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NSF independent fusion of *Salmonella*-containing late phagosomes with early endosomes

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ABSTRACT

Initial characterizations of live-*Salmonella*-containing early (LSEP) and late phagosomes (LSLP) in macrophages show that both phagosomes retain Rab5 and EEA1. In addition, LSEP specifically contain transferrin receptor whereas LSLP possess relatively more rabaptin-5. In contrast to LSLP, late-*Salmonella*-containing vacuoles in epithelial cells show significantly reduced levels of Rab5 and EEA1. Subsequent results demonstrate that both phagosomes efficiently fuse with early endosomes (EE). In contrast to LSEP, fusion between LSLP and EE is insensitive to ATPγS treatment. Furthermore, LSLP fuses with EE in absence of NEM-sensitive fusion factor (NSF) as well as in the presence of NSF:D1EQ mutant demonstrating that LSLP fusion with EE is NSF independent.

1. Introduction

Phagosome maturation depends on the fusion with various intracellular compartments which is regulated by distinct sets of Rabs and SNARE proteins [1,2]. During phagosome maturation, these proteins are removed and replaced by different sets of similar molecules required for the fusion with downstream compartments [1–5]. Rab proteins along with their effector molecules activate specific SNAREs (soluble-NSF-attachment-receptor) and regulate the fusion between vesicles [6,7]. One of the ubiquitous proteins required for multiple vesicular fusion events is NEM-sensitive fusion (NSF) protein [8], which has both ATP binding and hydrolyzing activity [9,10].

Current model of vesicular fusion suggests [7,11] that intracellular membrane fusion events are mediated by complementary sets of SNAREs that are localized on the membranes destined to fuse. However, due to the dynamic nature of the fusion process, complementary SNAREs are usually present in a paired configuration (cis-SNARE) in the resultant compartment rendering them fusion incompetent. Subsequently, ATP hydrolysis of NSF dissociates the SNARE complex to an active unpaired (trans-SNARE) configuration. Finally, activated SNAREs residing on the donor vesicle binds with the cognate SNAREs present on the target membrane (trans-SNARE pairing) leading to fusion. Thus, the separation of cis-SNARE complex by ATP hydrolysis of NSF is prerequisite for vesicular fusion.

Consequently, it has been shown that fusion of early endosomes with phagosomes also requires ATP and NSF, suggesting that NSF–SNAP–SNARE complex is involved in phagosome maturation [12]. Though, large numbers of pathogens are shown to modulate the function of Rabs during maturation in the host cells [1–5], however, not much is known about the role of NSF in the intracellular trafficking of pathogens. Previously, we have shown that *Salmonella*-containing early phagosomes recruits Rab5 and NSF and promotes fusion with early endosomes [3,13]. In the present investigation, we have characterized the role of NSF at different stages of the maturation of *Salmonella*-containing phagosomes in macrophages and shown that fusion between *Salmonella*-containing late phagosomes and early endosomes does not require the function of NSF.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Tissue culture supplies were obtained

Abbreviations: LSEP, live *Salmonella*-containing early phagosomes; LSLP, live *Salmonella*-containing late phagosomes; NEM, *N*-ethylmaleimide; NSF, NEM-sensitive fusion factor; HRP, horse radish peroxidase; IM, internalization medium; HB, homogenization buffer; PNS, post-nuclear supernatant

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from the Grand Island Biological Co. (Grand Island, NY). *N*-Hydroxy succicinimidobiotin (NHS-biotin), avidin-horseradish peroxidase (Avidin-HRP), avidin were purchased from Vector laboratories, Burlingame, CA. ECL reagents were procured from Amersham International (Amersham, UK). Gel filtered cytosol used in the fusion assay was prepared from J774E cells as described previously [15].

2.2. Antibodies and recombinant proteins

Antibodies against EEA1 and Rabaptin 5 were received as kind gift from Dr. M. Zerial (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany). Anti-NSF and anti-transferrin receptor antibodies were purchased from Synaptic Systems (Gottingen, Germany) and Zymed (Carlsbad, CA), respectively. Anti-Rab5 antibody was kindly provided by Dr. A. Wandinger-Ness (University of New Mexico, Albuquerque) and NSF constructs were received from Dr. S.W. Whiteheart (University of Kentucky, Lexington, KY). All the secondary antibodies labeled with HRP and Alexa-546 were purchased from Jackson Immunoresearch Laboratories (West Grove, PA) and Molecular probes (Carlsbad, CA), respectively. *Salmonella* H antiserum which predominantly detects *Salmonella* flagellin was purchased from Becton Dickinson and Company (Sparks, Maryland).

2.3. Cells

J774E macrophage and HeLa cells were maintained in RPMI-1640 and DMEM medium, respectively, supplemented with 10% fetal calf serum and gentamycin (50 μg/ml) at 37 °C in 5% CO_2-95% air atmosphere.

