cytosol is thawed and dispensed onto the surface of the washed G-25 column. The cytosol is gel filtered by centrifuging the column at 1150*g* at 4°C using Eppendorf centrifuge rotor F34-6-38.
8. The protein content of the gel-filtered cytosol is estimated by bicinchoninic acid (BCA) method according to the maufacturer's instructions.

3.2.4. In Vitro Phagosome-Endosome Fusion Assay

To determine the interaction of *Salmonella*-containing phagosomes with early endosomes, we have developed an in vitro fusion assay by mixing *Salmonella*-containing phagosomes with early endosomes in the presence of host cytosol at appropriate temperature. This assay essentially measures the

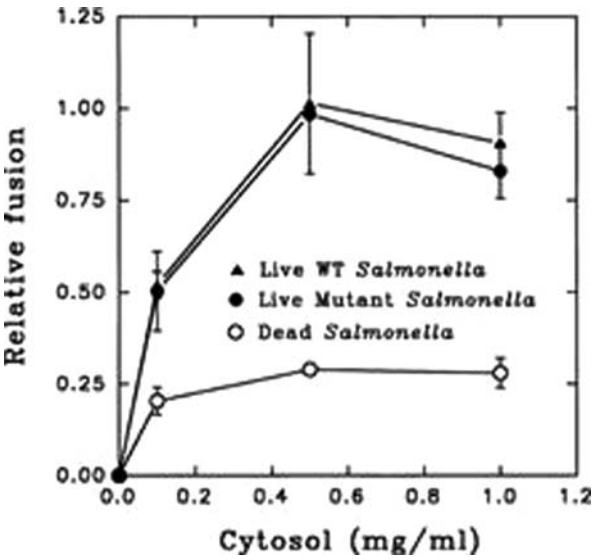

Fig. 2. Cyotosol-dependent fusion of endosomes with phagosomes containing dead or live *Salmonella*. Early endosomes containing AHRP are incubated with phagosomes containing dead or live biotinylated *Salmonella* in ATP regenerating fusion buffer supplemented with different concentrations of gel-filtered cytosol for 5 min at 37°C. Maximum fusion of LSP with early endosomes was observed at 0.5 mg/mL of cytosol concentration which was normalized to one unit, and the results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to ~10.7 ng of HRP activity/per mg of protein in the fusion assay containing live wild-type *Salmonella* phagosomes. The aro A mutant *Salmonella* are used as control. (From **ref. *15***.)

HRP activity associated with biotinylated *Salmonella* after the fusion reaction **(Fig. 3)**.

1. Purified phagosomes (20 µg) containing live or dead biotinylated *Salmonella* are mixed with AHRP-loaded early endosomes (20 µg) in 40 µL fusion buffer supplemented with ATP regenerating system and gel-filtered cytosol **(Fig. 2)** in a clean 0.5 mL centrifuge tube placed on ice (*see* **Note 21**).
2. Fusion is carried out by incubating the above reaction mixtures in a water bath maintained at 37°C for 5 min. The reaction is stopped by promptly chilling the tubes on watery ice.
3. After the fusion reaction, vesicles are solubilized by addition of 40 µL of 2X SB supplemented with 0.25 mg/mL avidin as scavenger (*see* **Note 9**) and incubating on ice for 30 min.
4. The bacteria-biotin-AHRP complexes formed as a result of phagosome-endosome fusion are recovered by centrifugation (10,000*g*, 5 min, 4°C in an Eppendorf centrifuge using rotor F-45-30-11). The resulting pellet is resuspended in 40 µL of PBS by gentle tapping and transferred to a 96-well microtiter plate.
5. The enzyme activity of HRP associated with the biotinylated bacteria is measured as described in **Subheading 3.1.5**.

