Live *Salmonella* Recruits *N*-Ethylmaleimide–sensitive Fusion Protein on Phagosomal Membrane and Promotes Fusion with Early Endosome

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Abstract. To understand intracellular trafficking modulations by live *Salmonella*, we investigated the characteristics of in vitro fusion between endosomes and phagosomes containing live (LSP) or dead *Salmonella* (DSP). We observed that fusion of both DSP and LSP were time, temperature and cytosol dependent. GTPγS and treatment of the phagosomes with Rab-GDI inhibited fusion, indicating involvement of Rab-GTPases. LSP were rich in rab5, α-SNAP, and NSF, while DSP mainly contained rab7. Fusion of endosomes with DSP was inhibited by ATP depletion, *N*-ethylmaleimide (NEM) treatment, and in NEM-sensitive factor (NSF)–depleted cytosol. In contrast, fusion of endosomes with LSP was not inhibited by ATP depletion or NEM treatment, and occurred in NSF-depleted cytosol. However, ATPγS inhibited both fusion events. Fusion of NEM-treated LSP with endosomes was abrogated in NSF-depleted cytosol and was restored by adding purified NSF, whereas no fusion occurred with NEM-treated DSP, indicating that NSF recruitment is dependent on continuous signals from live *Salmonella*. Binding of NSF with LSP required prior presence of rab5 on the phagosome. We have also shown that rab5 specifically binds with Sop E, a protein from *Salmonella*. Our results indicate that live *Salmonella* help binding of rab5 on the phagosomes, possibly activate the SNARE which leads to further recruitment of α-SNAP for subsequent binding with NSF to promote fusion of the LSP with early endosomes and inhibition of their transport to lysosomes.

Key words: phagocytosis • reconstitution • fusion • phagosome • Rab

Introduction

Intracellular pathogens use various strategies to ensure survival within the intravacuolar environment (Portillo and Finley, 1995a,b). For example, *Mycobacterium tuberculosis*, *Legionella pneumophilia*, and *Toxoplasma gondii* survive and proliferate in the vacuolar compartments that do not mature into phagolysosomes (Clemens and Horwitz, 1995; Sturgill-Koszycki et al., 1994; Xu et al., 1994), whereas *Trypanosoma cruzi* (Hall et al., 1992), *Shigella flexneri* (High et al., 1992), and *Listeria monocytogenes* (Portnoy et al., 1988) lyse the phagosomal membranes to reside in the cytoplasm. *Coxiella burnetti* (Maurin et al., 1992) and *Leishmania* (Russell et al., 1992) survive even in the acidified phagosomes. So far, very little is known about the mechanism of intracellular survival.

Salmonella species cause enteric fever and gastroenteritis, in both human and animal hosts (Keusch, 1994), and the pathogenesis is related to the survival of the bacteria in phagocytes. However, the mechanism *Salmonella* uses to modulate intracellular survival remains to be explored. Previous studies have shown that phagosomes containing *S. typhimurium* are unusually large and less acidified than the phagosomes containing inert particles (Alpuche-Aranda et al., 1994). Phagosomes containing *S. typhimurium* fuse with the compartment containing lysosomal glycoprotein (lgp), bypassing compartments containing cation-dependent mannose 6-phosphate receptors (CD-M6PR) or cation-independent mannose 6-phosphate receptors (CI-M6PR), which are normally encountered along the endocytic route (Portillo and Finlay, 1995a,b). However, there are conflicting reports regarding the maturation of *Salmonella*-containing phagosomes. Contrary to an earlier report suggesting a delay in phagosomal acidification (Alpuche-Aranda et al., 1992), Rathman et al. (1996) reported that *Salmonella* reside in an acidic (pH 4–5) phagosome. Oh et al. (1996) showed that *Salmonella*-containing phago-

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somes mediate rapid and complete fusion with lysosomes, in contrast to the inhibition of fusion of *Salmonella* phagosomes with lysosomes reported by Buchmeier and Heffron (1991). These results suggest that *Salmonella*-containing phagosomes may have the capacity to selectively fuse with vesicles carrying different markers in order to create phagosomal environments that allow their survival.

It has been shown that ras-related rab GTPases regulate the intracellular trafficking through vesicle fusion (Balch, 1990; Zerial and Stenmark, 1993; Rothman and Sollner, 1997; Lupashin and Waters, 1997; Schimmoller et al., 1998). Similarly, recent studies have shown that fusion of endocytic vesicles with phagosomes containing inert particles require cytosol, ATP, and are regulated by Rab-GTPases (Pitt et al., 1992; Desjardins et al., 1994a,b; Jahraus et al., 1998). A series of generic and compartment-specific proteins, the rabs (Mayorga et al., 1991; Beron et al., 1995), *N*-ethylmaleimide–sensitive fusion protein (NSF)[1], soluble NSF attachment protein (SNAP), etc. (Sollner et al., 1993; Soggard et al., 1994; Weber et al., 1998; Pfeffer, 1999), further regulate the docking and the fusion of the vesicles. We sought to delineate whether *Salmonella* alter the function of any of these proteins to avoid or induce the specific interactions of phagosomes with other vacuolar compartments.

In this investigation, we have shown that in vitro fusion of phagosomes containing live or dead *Salmonella* with endosomes is regulated by rab GTPases, and both fusion events require cytosolic proteins. Our results also indicate that phagosomes containing live *Salmonella* specifically recruit rab5, α-SNAP, and NSF on the phagosomal membrane and promote efficient fusion with the early endosomes.

Materials and Methods

Materials

Unless otherwise stated, all reagents were obtained from Sigma Chemical Co. Tissue culture supplies were obtained from the Grand Island Biological Co. *N*-hydroxy succicinimidobiotin (NHS-biotin), avidin-horseradish peroxidase (Avidin-HRP), avidin, and bicinchoninic acid (BCA) reagents were purchased from Pierce Biochemicals. Goat anti–rabbit IgG conjugated with 20-nm colloidal gold and goat anti–mouse IgG conjugated with 12-nm colloidal gold were purchased from Jackson ImmunoResearch Laboratory. ECL reagents were procured from Amersham International. Other reagents used were of analytical grade. Cytosol was obtained from J774 E cells after high-speed centrifugation of cell homogenate (Mayorga et al., 1989). Cytosol (0.1 ml) was gel filtered through 1 ml of G-25 Sephadex spin column just before use in the fusion assay.

Antibodies and Recombinant Proteins

Monoclonal antibody (mAb), 4F11, a mouse IgG_{2ak} mAb specific for the carboxy-terminal of mouse rab5 (Qiu et al. 1994) and an affinity-purified rabbit polyclonal antibody which recognizes carboxy-terminal domain of rab7 were generously provided by Dr. A. Wandinger-Ness (Northwestern University, Evanston, IL). A rabbit polyclonal anti-Rab5 antibody was received as a gift from Dr. J. Gruenberg (EMBL, Heidelberg, Germany). Affinity-purified rabbit polyclonal antibodies against native NSF, recombinant NSF and dominant negative NSF (D1E-Q, Glu^3 29 to Gln) fusion proteins were received as a kind gift from Dr. S.W. Whiteheart (University of Kentucky, Lexington, KY). Recombinant GDI and Rab 5 fusion proteins were kindly provided by Dr. Philip Stahl (Washington University School of Medicine, St. Louis, MO). Anti-*Salmonella* antibodies (anti-SopE, anti-SopB, and anti-SipC) were kindly provided by Dr. E.E. Galyov from Institute for Animal Health (Berkshire, UK). Mouse anti-actin and anti-transferrin receptor antibodies were purchased from Calbiochem and Zymed Laboratory, respectively. Anti–α-SNAP and all the second antibodies labeled with HRP were purchased from Santa Cruz Biotechnology.

Cells

J774E clone, a mannose receptor positive macrophage cell line was kindly provided by Dr. Philip Stahl (Washington University School of Medicine, St. Louis, MO). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and gentamycin (50 μg/ml) and were grown at 37°C in 5% CO_2 95% air atmosphere.

Bacterial Strains

The virulent wild-type (WT) *S. typhimurium* (a clinical isolate from Lady Harding Medical College, New Delhi, India) and the auxotropic mutant (aro A) of *S. typhimurium* (SL3235 from Dr. K. Sanderson of *Salmonella* Genetic Stock Centre, Calgary, Canada) were obtained from Dr. Vineeta Bal of National Institute of Immunology (New Delhi, India). Bacteria were grown overnight in Luria broth (LB) at 37°C with constant shaking (300 rpm), washed twice in PBS, and then used in LSP preparation. For preparing DSP, bacteria were first fixed with 1% glutaraldehyde at 4°C for 30 min and subsequently incubated at 65°C for 45 min (Rathman et al., 1996). Complete loss of viability of the bacteria was confirmed by the absence of colony formation on LB agar plates.