The *Salmonella typhimurium* (SL1344 strain) was obtained from Dr. David W. Holden of Imperial College of Science, London. *Salmonella* were also transformed with pFPV25.1 vector (provided by Dr. Raphael Valdivia, Duke Centre for microbial pathogenesis, Durham, NC) for constitutive expression of GFP. Bacteria were routinely grown overnight in Luria Broth containing appropriate antibiotics at 37 °C and log phase cells were harvested by centrifugation for phagosome preparation [14].

2.4. Purification of Salmonella-containing phagosomes

Salmonella-containing phagosomes (live and dead) were purified from J774E cells as described previously [13]. Briefly, J774E clone macrophages (1×10^8) were incubated with 1×10^9 biotinylated-*Salmonella* at 4 °C for 1 h and then the cells were shifted to pre-warmed medium and incubated for 5 min at 37 °C to restrict their entry primarily into the early compartment. Cells were washed (300×*g* for 6 min) three times to remove uninternalized bacteria. One aliquot of the cell suspension was processed for the *Salmonella*-containing early phagosome preparation. The other half of the cell suspension was further incubated for 85 min at 37 °C to chase the bacteria to the late compartments. Subsequently, cells were washed and homogenized in homogenization buffer (HB: 250 mM sucrose, 0.5 mM EGTA and 20 mM Hepes-KOH, pH 7.2 containing protease inhibitors) and post-nuclear supernatants (PNS) were prepared by low speed (400×*g* for 10 min) centrifugation. Finally, phagosomes were purified from PNS using 12% sucrose cushion as described previously [13,14] and used for *in vitro* fusion assay.

2.5. Characterization of Salmonella-containing early and late phagosomes

To characterize the live-*Salmonella*-containing early (LSEP) and late phagosomes (LSLP), respective phagosomes containing non-biotinylated-*Salmonella* were purified as described in the previous section. Subsequently, phagosomal proteins (40 μg of protein) from each time point were resolved on a SDS–PAGE and Western blot analyses were carried out using indicated specific antibodies.

2.6. Determination of the intracellular localization of Salmonella by confocal microscopy

To determine the localization of *Salmonella* at different stages of their maturation in macrophages, J774E cells (5×10^5) were plated on sterile glass coverslips placed in a six-well tissue culture plate and directly incubated with live GFP-*Salmonella* (5×10^6 cells) for 5 min at 37 °C to restrict them into early compartments. Cells were washed three times to remove unbound bacteria. In another set of experiment, infected cells were further incubated for 85 min at 37 °C to chase the bacteria to the late compartments. At indicated time points, cells were washed three times with cold PBS and fixed with 4% paraformaldehyde. Subsequently, cells were permeabilized with 0.2% saponin for 20 min and blocked with 2% BSA for 1 h. Cells were further probed with specific antibodies against Rab5 or EEA1 in PBS containing 0.1% saponin and 2% BSA for 1 h at 37 °C. Cells were washed thrice with PBS and incubated with Alexa Fluor 546-labeled goat anti-mouse secondary antibody (1:500) in the same buffer for 1 h at 37 °C. Slides were mounted in prolong gold antifade reagents (Molecular probes) and viewed in an LSM 510 Meta confocal microscope using an oil immersion objective. Identical experiments were carried out in HeLa cells.

2.7. In vitro fusion between Salmonella-containing early or late phagosomes and early endosomes

Early endosomes containing avidin-HRP were prepared as described previously [13]. To determine the fusion between LSEP or LSLP and EE, appropriate phagosomal preparations containing biotinylated-*Salmonella* were mixed with avidin-HRP loaded EE 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) containing ATP regenerating system (1 mM ATP, 8 mM creatine phosphate, 31 units/ml creatine phosphokinase and 0.25 mg/ml avidin as scavenger) supplemented with gel filtered cytosol and incubated for 60 min at 37 °C. The fusion reaction was stopped by chilling on ice. The HRP-avidin–biotin-bacterial complex was recovered by centrifugation (10000×*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 a fusion unit. Specific fusion value was determined by subtracting the values corresponding to HRP activity obtained when the endosomes and phagosomes were mixed in fusion buffer without cytosol. Results are expressed as relative fusion in comparison to control.

To determine the role of NSF in fusion between EE and respective phagosomes, fusion was carried out with *N*-ethylmaleimide (NEM) treated phagosomes in the presence of NSF-depleted cytosol as well as NSF-depleted cytosol containing dominant negative mutant of NSF. NSF was immunodepleted from the macrophage cytosol using the procedure described previously [13].

3. Results

3.1. Characterization of Salmonella-containing early and late phagosomes

Live or dead *Salmonella*-containing purified phagosomes at different stages of their maturation in macrophages were analyzed to