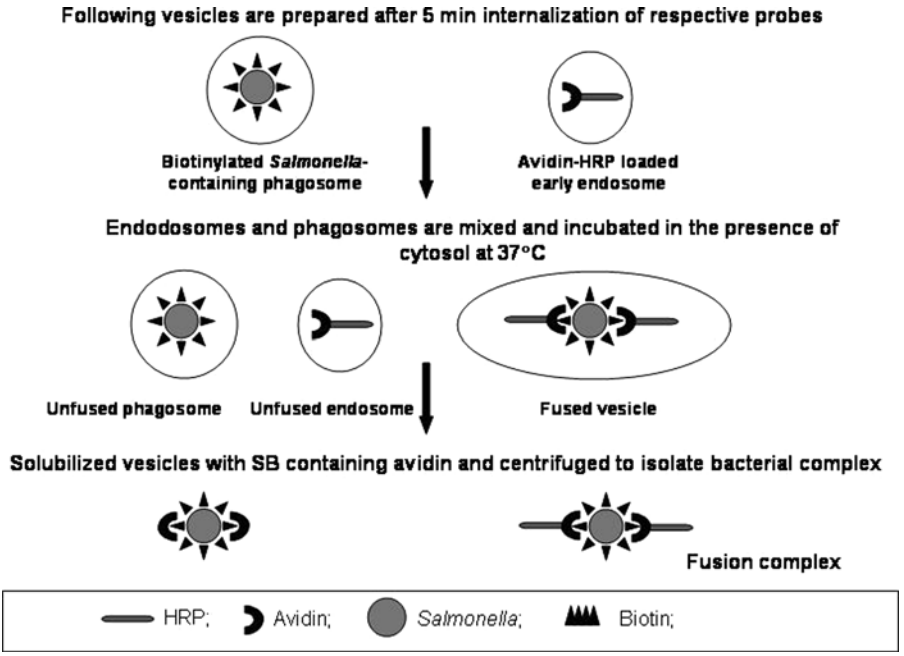

Fig. 3. Schematic representation of in vitro fusion between AHRP-loaded early endosomes and biotinylated *Salmonella*-containing phagosomes.

6. Two control reactions are included in this assay to estimate the total and nonspecific fusion. Total fusion is measured by solubilizing the fusion reaction in SB without any added avidin as scavenger. Nonspecific fusion corresponding to bacteria-associated HRP activity is obtained when the endosomes and phagosomes are mixed in fusion buffer without cytosol or with cytosol at 4°C. These nonspecific values are low and are subtracted from the corresponding values to determine specific fusion.
7. From the data in **Fig. 2** it is evident that under our assay conditions, endosome-phagosome fusion is dependent on the concentration of cytosol and maximum fusion occurs at 0.5 mg/mL of cytosol. This suggests that host cytosolic factor(s) might regulate the enhanced fusion between early endosomes and LSP. It is also evident that LSP fuse more efficiently with early endosomes than DSP suggesting the probable role of bacterial effector protein(s) in recruiting the host cytosolic factor(s).

3.3. Recruitment of Host Rab5 on Salmonella Containing Phagosome

Our observation that fusion between early endosomes and LSP is dependent on cytosol indicates that some host factors are involved in driving the fusion process. As it is well demonstrated that vesicle fusion is regulated by specific Rab proteins, it is logical to expect that some endocytic Rab proteins from the host cells might play a role in the fusion between *Salmonella*-containing phagosomes and early endosomes. In the endocytic pathway, Rab5 specifically regulates early endosome fusion and Rab7 drives transport of cargo towards the late/lysosomal compartments ***(9–12)***. Thus, we investigate the presence of host endocytic Rab5 and Rab7 on the LSP and DSP by Western blotting using specific antibodies. Since the LSP and DSP differ in their ability to fuse with early endosomes, we investigated the presence of these regulatory molecules on the surface of *Salmonella*-containing phagosomes by immuno-electron microscopy ***(18)***.

3.3.1. Detection of Endocytic Rabs on Salmonella-Containing Phagosomes by Western Blotting

1. To detect the presence of host Rab5 and Rab7 on LSP and DSP, highly purified phagosomes are prepared as detailed in **Subheading 3.2.2.** and 40 µg of each sample is mixed with SDS sample buffer and incubated in a boiling water bath for 5 min. Samples are analyzed by 12% SDS-PAGE at constant current of 0.02 amperes for 70 min, using a Bio-Rad MiniProtean II apparatus.
2. The gel is then immersed in chilled transfer buffer (TB) for 15 min. Proteins are transferred onto nitrocellulose membrane at 10 V for 30 min using a Bio-Rad semi-dry transfer apparatus (*see* **Note 22**).