Preparation of Phagosomes Containing Live or Dead Salmonella

Biotinylated *Salmonella* were used as a phagocytic probe for the phagosomes. Essentially, WT and mutant *Salmonella* were grown in LB as described previously. Bacteria were biotinylated as described (Zurzolo et al., 1994). In brief, both strains of bacteria were incubated with NHS-biotin (0.5 mg/ml) in PBS-CM (0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ in 10 mM PBS, pH 8) for 1 h at 4°C. Then, the cells were sequentially washed with PBS and 50 mM NH_4Cl to quench excess free biotin and resuspended in PBS. Viability of the biotinylated bacteria was determined by plating the cells in LB agar plate. An aliquot of live biotinylated bacteria was killed by glutaraldehyde followed by heat treatment. To determine the biotinylated bacterial proteins in dead and live *Salmonella*, 1×10^7 bacteria were boiled in SDS sample buffer and subjected to SDS-PAGE analysis. Subsequently, proteins were transferred to nitrocellulose membrane and probed with avidin-HRP. Multiple proteins were biotinylated in both the preparations and showed essentially identical profiles.

Biotinylated WT (live and dead) and mutant *Salmonella* were used in phagosome preparation using a method described previously (Alvarez-Dominguez et al., 1996). J774E clone macrophages (1×10^8) were incubated in suspension with 1×10^9 bacteria at 4°C for 1 h in RPMI-1640 medium containing 5% FCS and bacterial infection was synchronized by centrifugation at low speed. Then the cells were shifted to prewarmed medium and incubated for 5 min at 37°C. The uptake was stopped by the addition of ice-cold medium. Cells were washed three times to remove unbound bacteria by centrifugation at low speed (300 *g* for 6 min). Subsequently, cells were 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) and homogenized in a ball bearing homogenizer (Pitt et al., 1992) at 4°C. Homogenates were centrifuged at a low speed (400 *g* for 5 min) at 4°C to remove nuclei and unbroken cells. The postnuclear supernatant (PNS) was quickly frozen in liquid nitrogen and stored at −70°C. To obtain the phagosomal fraction, the PNS was quickly thawed and diluted with HB (1:3), and centrifuged at 12,000 *g* for 6 min at 4°C as reported earlier (Mayorga et al., 1991; Pitt et al., 1992). The resultant pellet containing phagosomes was used for in vitro fusion assay. The viability of bacteria in the phagosomes was determined by selective lysis of the phagosomal membrane using PBS containing 0.5% Triton X-100 followed by cultivation of bacteria in LB agar plate. The integrity of the phagosomes was

[1]*Abbreviations used in this paper:* ATPγS, adenosine 5′-0-thiotriphosphate; DSP, dead *Salmonella*-containing phagosome; GDI, GDP dissociation inhibitor; GTPγS, guanine 5′-3-0-(thio) triphosphate; HB, homogenization buffer; LSP, live *Salmonella*-containing phagosome; NEM, *N*-ethylmaleimide; NSF, NEM-sensitive fusion protein; PNS, postnuclear supernatant; SNAP, soluble NSF attachment protein; WT, wild-type.

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checked by measuring the biotin associated with bacteria using avidin-HRP before and after quenching the biotinylated bacteria in broken phagosomes with avidin. About 70% of the phagosomes were estimated to be intact.

Preparation of Endosome

Early endosomes containing avidin-HRP were prepared as described previously (Diaz et al., 1988). 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 h to allow cell surface binding. Internalization was carried out by the addition of prewarmed medium and incubation for 5 min at 37°C to label the early endosomal compartment and uptake was stopped by the addition of ice-cold medium. Similarly, for the preparation of late endosomes, internalization was carried out for 25 min at 37°C. Avidin-HRP is essentially endocytosed via the mannose receptor (Lang and de Chastellier, 1985). Cells were washed with ice-cold medium and homogenized in HB at 4°C and 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 min at 4°C. The supernatant was again centrifuged at 50,000 *g* for 5 min at 4°C. The resultant pellet enriched in early endosomal vesicles was used for in vitro fusion assay. More than 70% of the avidin-HRP activity was recovered in the vesicle preparation suggesting that avidin-HRP is retained in the vesicles.

In Vitro Fusion Assay

Phagosomal fractions containing the biotinylated dead or live *Salmonella* and early endosomes containing avidin-HRP were mixed 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 the scavenger) supplemented with gel-filtered cytosol (Mayorga et al., 1991). Fusion was carried out for indicated periods of time 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 min) 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 fusion unit. Both phagosomes added in the fusion reaction were quantified by protein estimation and same amount of each of the phagosomes (containing dead or live bacteria) was used in a single reaction. Two controls were included in each experiment to determine the total and background activity. Total activity was measured by solubilizing the fusion reaction without avidin as the scavenger. Background values corresponding to bacteria associated HRP activity when the endosomes and phagosomes were mixed in fusion buffer without cytosol or with cytosol but at 4°C were low and were subtracted from the corresponding values to determine specific fusion. The maximum fusion between endosomes and phagosomes was observed at 0.5 mg/ml of cytosol concentration for all the phagosomes, which was expressed as one unit of relative fusion. HRP activity corresponding to one unit in each experiment is mentioned in the figure legends.

Measurement of HRP Activity

HRP activity was measured in a 96-well microplate (Costar Co.) using *O*-phenylenediamine as the chromogenic substrate (Gruenberg et al., 1989). In brief, the final pellet after the fusion reaction was resuspended in 20 μl of PBS and transferred to microplates. The reaction was initiated by adding 100 μl of 0.05 N sodium acetate buffer, pH 5.0, containing *O*-phenylenediamine (0.75 mg/ml) and 0.006% H_2O_2. After 20 min, the reaction was stopped by adding 100 μl of 0.1 N H_2SO_4 and absorbance was measured at 490 nm in an ELISA reader.

Treatment of Phagosomes with GDI

To determine the role of rab protein in phagosome and endosome fusion, phagosomes were treated with rab-GDP dissociation inhibitor (GDI) as described previously (Garret et al., 1994). DSP or LSP (150 μg each) was preincubated with fusion buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml of aprotinin) for 20 min at room temperature in the presence of 1 mM GDP. Subsequently, 6 μg of the purified GDI was added to one set of phagosomes in the reaction mixture and incubation was carried out for another 10 min at room temperature. Phagosomes were sedimented by centrifugation (10,000 *g* for 5 min) and the supernatants were assayed for the presence of rab proteins by Western blot analysis using anti-rab5 antibody as an indicator. The pellet containing the rab-stripped phagosomes was washed with PBS and used for in vitro fusion reaction.

Preparation of Highly Purified Phagosomes

To characterize the phagosomal proteins, phagosomes were further purified as described (Sturgill-Koszyeki et al., 1994). In brief, the phagosomal fraction was resuspended in 100 μl of HB containing protease inhibitors and loaded on 1 ml 12% sucrose cushion. Samples were centrifuged at 1,700 *g* for 45 min at 4°C, and the purified phagosomes were recovered from the bottom of the tube.

The purity of the phagosomes was checked by biochemical analysis to determine the contamination with other cellular component. Plasma membrane contamination was measured as previously described (Desjardins et al., 1994a,b). First the bacteria were internalized for 5 min and washed extensively. Subsequently, the cell surface was labeled with HRP (500 μg/ml) for 30 min at 4°C which is recognized by mannose receptor. Cells were washed and phagosomes were purified. No HRP activity was detected in the purified phagosomes indicating no plasma membrane contamination. Similarly, J774E cells were incubated with HRP for 30 min at 4°C, washed and chased for 90 min to label the lysosome (Ward et al., 1997). Cells were washed and the bacteria were internalized for 5 min. Finally, HRP activity in the purified phagosomes were determined to measure the lysosomal contamination. Most of the HRP activity was found to be present in the lysosomal fraction which showed ~97% of total β-galactosidase activity and no HRP activity was detected in the purified phagosome. We have also measured the β-galactosidase activity in the purified phagosome, which was found to be 3% of the total activity indicating no lysosomal contamination (Ward et al., 1997). The endosome contamination was determined by mixing an aliquot of PNS after bacterial uptake and an aliquot of PNS after 5 min uptake of HRP at 4°C (Alvarez-Dominguez et al., 1996). Phagosomes were purified and endosomal contamination was measured as a percentage of HRP activity present in the phagosome compared with the total activity present in the PNS. Less than 0.2% of the HRP activity in the phagosomal fraction indicates the purity of the phagosome. The galactosyltransferase activity (Bole et al. 1986) was measured to check the Golgi contamination using [^{3}H]UDP-galactose which is found to be ~3% of the total activity in the purified phagosome.

Purified phagosomes (40 μg of protein) were analyzed by 12% SDS-PAGE. The proteins were transferred onto nitrocellulose membrane and checked for the presence of transferrin receptor, rab5, rab7, α-SNAP, and NSF using respective antibodies. Proteins were visualized using appropriate HRP-labeled second antibody and ECL.

Removal of NSF from the Cytosol

To remove NSF from the cytosol, first 100 μl of protein A/G plus–agarose (Santa Cruz Biotechnology) was incubated with 10 μl of anti-NSF antibody (R3230) in PBS overnight at 4°C. The antibody-protein A/G–agarose complex was washed, and centrifuged at 10,000 *g* for 5 min at 4°C. Subsequently, 100 μl of J774E cytosol (600 μg) was added to the protein A/G–agarose anti-NSF complex and incubated for 2 h at 4°C to deplete the NSF from the cytosol. Subsequently, NSF-depleted cytosol was separated from the agarose beads by centrifugation. Immunodepletion of NSF from the cytosol was confirmed by Western blot analysis using an anti-NSF antibody. NSF-depleted cytosol was used for the in vitro fusion assay.

GTP Binding Overlay Assay

DSP and LSP (800 μg protein each) were resuspended in 100 μl of 20 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, and 0.6% CHAPS and incubated for 30 min at 4°C. Subsequently, these were sonicated for 10 s three times at 1-min intervals. Finally, lysates were centrifuged at 100,000 *g* for 1 h (Kikuchi et al, 1995). Rab5 was immunoprecipitated from the resultant supernatant with anti-rab5 antibody conjugated with protein A/G plus–agarose (Santa Cruz Biotechnology) as described in the previous section for NSF. Subsequently, the absorbed proteins on the gel were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes. GTP-binding state of the rab5 was detected in an overlay assay with 1 Ci/ml of α-[^{32}P]GTP (3,000 Ci/mM, NEN) in 50 mM phosphate buffer, pH 7.5, containing 5 mM $MgCl_2$, 1 mM EGTA, and 0.3% Tween 20 as described (Via et al., 1997), and visualized by autoradiography. GTP binding was quantitated in a PhosphorImager.

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Immunolabeling of rab5 and NSF on Dead and Live Salmonella-containing Phagosomes

The NSF content of purified DSP and LSP was determined by immunogold labeling. The phagosomes were washed five times with ice-cold homogenization buffer and sedimented by centrifugation. Samples were then processed for immunolabeling using negative staining technique (Colombo et al., 1996). First, glow-discharged formver and carbon-coated nickel grids were overturned on a drop of vesicle suspension for 2 min. Excess fluid was removed from the grid with filter paper. The specimens were quickly rinsed twice on homogenization buffer (HB) and incubated for 30 min on HB containing 3% skim milk and 0.1% gelatin (blocking buffer). The samples were then incubated for 2 h with mouse anti-NSF antibody (raised against purified recombinant NSF) diluted 1:100 in blocking buffer. Subsequently, the specimens were rinsed three times (5 min each) with blocking buffer and were incubated for 1 h goat anti–mouse conjugated with 12-nm colloidal gold at a 1:20 dilution. The samples were washed twice with HB and fixed in 1% glutaraldehyde in HB for 10 min. Finally, samples were sequentially washed with HB and distilled water, stained with 0.5% aqueous uranyl acetate for 1 min, blotted on filter paper, and air dried. Similarly, we have immunolocalized rab5 on both LSP and DSP using polyclonal rabbit anti-rab5 antibody (1:200 dilution) followed by treatment with goat anti–rabbit IgG conjugated with 18-nm colloidal gold.

Binding of NSF and rab5 on Salmonella-containing Phagosomes

Binding of NSF to the phagosomes was carried out essentially using the procedure described by Colombo et al. (1996). In brief, PNS was diluted with HB containing 0.5 M KCl and incubated for 15 min at 4°C followed by 5 min incubation at 37°C to deplete the endogenous NSF. Subsequently, phagosomes were purified and incubated at 37°C for 10 min in fusion buffer containing 1 mg/ml of cytosolic proteins supplemented with 60 ng of purified NSF. Finally, phagosomes were washed and the samples were analyzed by Western blot using anti-NSF antibody.

To determine the binding of rab proteins, phagosomes were treated with Rab-GDI as described earlier. Recruitment of the rab5 by the respective phagosomes was carried out by incubating the phagosomes at 37°C for 10 min in fusion buffer containing 1 mg/ml of cytosolic proteins supplemented with 30 ng of purified GST-Rab5. Presence of rab5 on the phagosomes was determined by Western blot analysis using anti-rab5 antibody.

Detection of rab5-binding Protein from Salmonella

To detect the rab5 binding protein from *Salmonella*, the bacteria were grown overnight at 37°C with constant shaking (300 rpm) in Luria broth (LB) containing 300 mM NaCl to induce the secretion of *Salmonella* secretory proteins (Chen et al., 1996). Subsequently, the cells were removed by low-speed centrifugation and the medium was collected. Medium was concentrated using Amicon membrane (10 kD cut off) at 4°C. GST-Rab5 (200 μg) was immobilized with glutathione beads and incubated in the presence of concentrated spent medium (300 μg) for 10 h at 4°C. Beads were washed (10,000 *g* for 5 min) three times to remove unbound proteins. Subsequently, the proteins were separated by 12% SDS-PAGE and were transferred onto nitrocellulose membrane. The *Salmonella* proteins were detected by Western blot analysis using respective antibodies against SopE, SopB, and SipC. Proteins were visualized using appropriate HRP-labeled second antibody by ECL. Similarly, GST-Rab7 and free GST were used as control.

Results

In Vitro Fusion of Endosomes with Phagosomes Containing Live and Dead Salmonella

The results presented in Fig. 1 a show a typical in vitro fusion experiment in which phagosomes containing dead or live bacteria (WT and mutant *Salmonella*) were incubated for 5 min with endosomes loaded with avidin-HRP at 37°C, in presence of different concentrations of cytosol in ATP regenerating system. The extent of fusion of early endosomes with phagosomes containing live WT or live mutant *Salmonella* were similar in the presence of cytosol and reached a maximum at ~0.5 mg/ml cytosol. In contrast, <40% fusion was observed between early endosomes and DSP in comparison to fusion of LSP with early endosomes under similar conditions.

The data presented in Fig. 1 b compare the time course of the fusion between the early endosomes and LSP or DSP. LSP revealed a much faster fusion rate where ~70% fusion was achieved in 5 min. In contrast, DSP showed ~30% of the fusion activity at a similar time point. DSP required ~20 min to achieve the same fusion activity as exhibited by LSP after only 5 min. However, fusion of both LSP and DSP with early endosomes reached a similar steady-state at ~60 min presumably due to relatively slow, nonspecific acquisition of the fusion factors from the cytosol in the incubation mixture by DSP during the prolonged incubation period. The results presented in Fig. 1 c show that both LSP and DSP fuse with early endosomes with similar efficiency in a 60-min fusion assay. In contrast, relatively more fusion was observed between late endosomes with DSP in comparison to the fusion between late endosomes with LSP under similar conditions (Fig. 1 c). To characterize the fusion of LSP or DSP with early endosomes at the same level, we have chosen 60-min time point for the subsequent fusion assay.

Fusion between early endosomes and DSP or LSP at high cytosol concentration (1.2 mg/ml) was inhibited by ~75% by GTPγS (25 μM), a nonhydrolyzable analogue of GTP, indicating that GTP hydrolysis is required for the fusion (Fig. 1 d). No fusion was observed when these preparations were incubated in ATP regenerating system at 4°C (data not shown). Moreover, pretreatment of the DSP or LSP with trypsin totally abrogated fusion indicating the role of proteins present on the phagosomal membrane (data not shown).