3. The nitrocellulose membranes are incubated in PBST (PBS containing 0.1% Tween-20) containing 2% BSA for 3 h at room temperature on a rotary shaker with gentle agitation to block the membrane.
4. To detect the presence of respective Rabs, membranes are incubated with specific polyclonal antibodies against Rab5, Rab7, or Transferrin receptor for 1 h at room temperature on a rotary shaker. The antibodies are diluted 1:2000 in PBST.
5. Subsequently the primary antibodies are decanted and the membranes are washed in excess PBST for 10 min with agitation, giving three buffer changes (*see* **Note 23**).
6. The membranes are incubated with appropriate HRP-conjugated secondary antibodies diluted 1:10000 in PBST for 1 h at room temperature with gentle shaking after which they are washed three times in PBST.
7. The membranes are developed by the addition of ECL reagent (Amersham Biosciences) according to manufacturer's recommendations. The signals are captured by exposing the membranes to photographic film followed by developing and fixing.
8. Our data in **Fig. 4** indicate that LSP contain about fivefold more host Rab5 in comparison to DSP (*see* **Note 24**) In contrast, DSP, which are efficiently targeted to the lysosomes, contain significantly higher amounts of host Rab7 as compared to LSP. Since LSP have more Rab5 than DSP, it suggests that some effector molecule(s) from live *Salmonella* might be involved in the recruitment of host Rab5 on the phagosomes. Enhanced content of Rab5 on LSP promotes their fusion with early endosomes, and this possibly evades their targeting to lysosomes.

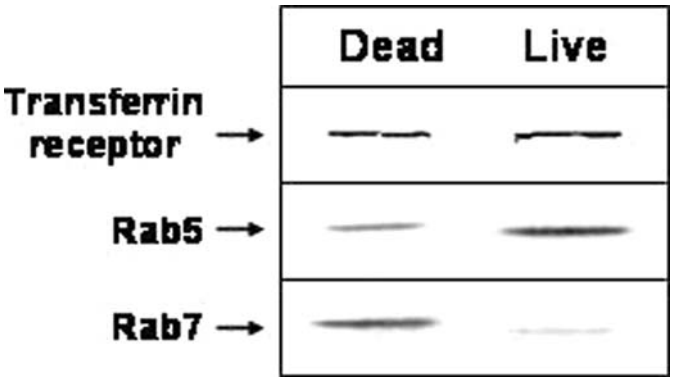

Fig. 4. Dead or Live *Salmonella*-containing phagosomes (40 µg protein each per lane) is electrophoresed and transferred to nitrocellulose membranes. After incubation with specific antibodies against Rab5, Rab7, and Transferrin receptor, the proteins are visualized using appropriate HRP-conjugated secondary antibodies and ECL. (From **ref. *15***.)

3.3.2. Detection of Rab5 on Salmonella-Containing Phagosomes by Electron Microscopy

1. To specifically demonstrate the presence of host Rab5 on the surface of the phagosome, we have performed immuno-electron microscopy using specific antibodies.
2. Highly purified LSP and DSP are prepared as detailed in **Subheading 3.2.2.** and washed five times in ice-cold HB by centrifugation at 12,000*g* for 6 min in an Eppendorf centrifuge using rotor F-45-30-11.
3. Glow-discharged formver and carbon-coated nickel grids (Electron Microscopy Sciences, Fort Washington, PA) are gently held with fine forceps (*see* **Note 25**) and inverted on a 20 µl drop of LSP or DSP suspension for 2 min to enable vesicles to adhere to the coated side. Excess fluid is decanted from the edge of the grids on filter paper.
4. Grids are rinsed twice by placing the coated surface on a 20 µL drop of HB for 2 min.
5. Subsequently, grids are blocked for 30 min by inverting on a 20 µL drop of blocking buffer (BB, HB containing 3% skim milk and 0.1% gelatin.).
6. The grids are then incubated for 2 h in polyclonal rabbit anti-Rab5 antibody (diluted 1:200 in BB), rinsed three times in BB, and incubated for 1 h in goat anti-rabbit IgG conjugated with 20 nm colloidal gold (purchased from Jackson ImmunoResearch Laboratories). The secondary antibody is diluted 1:20 in BB. In control preparations, incubation of grids with primary antibody is omitted.
7. The grids are rinsed twice in BB and incubated in 1% glutaraldehyde (prepared fresh in HB) for 10 min to fix samples.
8. Finally, the grids are washed twice sequentially in HB and distilled water and negatively stained with 0.5% aqueous uranyl acetate for 1 min. Excess fluid is drained on filter paper and the grids are briefly air dried and examined by electron microscopy (JEOL 1200 EX 11).
9. Electron micrographs of LSP show a much higher accumulation of Rab5-associated 20 nm gold particles as compared to DSP **(Fig. 5)**. This confirms that LSP recruit host Rab5 to promote their fusion with early endosomes.