Role of rab Proteins in Fusion between LSP or DSP with Early Endosomes

Since vesicle fusion is regulated by ras-related rab GTPases, we determined the role of rab GTPases in the fusion of endosomes with LSP or DSP. Treatment of phagosomes with rab-GDI in presence of GDP selectively depleted the rab proteins from the phagosomal membrane and released them in the supernatant as shown in Fig. 2 a, indicating the removal of rab proteins by GDI-GDP treatment (Ikonen et al., 1995; Dirac-Svejstrup et al., 1994). In contrast, transferrin receptor was retained on the phagosomes under the same treatment. But the treatment of the phagosomes with GDP alone was unable to remove the rab protein and the transferrin receptor from the phagosomes (Fig. 2 a). Thus, GDI-GDP treatment selectively removed the rab proteins from the phagosomes. Subsequently, rab-depleted DSP or LSP were used in the fusion assay. As shown in Fig. 2 b, ~80% of the fusion was inhibited when the rab proteins were selectively depleted from the phagosomes.

To characterize the phagosomes, purified LSP and DSP were analyzed for the presence of early endocytic markers, transferrin receptor and two endocytic rabs, viz., rab5 and rab7 (Grovel et al., 1991; Barbieri et al., 1994; Feng et al.,

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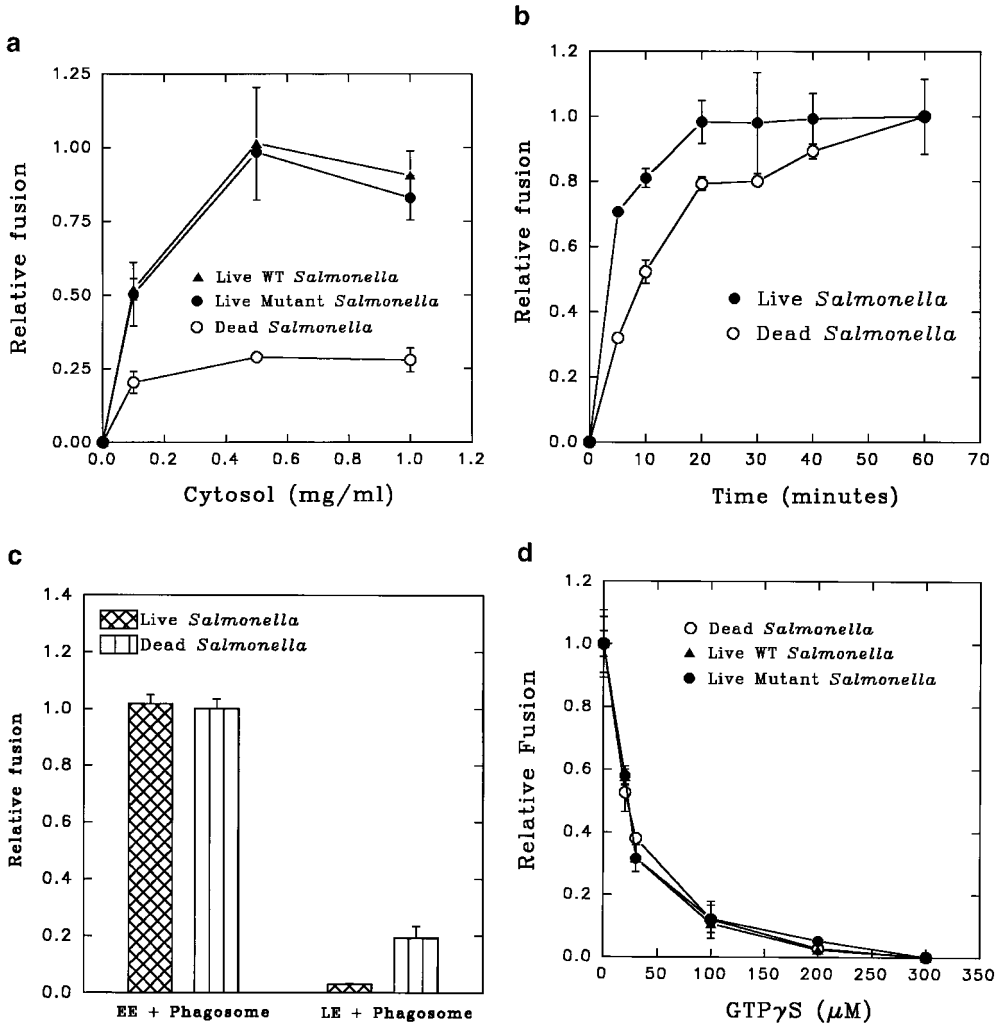

Figure 1. Cytosol-dependent fusion of endosomes with phagosomes containing dead or live *Salmonella.* (a) Early endosomes containing avidin-HRP were incubated with phagosomes containing dead or live biotinylated *Salmonella* in ATP regenerating fusion buffer supplemented with different concentrations of gel-filtered cytosol for 5 min at 37°C. Fusion was measured as indicated in Materials and Methods. 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 WT *Salmonella* phagosomes. (b) Fusion assays were performed as described under Materials and Methods using 0.5 mg/ml cytosol in ATP regenerating system. At indicated time, fusion was stopped by chilling on ice and measured as described. Maximum fusion obtained in LSP or DSP assay was chosen as one unit and the results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to ~8.3 and 9.6 ng HRP activity/mg of protein in the fusion assay containing LSP and DSP, respectively. (c) Late endosomes containing avidin-HRP were incubated with phagosomes containing dead or live biotinylated *Salmonella* in ATP regenerating fusion buffer supplemented with 0.5 mg/ml gel-filtered cytosol for 60 min at 37°C. Fusion was measured as indicated in Materials and Methods. Fusion observed with early endosomes with LSP was normalized to one unit and 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. EE, early endosome; LE, late endosome. (d) Fusion between endosomes and phagosomes containing live or dead *Salmonella* were carried out in ATP regenerating system in the presence of different concentrations of GTPγS at high cytosol concentration (1.2 mg/ml). Fusion obtained in the absence of GTPγS was chosen as one unit and the results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to ~10.7 ng, 10.2 ng, and 9.6 ng of HRP activity/mg of protein in the fusion assay containing live WT, live mutant and dead *Salmonella* phagosomes, respectively, in the absence of GTPγS.

1995; Mukhopadhyay et al., 1997a). Data presented in Fig. 2 c demonstrate that LSP recruit more rab5, an early endosomal marker, than the DSP. In contrast, DSP recruit more rab7, a late endosomal marker, than the LSP. Moreover, both LSP and DSP expressed relatively equivalent amount of transferrin receptors suggesting that both bacteria remain in early compartment. Quantitation of rab5 and rab7 on LSP and DSP by densitometry indicated that 275 ± 9.7 arbitrary units of rab5 and 132 ± 9.16 arbitrary units of rab7 are present on LSP compared with 57 ± 5.7 arbitrary units of rab5 and 306 ± 15.5 arbitrary units of rab7 are present on DSP. Moreover, the results presented in Fig. 2 d also confirmed that LSP recruits more rab5 in the GTP-bound state than DSP. Quantitation of the rab5 in GTP-bound state on LSP and DSP by PhosphorImager indicated that ~2.5-fold of rab5 in GTP-bound form is present on LSP compared with rab5 on DSP. Immunolocalization studies also revealed presence of higher amounts of rab5 on LSP than on DSP (Fig. 2 e).

Role of ATP in the Fusion of Endosomes with LSP and DSP

To analyze the energy requirements in the fusion between the endosomes with LSP or DSP, fusion was carried out for 60 min in ATP depleting system (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 containing 5 mM glucose and 25 units/ml hexokinase, and 0.25 mg/ml avidin as the scavenger). Under these conditions, ATP was completely de-

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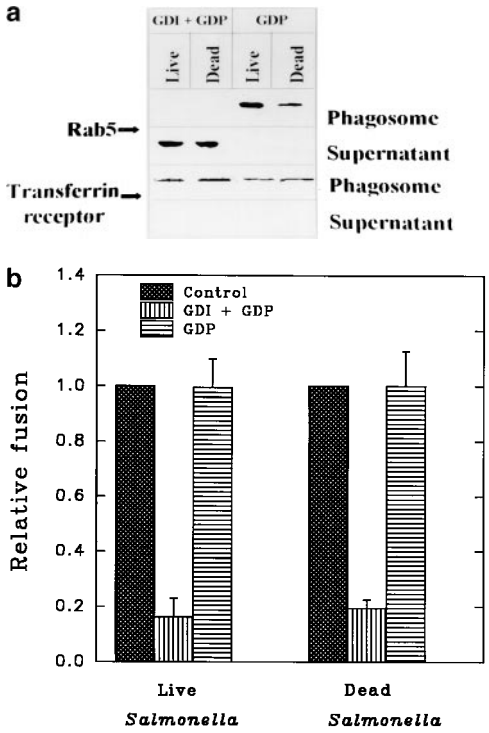

pleted from the fusion system as determined by luciferase assay (data not shown). Data presented in Fig. 3 indicate that the fusion of endosomes with DSP was sensitive to ATP depletion. However, the fusion between endosomes and LSP was insensitive to ATP depletion. In contrast, fusion of endosomes with both LSP and DSP were inhibited by ATPγS in ATP regenerating system (Fig. 3), indicating that ATP hydrolysis is required for both fusion events.

Role of NSF in Fusion of Endosomes with LSP and DSP

The fusion of endocytic vesicles involves stepwise maturation and recruitment of several factors from the cytosol. One of these, a NEM-sensitive factor (NSF), is a ubiquitous protein required for multiple vesicular transport events (Beckers et al., 1989; Diaz et al., 1989). We determined the role of NSF by treating LSP or DSP and cytosol separately with NEM and using them in various combinations in the fusion assay under steady state conditions (60 min) when both LSP and DSP fused with early endosomes to the same extent. The results presented in Fig. 4 a show that NEM treatment of cytosol or DSP inhibited fusion of DSP with early endosomes. In contrast, fusion of LSP (WT and mutant) with early endosomes was not inhibited

Figure 2. Role of rab proteins in fusion of LSP or DSP with early endosomes. (a) LSP or DSP were treated either with GDP (1 mM) alone or with GDI (6 μg/ml) as described in Materials and Methods. Subsequently, treated phagosomes and resultant supernatants were assayed for the presence of rab proteins by Western blot analysis using anti-rab5 antibody. Transferrin receptor was used as a control. (b) LSP or DSP treated either with GDP or GDP-GDI were analyzed in in vitro fusion assay with endosomes. Fusion obtained with untreated phagosomes was chosen as one unit and the results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to ~25.3 ng and 26.5 ng of HRP activity/mg of protein in the fusion assay containing live and dead *Salmonella* phagosomes, respectively. (c) DSP or LSP (40 μg protein each per lane) was electrophoresed and transferred to nitrocellulose membranes. After incubation with specific antibodies against rab5, rab7, and transferrin receptor, the proteins were visualized using appropriate HRP-conjugated second antibodies and ECL. (d) DSP and LSP (800-μg protein each) were solubilized and rab5 were immunoprecipitated from both LSP and DSP as described in Materials and Methods. GTP-binding state of the rab5 was detected on LSP or DSP by an α-[^{32}P]GTP overlay assay and visualized by autoradiography. (e) DSP or LSP was 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 as described in Materials and Methods. (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.

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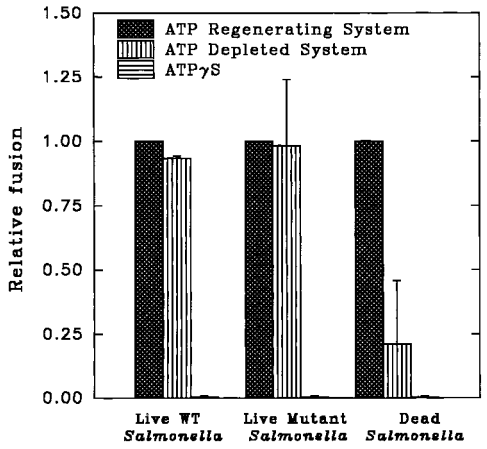


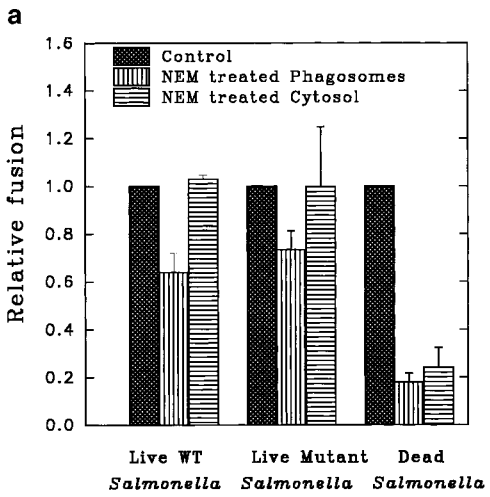
Figure 3. Role of ATP in the fusion of endosomes with LSP or DSP. In vitro fusion between live or dead *Salmonella*-containing phagosomes were carried out either in the presence of ATP regenerating system (control) or in the presence of ATP-depleted system as indicated in Materials and Methods. Fusion of respective phagosomes with endosomes was also analyzed in the presence of ATPγS (30 μM) in fusion buffer containing ATP regenerating system. Fusion obtained with untreated phagosomes was chosen as one unit and the results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to ~14.4, 13.4, and 14.7 ng of HRP activity/mg of protein in the fusion assay with phagosomes containing live WT, live mutant and dead *Salmonella*, respectively.

by NEM-treated cytosol or by treatment of respective phagosomes with NEM.

To further substantiate the requirement of NSF, NSF immunodepleted cytosol was used in the fusion assay. About 80% of the fusion between DSP with early endosomes was inhibited when the fusion was carried out using either untreated phagosomes in NSF-depleted cytosol or the phagosomes treated with NEM in the presence of normal cytosol. Almost complete inhibition of fusion was observed when DSP were treated with NEM and the fusion was carried out in NSF-depleted cytosol (Fig. 4 b). In contrast, fusion of LSP with endosomes in NSF-depleted cytosol showed only 20% inhibition. Moreover, when NSF on the LSP was inactivated by NEM treatment and the fusion with early endosomes was analyzed in the presence of normal cytosol, ~65% fusion was observed. However, when LSP were treated with NEM, the fusion with early endosomes in the presence of NSF-depleted cytosol was totally abrogated (Fig. 4 b).

Recruitment of NSF by LSP

To determine the role of NSF recruitment in the fusion between LSP or DSP with early endosome, first the NSF activity on the respective phagosomes was inactivated by NEM treatment and then the fusion was determined in the presence of NSF-depleted cytosol containing different concentrations of NSF:WT or NSF:D1EQ (Whiteheart et al., 1994) mutant proteins. In the absence of NSF, phagosome-endosome fusion was totally abrogated. However, ~80% of the fusion between LSP and early endosomes was restored by adding 100 ng/ml of NSF:WT protein. In contrast, same concentration of NSF:WT protein stimulated only ~10% of the fusion between DSP and early endosomes. Maximum fusion between LSP with early endosomes was observed at ~250 ng/ml of NSF, whereas same extent of fusion between DSP with early endosomes was achieved at ~500 ng/ml of NSF. Addition of negative mutant of NSF (NSF:D1EQ) did not stimulate the fusion in either system (Fig. 5 a). More efficient recovery of fusion between LSP and endosomes by the addition of NSF may



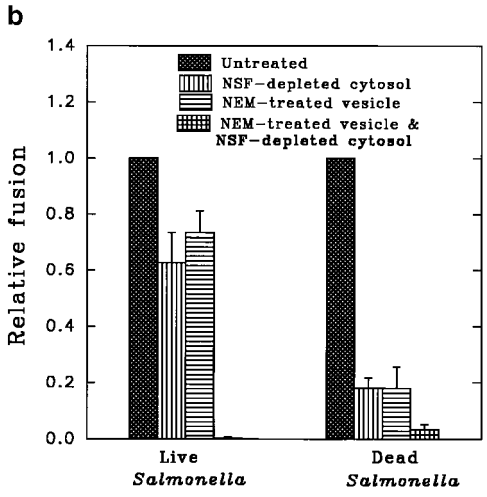


Figure 4. Role of NSF in the endosome fusion with live or dead *Salmonella*-containing phagosomes. (a) DSP, LSP, and cytosol preparations were treated separately with NEM (3 mM; 30 min at 4°C). Before using in fusion assay, excess NEM was quenched with 3 mM dithiothreitol. Fusion was carried out in ATP regenerating system. Untreated phagosomes in normal cytosol (▩), NEM-treated phagosomes in normal cytosol (▥), and untreated phagosomes in NEM-treated cytosol (▤). Fusion obtained with untreated phagosomes was chosen as one unit and the results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to ~15.6, 17.3, and 16.8 ng of HRP activity/mg of protein in the fusion assay with phagosomes containing live WT, live mutant, and dead *Salmonella*, respectively. (b) Fusion assay was carried out in the presence of NSF immunodepleted cytosol as indicated in the Materials and Methods. Untreated phagosomes in normal cytosol (▩), untreated phagosomes in NSF-depleted cytosol (▥), NEM-treated phagosomes in normal cytosol (▤), and NEM-treated phagosomes in NSF-depleted cytosol (⊞) using DSP or LSP. Fusion obtained in control assay was chosen as one unit and the results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to ~13.6 and 14 ng of HRP activity/mg of protein in the fusion assay containing LSP or DSP, respectively.