3.4. SopE, a Type III Secretary Protein of Salmonella, Binds Host Rab5

The presence of live *Salmonella* in the phagosomes is essential both for the recruitment of host Rab5 on the phagosomal membrane **(Fig. 5)** as well as for enhanced fusion of LSP with early endosomes **(Fig. 2)**, suggesting the involvement of some effector protein actively produced by live bacteria in the regulation of these processes. In order to identify the bacterial effector which may bind Rab5, GST-Rab5 is used as a bait to pull out the putative interacting protein from a preparation of *Salmonella*-secreted proteins. The interacting protein is subsequently identified by Western blotting using antibodies specific to some S*almonella*-secreted proteins.

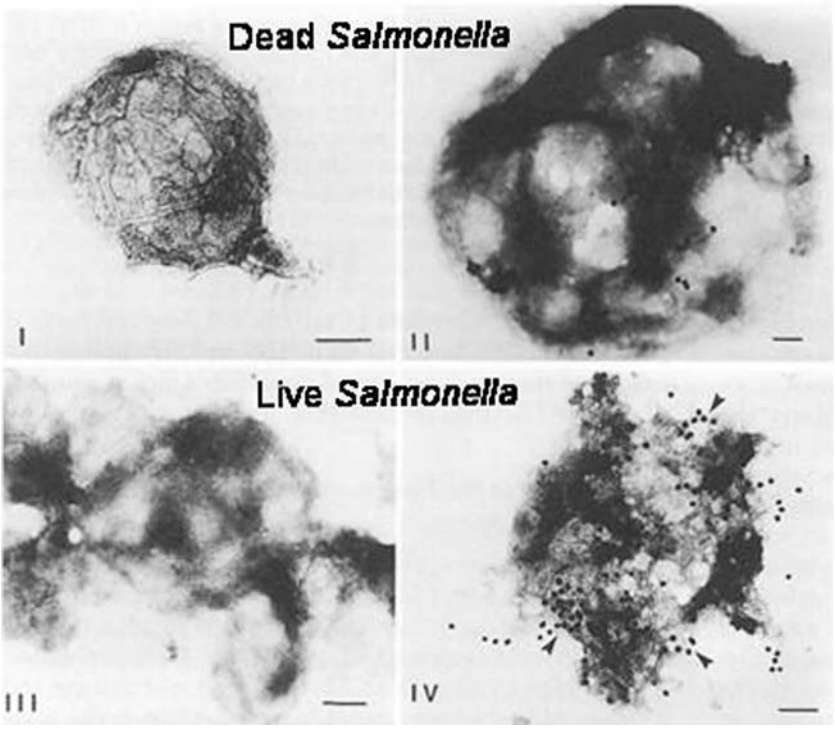

Fig. 5. DSP or LSP are incubated with rabbit anti-Rab5 antibody for 2 h at room temperature followed by treatment with goat anti-rabbit antibody conjugated with 20-nm colloidal gold particles. (**I** and **II**) DSP; (**III** and **IV**) LSP. In **I** and **III**, phagosomes were processed for the negative staining without primary anti-Rab5 antibody. Arrow in **IV** shows the presence of Rab5 on the live *Salmonella* containing phagosomes as revealed by 20 nm gold particles. Bars, 100 nm. (From **ref. *15***.)