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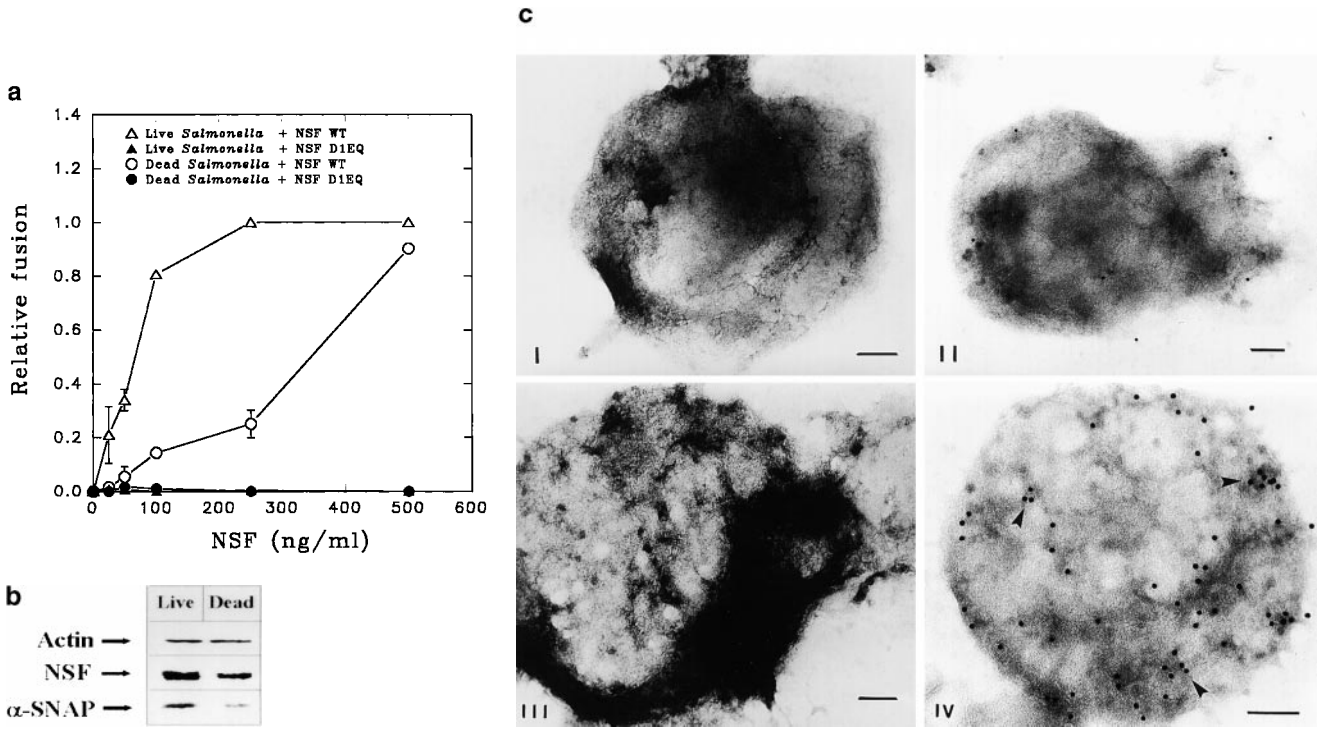

Figure 5. Recruitment of NSF by live *Salmonella*-containing phagosomes. (a) NSF on LSP or DSP were inactivated by NEM treatment and the fusion was measured in the presence of NSF-depleted cytosol containing different concentrations of NSF:WT or NSF:D1EQ mutant proteins. Maximum fusion obtained in live *Salmonella* containing phagosome assay was chosen as one unit and the results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to ~13.6 and 14 ng of HRP activity/mg of protein in the fusion assay containing LSP or DSP, respectively. (b) DSP or LSP (40 μg protein each per lane) was electrophoresed and transferred to nitrocellulose membranes and incubated with anti-NSF, anti-αSNAP, or anti-Actin antibody followed by HRP-conjugated second antibodies and developed using ECL. (c) DSP or LSP was incubated with specific mouse anti-NSF antibody for 2 h at room temperature followed by treatment with goat anti–mouse antibody conjugated with 12-nm colloidal gold particles as described in Materials and Methods. (I and II) DSP; (III and IV) LSP. In I and III, phagosomes were processed for negative staining without primary anti-NSF antibody. Arrow in IV shows the presence of NSF on LSP as revealed by 12-nm gold particles. Bar, 100 nm.

be due to efficient recruitment of NSF on phagosomes by the live *Salmonella.* Therefore, the amount of NSF and their receptor, α-SNAP, on the phagosomal membranes was compared by Western blot analysis using specific antibodies. Western blot analysis revealed the presence of more NSF and α-SNAP on the LSP than on DSP but the amount of actin remains same indicating that similar amount of LSP and DSP have differential level of NSF and α-SNAP (Fig. 5 b). Quantitation of NSF and α-SNAP on LSP and DSP by densitometry indicated that 237 ± 21 arbitrary units of NSF and 153 ± 11 arbitrary units of α-SNAP are present on LSP compared with 97 ± 5 arbitrary units of NSF and 38 ± 10 arbitrary units of α-SNAP present on DSP. These observations were further strengthened by immunolocalization of more NSF on LSP than DSP using second antibody labeled with colloidal gold (Fig. 5 c).

NSF Recruitment Is Dependent on the Viability of Salmonella in Phagosomes

To determine whether presence of viable *Salmonella* in the phagosomes drives more efficient NSF-dependent fusion, LSP were treated with ciprofloxacin (500 μg/ml in HB) at 4°C for 30 min to kill the resident bacteria. The ciprofloxacin-treated phagosomes (CF-LSP) fused with early endosomes as efficiently as LSP or DSP in cytosol containing ATP regenerating system (data not shown). When CF-LSP were treated with NEM and analyzed in NSF-depleted cytosol, the efficiency of the fusion was significantly lower than that with the LSP. Only 20% of the fusion between endosomes with CF-LSP were recovered by the addition of 100 μg/ml of NSF:WT protein while the same concentration of NSF:WT promoted ~90% of the fusion between endosomes and untreated LSP under similar conditions (Fig. 6 a). The fusion obtained with CF-LSP closely resembled the fusion observed with DSP indicating that presence of viable *Salmonella* is required for efficient recruitment of NSF. The results presented in Fig. 6 b show that CF-LSP were unable to retain rab5 and NSF on the phagosomal membrane suggesting that the continuous signal from the viable organism is required for the recruitment of these proteins on the phagosomes.

Binding of NSF and rab5 on Salmonella-containing Phagosomes

To determine the binding of NSF to the phagosomes, KCl-

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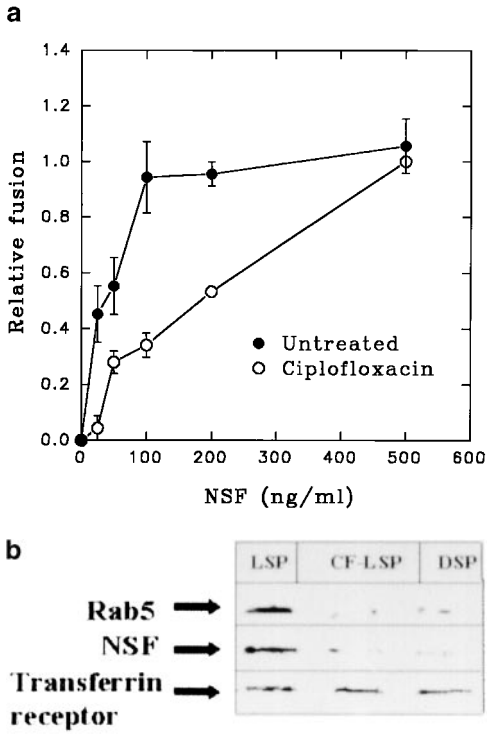

Figure 6. NSF recruitment is dependent on the presence of live *Salmonella* in phagosome. (a) LSP were prepared as described and treated with ciprofloxacin (500 μg/ml) at 4°C for 30 min. NSF on untreated and antibiotic-treated phagosomes were determined as described in Fig. 5 a. Maximum fusion obtained in LSP assay was chosen as one unit and the results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to ~8.92 and 10.3 ng of HRP activity/mg of protein in the fusion assay containing live and dead *Salmonella* phagosomes, respectively. (b) Determination of rab5 and NSF on different *Salmonella*-containing phagosome. LSP and DSP were prepared as indicated and bacteria in LSP was killed by ciprofloxacin inside the phagosome (CF-LSP). Phagosomes were washed and the presence of rab5 and NSF on different phagosomes were determined by Western blot analysis using specific antibodies.

treated LSP, CF-LSP, and DSP were incubated in the presence of purified NSF in fusion buffer containing cytosol for 10 min at 37°C. Treatment of the phagosomes by 0.5 M KCl selectively stripped the endogenous NSF leaving the rab5 on the phagosomal membrane as revealed by Western blot analysis (data not shown). The result presented in the Fig. 7 (upper panel) shows efficient binding of NSF by the LSP. No detectable binding of NSF was observed with CF-LSP or with DSP.

To understand the mechanism of NSF recruitment by LSP, the phagosomes were treated with Rab-GDI, which deplete the endogenous rabs as well as NSF, as determined by Western blot analysis (data not shown). Data presented in Fig. 7 (second panel) show that Rab-GDI–treated LSP were unable to bind NSF indicating that the presence of rab protein is required for NSF binding. In contrast, the result presented in Fig. 7 (third panel) shows efficient binding of rab5 by the LSP in comparison to CF-LSP as well as DSP. Moreover, addition of rab5:S34N, a dominant-negative mutant that is locked in GDP form, was unable to bind LSP, whereas Rab5:Q79L, a GTPase-defective mutant, efficiently binds with LSP (data not shown). In contrast, no significant binding of rab7 was observed under similar conditions (Fig. 7, lower panel).

Detection of rab5-binding Protein from Salmonella

Salmonella have evolved a complex protein secretion system termed type III to deliver the bacterial effector proteins into the host cells that modulate the host cellular function (Galan and Collmer 1999; Uchiya et al., 1999). To detect the rab5-binding protein from *Salmonella*, immobilized GST-Rab5, GST-Rab7, or GST alone was incubated in the presence of concentrated spent medium for 10 h at 4°C. Subsequently, *Salmonella* proteins bound to respective beads were detected by Western blot analysis using antibodies against SopE, SopB and SipC. The results presented in Fig. 8 a show that rab5 specifically binds with SopE but not with SopB and SipC (middle panel). In contrast, rab7 (upper panel) and GST (lower panel) alone were unable to bind any of these secretory proteins from the *Salmonella* extract. Moreover, when immobilized GST-Rab5 was incubated with a *Salmonella* lysate obtained after growing the cells in the presence of ^{35}S-methionine, specifically a 30-kD protein associated with immobilized GST-Rab5 was detected by autoradiography (data not shown). Western blot analysis of cytosols from uninfected or *Salmonella*-infected macrophages with anti-SopE antibody revealed the presence of SopE only in the infected cytosol (Fig. 8 b).

Discussion

The intracellular trafficking of phagosomes depends on the membrane composition as well as intravesicular content. Recent studies have shown that the process of phagosome maturation is complex and requires extensive re-

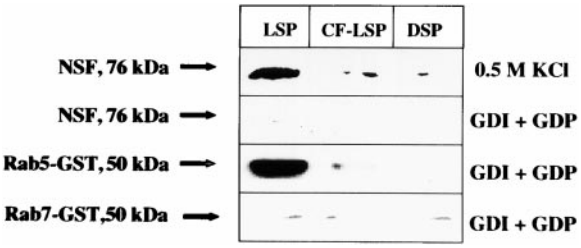

Figure 7. Binding of NSF and rab5 on different phagosomes. (Top) Phagosomes stripped of endogenous NSF by 0.5 M KCl treatment were incubated in presence of purified NSF in the fusion buffer containing cytosol as described in Materials and Methods. Presence of NSF on the phagosomes was determined by Western blot using specific antibody using ECL. Phagosomes were treated with Rab-GDI to remove endogenous rab5 and NSF and incubated in presence of purified NSF, rab5, or rab7 in the fusion buffer containing cytosol as described in Materials and Methods. Presence of NSF (second row), rab5 (third row), and rab7 (bottom) on respective phagosomes was determined using specific antibodies by Western blot using ECL. LSP, live *Salmonella*-containing phagosome; CF-LSP, LSP treated with ciprofloxacin; DSP, dead *Salmonella*-containing phagosome.

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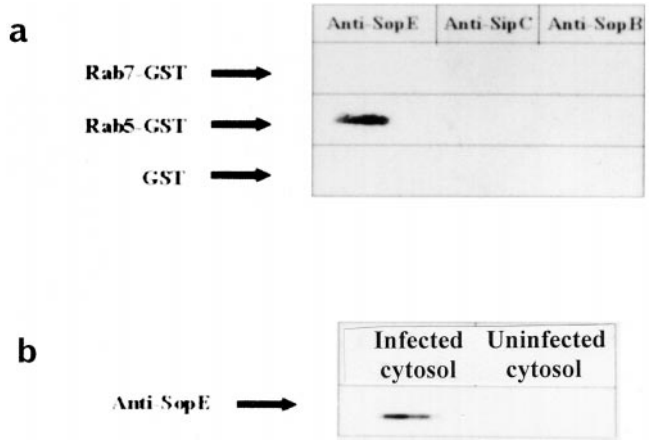

Figure 8. Detection of rab5-binding protein from *Salmonella*. (a) To detect the rab5-binding protein, GST pull-out assay was carried out with GST-Rab5 (middle), GST-Rab7 (top), or GST alone (bottom) in the presence of concentrated *Salmonella* spent medium as described in Materials and Methods. The *Salmonella* proteins associated with respective beads were detected by Western blot analysis using antibodies against SopE, SopB, and SipC. (b) J774E macrophages were infected with live *Salmonella* as described in Materials and Methods. Cells were washed and incubated for 10 h, and subsequently, cytosol was purified from the infected cells. Western blot analysis was carried out to determine the presence of Sop E in the respective cytosol using specific antibody.

modulation of the phagosomal membrane (Kornfeld and Mellman, 1989; Desjardins et al., 1994a,b; Beron et al., 1995). Some of these changes are mediated by fusion with the other endocytic vesicles which live organisms often use for their survival. For instance, some microorganisms modulate this process to avoid transport to lysosomes for their survival in phagocytes (Portillo and Finlay, 1995a,b).

To understand the sorting events in the early compartments that modulate the intracellular destiny of *Salmonella* in macrophages, we used a biochemical assay to determine the in vitro fusion of endosomes with LSP or DSP. We found that LSP fuses with early endosomes more efficiently (within 5 min) than DSP (Fig. 1 a) due to efficient recruitment of the fusion factors like rab5 and NSF on LSP (Figs. 5 a and 7). However, by 60 min (Fig. 1 b) the fusion of LSP or DSP with early endosomes was found to be similar presumably reflecting the relatively slow, nonspecific acquisition of the fusion factors (Figs. 5 a and 7) by DSP during prolonged incubation period. Moreover, no significant fusion was observed between LSP with late endosomes (Fig. 1 c). The difference in the efficiency of fusion between LSP or DSP with early endosomes was not due to differential rates of uptake of live or dead *Salmonella* by macrophages, which were found to be similar (data not shown).

GTPγS, a nonhydrolyzable analogue of GTP, inhibits the fusion of phagosomes with early endosomes at a high cytosol concentration irrespective of whether LSP or DSP were used, suggesting that one or more GTPases may be regulating this process (Fig. 1 d). Selective removal of the rab proteins from both LSP and DSP (Fig. 2 a) by GDI in the presence of GDP, inhibited the fusion of phagosomes with early endosomes by ~80% compared with the untreated control (Fig. 2 b). These results are consistent with previous observation that phagosome-lysosome fusion is inhibited by GDI-GDP treatment but not with GDP alone (Funato et al., 1997). Since Rab-GDI is known to inhibit several vesicular transport pathways by removal of the rab proteins from the intracellular vesicles (Dirac-Svejstrup et al., 1994; Ullrich et al., 1994), our results indicate the requirement of rab-GTPase in the fusion of *Salmonella* containing phagosomes with endosomes.