3.4.1. Purification of Salmonella-Secreted Proteins

1. A single colony of *Salmonella* is inoculated into 5 mL of LB and grown overnight at 37°C with constant shaking (300g). This preinoculum is added into 2 L of fresh LB containing 300 m*M* NaCl and grown for an additional 16 h at 37°C with shaking. The high-salt medium induces the secretion of *Salmonella* secretory proteins ***(19)***.
2. Subsequently, the spent medium containing *Salmonella*-secreted proteins is separated from the bacterial cells by centrifugation (*see* **Note 3**) and concentrated using Amicon membrane (10 kDa cutoff) by centrifugation at 3000g, 4°C in an Eppendorf centrifuge using F34-6-38 rotor (*see* **Note 26**).

3. After concentration of the spent medium, the protein content is estimated by BCA and protein is quickly frozen in liquid nitrogen and stored as 0.5 mL aliquots at –70°C.

3.4.2. Detection of Rab5-Interacting Protein from Salmonella

1. To detect the Rab5-interacting protein from *Salmonella*, 200 µg of GST-Rab5 purified protein is incubated with 100 µL bed volume of prewashed glutathione-agarose beads for 1 h at room temperature with gentle agitation on a rotary shaker. The beads are washed three times with chilled PBS and recovered by centrifugation at 130*g*, 3 min, 4°C in an Eppendorf centrifuge using F45-30-11 rotor and supernatant carefully aspirated without disturbing compacted beads.
2. Subsequently, the GST-Rab5 immobilized beads (100 µL) are incubated with 300 µg of concentrated spent medium (100 µL) containing protease inhibitors. The suspension is incubated on a rotary shaker at 4°C for 10 h after which beads are washed three times with chilled PBS to remove any unbound proteins.
3. A 25 µL aliquot of the beads is boiled with SDS loading buffer. Proteins are resolved by 12% SDS-PAGE and subsequently transferred to a nitrocellulose membrane as described in **Subheading 3.3.1**.
4. The *Salmonella*-secreted proteins which bind GST-Rab5 are detected by Western blotting as described in **Subheading 3.3.1.** using primary antibodies against some *Salmonella* secretory proteins: SopE, SopB, and SipC.
5. To identify the *Salmonella*-secreted protein that specifically interacts with host Rab5, GST-Rab7 and free GST are used as controls.
6. The data in **Fig. 6** indicate that Rab5 specifically interacts with a *Salmonella*-secreted protein, SopE but not with SopB or SipC. This interaction of Rab5 with SopE is specific because Rab7 or GST does not bind with SopE.

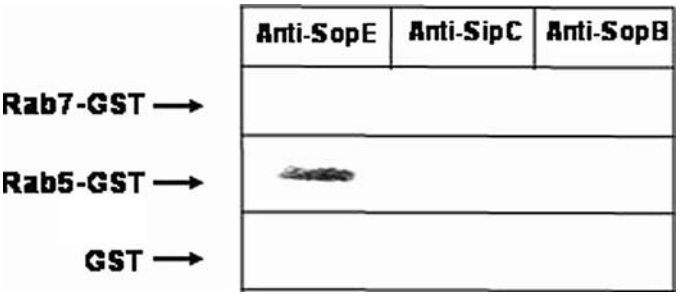

Fig. 6. Detection of Rab5-binding protein from *Salmonella*. To detect the Rab5-binding protein, GST pull-out assay was carried out with GST-Rab5 (middle), GST-Rab7 (top), or GST alone (bottom) in the presence of concentrated *Salmonella* spent medium. The *Salmonella* proteins associated with respective beads were detected by Western blot analysis using antibodies against SopE, SopB, and SipC. (From **ref. *15***.)