Distinct rab proteins, rab5 and rab7, are known to be associated with early and late endosomes, respectively. Rab5 mediates the homotypic fusion among early endosomes and phagosomes (Grovel et al., 1991; Bucci et al., 1992; Desjardins et al., 1994a,b; Barbieri et al., 1996; Jahraus et al., 1998) while rab7 on the late endosome regulates the transport between early to late compartments (Schimmoller and Riezman, 1993; Feng et al., 1995; Hass et al., 1995; Funato et al., 1997; Mukhopadhyay et al., 1997b). Moreover, rab5 function is upstream of rab7 in endocytosis (Mukhopadhyay et al., 1997a). By Western blot analysis with specific antibodies, we showed that LSP recruit more rab5, an early acting rab, than the DSP (Fig. 2, c and e). In contrast, DSP accumulate more rab7, the late acting rab, than LSP. Our results suggest that DSPs with increased rab7 content behave like phagosomes containing inert particles that are destined to fuse with lysosomes. We found that the presence of live *Salmonella* within phagosomes correlates with efficient recruitment of rab5 in GTP bound state on the phagosomal membrane (Figs. 2, c–e, and 7) which promotes efficient fusion with early endosomes (Fig. 2, a and b), thereby inhibiting maturation of LSP towards the lysosomal pathway. We also noted that biotinylated dead *Salmonella* colocalizes with avidin-HRP preloaded lysosomes within 45 min, however, the colocalization was not observed with live *Salmonella* even when the bacteria was chased for 90 min (data not shown). It has been shown that *Salmonella* survive in relatively large membrane-bound vesicles (Alpuche-Aranda et al., 1994). It could be due to the rab5-mediated fusion of live *Salmonella*-containing phagosomes with early endosomal compartment as it has been shown that overexpression of the GTPase-defective mutant of rab5 led to the appearance of unusually large endocytic vesicles (Stenmark et al., 1994). Studies with *Listeria*- and *Mycobacteria*-containing phagosomes have also shown that live bacteria containing early phagosomes are enriched in rab5 (Alvarez-Dominguez et al., 1996; Via et al., 1997) and thereby inhibit their maturation.

Previous studies have shown that vesicle fusion is energy dependent. ATP is required for endosome-endosome fusion, endosome-phagosome fusion, fusion between the Golgi vesicles, ER to Golgi transport (Beckers et al., 1989; Diaz et al., 1989; Mayorga et al., 1991), as well as the transport of phagosomes to the lysosomes (Funato et al., 1997). However, the energy requirements for the fusion of early endosomes with LSP or DSP are quite different (Fig. 3). Similar to the earlier observations, fusion of DSP with the early endosomes was sensitive to ATP depletion. In contrast, fusion of LSP (WT or mutant) with early endosomes was not inhibited by ATP depletion. It is possible that the energy required for the fusion with LSP with endosomes is

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supplied by the live bacteria. However, we ruled out this possibility as no ATP activity was detected when LSP were incubated in the presence of gel-filtered cytosol containing ATP-depleted system (data not shown). Alternatively, live *Salmonella* somehow acquire ATP-sensitive factors (e.g., NSF) on the phagosomal membrane, which is required for the docking and the fusion. However, addition of ATPγS, a nonhydrolyzable analogue of ATP, completely abrogated fusion of endosomes with both phagosomes, suggesting that ATP hydrolysis is required for the fusion (Fig. 3).

NEM-sensitive fusion protein (NSF) restores the in vitro transport activity of the Golgi membrane fractions treated with NEM (Malhotra et al., 1988) and is required for intra-Golgi transport, endoplasmic reticulum-Golgi transport, as well as fusion between the early endosomes, suggesting that NSF is a general component of the fusion machinery (Beckers et al., 1989; Diaz et al., 1989). NSF is a homohexamer of 76-kD subunits and it has both ATP binding and hydrolyzing activity (Wilson et al., 1989; Tagaya et al., 1993, Whiteheart and Kubalek, 1995). Recent studies have shown that association of NSF to synaptic vesicles (Hong et al., 1994), clathrin-coated vesicles (Steel et al., 1996), and endosomes (Robinson et al., 1997) are independent of Mg^{2+}-ATP. Thus, NSF association to these membranes occurs via ATP-independent mechanisms. The ATP binding and hydrolyzing activity of NSF probably favors recruitment of NSF by the LSP, and thereby results in insensitivity to ATP depletion (Fig. 3). We, therefore, investigated the role of NSF in the fusion of LSP and DSP with endosomes (Fig. 4 a). The fusion of DSP with endosomes was inhibited either by inactivating the NSF on DSP by NEM treatment or by using NEM-treated cytosol in the fusion assay. In contrast, NEM treatment of cytosol did not affect the fusion of LSP with early endosomes suggesting that NSF present on LSP is sufficient to promote the fusion of LSP with early endosomes. No significant inhibition was observed when fusion was carried out with NEM-treated LSP with early endosomes in ATP regenerating system suggesting that live *Salmonella* selectively recruit NSF from the cytosol. To determine the role of NSF unequivocally, NSF-immunodepleted cytosol was used in the fusion between DSP or LSP with early endosomes (Fig. 4 b). NSF-depleted cytosol did not support the fusion between the DSP with the early endosomes. However, no significant inhibition was observed using LSP under similar conditions.

To demonstrate the recruitment of NSF from cytosol by LSP or DSP, we inactivated the NSF on the phagosomes by NEM treatment and carried out the fusion in NSF-depleted cytosol containing different concentrations of NSF:WT and NSF:D1EQ mutant proteins. No fusion of LSP with early endosomes was observed in the absence of NSF (Fig. 4 b). The results presented in Fig. 5 a show that the fusion of LSP with endosomes is quickly restored by low concentrations of NSF:WT protein suggesting faster recruitment of NSF by the LSP compared with that by the DSP (Fig. 7). Moreover, fusion between LSP with early endosomes saturates at a low concentration of NSF and the fusion between DSP with early endosomes is linear suggesting the possibility that nonspecific binding of NSF with DSP during prolonged incubation (60 min) has equated the fusion. However, ATP binding and hydrolysis is required as NSF:D1EQ mutant, which is known to inhibit the fusion/transport (Colombo et al., 1996; Mukhopadhyay et al., 1997b), does not restore this fusion. The content of NSF and α-SNAP on the LSP was also much higher than that on the DSP as is evident from Western blot analysis and immunoelectron microscopy (Fig. 5, b and c). It will be of interest to determine how live *Salmonella* recruit the NSF on the phagosomal membrane. The results presented in Fig. 7 show that KCl-treated LSP bind exogenous NSF more efficiently than DSP or LSP that was treated with ciprofloxacin after isolation (CF-LSP) to kill the bacteria. KCl treatment of the phagosomes selectively remove the NSF leaving rab5 on the phagosomes (Alvarez-Dominguez et al., 1996), suggesting that the NSF recruitment by the LSP depends on the prior recruitment of rab 5. The inefficient fusion of both DSP and CF-LSP with early endosomes (Fig. 6 b) correlates with their failure to retain rab5 and NSF on the phagosome (Fig. 6 b), suggesting that survival of the bacteria is required for retaining the rab and NSF on the phagosome. Since Rab-GDI–treated LSP are unable to bind NSF on the phagosome, we investigated the recruitment of rab5 by the LSP. The data in Fig. 7 show that LSP efficiently and specifically bind rab5 but not rab7. In contrast, no significant binding of rab5 was detected with DSP or CF-LSP indicating that live bacteria provide the signal for retaining rab5 and NSF on the phagosome. Our results have further shown that rab5 specifically binds with SopE, a type III secretory protein of *Salmonella* (Fig. 8 a), and we have also detected the same protein in *Salmonella*-infected macrophage cytosol (Fig. 8 b). These results are consistent with the previous observations that show the translocation of type III secretory proteins including SopE from the phagosomes to cytosol (Hardt et al., 1998; Galan and Collmer, 1999; Uchiya et al., 1999). Thus, it is possible that SopE present on the LSP efficiently recruits the rab5 in GTP form (Fig. 2 d), activates the SNARE that recruits more α-SNAP (Fig. 5 b), the NSF receptor, and thereby triggers the binding of NSF on LSP. A recent study by Hardt et al. (1998) has also shown that SopE stimulates GDP to GTP nucleotide exchange of several Rho GTPases. Thus, the retaining of rab5 on the LSP may be due to the inhibition of the rab5-GTPase activating protein (GAP) activity by the live organism as it has been shown that GAP increase the GTPase rate of the rab protein, converting it into its GDP form and triggers the release of the rab to the cytosol by GDI. Thus, the recruitment of the NSF by the live *Salmonella* phagosomes is found to be a downstream effect of the rab5.

In conclusion, our results represent the first documentation that live *Salmonella* selectively recruit rab5 in GTP form that possibly activates the SNARE to recruit more α-SNAP and NSF and thereby promote fusion with the early endosomal compartment. The live *Salmonella*-driven fusion with the early endosomal compartment may extend the period of bacterial residence in the less acidic early endosomal compartment, thereby inhibiting the transport of the live *Salmonella* to the lysosomes.

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