4. Notes

1. *Salmonella* glycerol stock is prepared by adding 1 mL of sterile 100% glycerol to 3 mL of an overnight grown culture of the bacteria and storing the cell suspension in 100 µL aliquots at –70°C.
2. *Salmonella* colonies can be easily distinguished from any contamination on SS agar, which is a differential medium. The *Salmonella* colonies have a brown-black center and decolorize the SS agar from pink to orange-yellow.
3. For harvesting *Salmonella* the cells are centrifuged at 4000*g* for 5 min in an Eppendorf centrifuge using F34-6-38 rotor at 25ºC.
4. Biotinylation did not affect the viability of the bacteria since equivalent amount of *Salmonella* before and after biotinylation formed similar number of colonies on SS agar plates.
5. J774E macrophages are seeded at 1×10^7 cells in 20 mL of RPMI-1640 in a T-175 culture flask. A confluent monolayer is obtained within 48 h ($3–4 \times 10^7$ cells). Cells are harvested at this stage for experimental purposes.
6. The cells should be scraped very gently by moving the scraper in only one direction after adding 5 mL of medium to cover the growth surface of the culture flask. The cell suspension recovered after scraping should be immediately put on ice to avoid activation of proteases secreted by broken and damaged cells.
7. J774E macrophages are harvested by centrifugation at 130*g* for 6 min in an Eppendorf centrifuge using F34-6-38 rotor at 4ºC.
8. Fusion reactions are extremely susceptible to even minor fluctuations in temperature. The indicated incubation time and the temperature should be strictly maintained to get reproducible results.
9. Free avidin should be present in SB and FB, which serves as a scavenger to quench any unreacted biotin present in the respective reaction mixture.
10. Following addition of substrate buffer, the plates should be incubated in the dark. The reaction should be stopped once a pale yellow color is observed to avoid overdeveloping.
11. IM should always be prewarmed to 37°C to facilitate efficient internalization of the endocytic probe. The time of internalization of probe must be critically monitored. Internalization should be stopped at appropriate time by immediately chilling cells on ice and addition of excess chilled medium. Uninternalized probes should be removed thoroughly by repeated washes so that they do not internalize during subsequent handling of the macrophages to form another subset of vesicles.
12. Cells to be homogenized are always suspended in HB containing protease inhibitors to prevent the degradation of proteins by proteases that will be released from the broken cells.
13. Homogenization should always be carried out on ice. The homogenizer and the syringe should be thoroughly rinsed with HB and chilled before homogenization of cells. Air bubbles should be avoided during homogenization.
14. The rotor and chamber of the ultracentrifuge should be cooled prior to centrifugation so as to avoid a temperature shock to the vesicles.

15. The phagosome pellet should first be incubated on ice with the appropriate buffer to loosen it and then resuspended by gentle tapping so as to avoid damage to the vesicles, followed by dilution in large volumes of buffer. Phagosomes are fragile and should always be pipetted using cut tips.
16. The supernatant should form a layer on the 12% sucrose cushion and not settle down immediately.
17. After centrifugation of phagosomal preparation through the sucrose cushion, the supernatant should be removed very carefully without disturbing the pellet, leaving about 100 μL buffer with the pellet. Usually a fluffy, pale brownish pellet is obtained as a purified phagosomal preparation.
18. The phagosome preparations are checked by lysing an aliquot of LSP or DSP in 0.5% Triton-X 100 for 30 min on ice and plating on SS Agar to check for the formation of *Salmonella* colonies. As expected, LSP lysates form colonies, whereas DSP lysates do not form any colonies.
19. Cytosol should be concentrated to an approximate final concentration of 6 mg/mL by centrifuging at 1150*g*, 4ºC using Eppendorf centrifuge rotor F34-6-38.
20. During packing the G-25 column the slurry must be dispensed in one go to avoid formation of any air pockets. While washing the matrix by applying vacuum, care must be taken to ensure that the matrix does not dry out at any stage.
21. FB is prepared as a concentrated stock (10X) to facilitate final addition (to 1X) in a small volume such that the fusion reaction does not become dilute.
22. Transfer of proteins onto nitrocellulose membrane using semi-dry method is done as per manufacturer's (BioRad) instructions, taking care not to introduce air bubbles between the gel and membrane. Chilled TB is used for improving the efficiency of transfer.
23. Insufficient washing can give rise to nonspecific background signals on the membrane.
24. The ECL signals are quantitated as arbitrary densitometric units using ImageJ (image analyses freeware).
25. The coated grids must be handled with great care, ensuring that the surface is never scratched during handling with forceps.
26. Spent medium containing *Salmonella*-secreted proteins should be concentrated approximately 100-fold so that adequate amounts of secreted proteins are present in a small aliquot, making it convenient for use in the pull-out assay using immobilized Rab5.

Acknowledgments

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