The mechanism of inhibition of phagosome-lysosome fusion by SpiC protein, an effector of type III secretion system in *Salmonella* serovar Typhimurium

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Introduction

Salmonella enterica serovar Typhimurium is one of the most prevalent causes of gastroenteritis, an inflammation of the intestine. These bacteria infect the hosts by translocating through the M cells located among the ephithelial cells, and then internalized by the macrophages that are associated with the M cells. *Salmonella* survive intracellularly inside the phagosome (4), in fact, the pathogenesis is related to this survival inside the macrophages since a mutant that is defective in this survival has its virulence highly attenuated.

Phagocytosis follows the endocytosis pathway: the phagosome fuses subsequently with early endosome, late endosome, and lysosome, resulting in sequential acquisition and loss of markers such as rab5, rab7 and lysosomal glycoproteins (LAMP) markers, respectively, during the maturation of phagosome. However, *Salmonella* is able to modulate the host's membrane traffic system and survive intracellularly by avoiding fusion between *Salmonella*-containing phagosome with the lysosomes, compartments with antimicrobial environment including low pH, toxic oxygen, and the presence of degradative enzymes (10, 12).

To understand the pathogenesis it is important to know how *Salmonella* interfere with the regulated process of vesicle fusion. The recent model of vesicle fusion shows that the first step involves tethering of the vesicle and the target by Rabs GTPase and an unknown fibrous protein. Then, in the step called docking, the proteins v-SNARE and t-SNARE/SNAP25 (soluble NSF attachment protein complex) that are present in the transport vesicle and their target, respectively, are paired up tightly (6). Therefore this step confers specificity. t-SNARE protector proteins (such as sec1p in yeast) binds to t-SNARE to prevent nonspecific pairing between the two SNARES, and once the right target vesicle is encountered, Rab proteins in their GTP-form would recruit docking factors from the cytosol to deprotect t-SNARE. Rabaptin-5 complex is one of the docking factors that have been reported. Rabex-5, a nucleotide exchange factor that is part of Rabaptin-5, activates Rab5, and further stimulates Rab5-Rabaptin5 association, which further promotes formation of prefusion complex during priming. Prefusion complex requires association of many v- and t-SNARE proteins and ATP hydrolysis by *N*-ethylmaleimide (NEM) sensitive factors (NSF) (13). Fusion is facilitated by the SNARE complex, by an unknown mechanism that may involve the ATPase activity by NSF. Finally, GTP hydrolysis converts Rabs to their GDP form, and GDI protein, a cytosolic protein, retrieves these GDP-bound Rab proteins from their fusion targets and recycles them back to their membrane of origin. NSF and α-, β-, and γ-SNAP catalyze dissociation of the prefusion complexes so that these proteins can be recycled (1).

Salmonella modulate the host's membrane trafficking by arresting fusion with early endosome, therefore delaying the maturation of phagosome into lysosome. Phagosome containing live *Salmonella* was shown to fuse with early endosome very efficiently with recruitment of Rab5 but not Rab7 even after 7 days incubation (8). Similar mechanism is also employed by *Mycobacterium tuberculosis* (2). During *Salmonella* infection, Rab5 was found to specifically bind to SopE, another type III

secretion system in *Salmonella*. In addition, NSF is recruited to the phagosome such that fusion with early endosome takes place even in the absence of cytosolic NSF. In this model, it was proposed that SopE might function to recruit rab5 in GTP form, activates the SNARE which in turn recruits more α–SNAP and triggers the binding of NSF to the phagosome. Because SopE stimulates GDP to GTP exchange of several Rho GTPases, it may inhibit the activity of GTPase activating protein (GAP), and prevents release of GDP-form Rab5 to the cytosol by GDI.

Uchiya et. al have shown the involvement of SpiC, the first host trafficking interfering factors (Htif) found in *Salmonella*, in the inhibition of fusion between phagosome and lysosome (10, 14). The author demonstrated that SpiC is sufficient for the inhibition since *spiC* mutant cannot inhibit fusion, and this inhibition is restored by addition of plasmid containing spiC into the *spiC* mutant. SpiA, a type III secretion system apparatus in *Salmonella*, exports this protein to the host cytosol. Both Spi proteins are encoded by Spi/Ssa system in SPI-2 pathogenicity island. Once in the cytosol, SpiC inhibits fusion of phagosome with lysosome as well as with early endosomes in macrophages. Purified SpiC protein inhibited endosome-endosome fusion in vitro to the same extent as a dominant-negative mutant of the GTPase Rab5 (S34N). Moreover, corelates with its localization in the cytosol, SpiC affects a more generalized modulation of host trafficking, since expressed SpiC in Sindbis virus interferes with normal trafficking of transferrin *in vivo*. Therefore, these data suggest that the target of SpiC might be a protein that is involved in the endosome-endosome fusion.

Although the role of SpiC in inhibiting the phagosome-lysosome fusion has been well demonstrated, the detailed mechanism of how this protein prevents fusion is still unknown. The lack of homology in the SpiC sequence with other proteins in the database further complicates the prediction of its function. We propose to investigate the cellular target of SpiC during inhibition of phagosome-lysosome fusion (Figure 1). In the confine of this proposal, we would determine whether SpiC is involved in docking or the actual fusion step using fluorescence labeling and electron microscopy, as well as the use of clostridial toxin. We would further determine which host factor acts as a target of this protein by looking at the effect of SpiC mutant on the recruitment of Rab5 and NSF, and also the binding of these proteins to SpiC using imunoprecipitation. Knowing the mechanism of how this protein interferes with the host's cellular trafficking is critical to our understanding of pathogenesis of *Salmonella*. This information would be useful not only in the development of new vaccines against *Salmonella* infection, but also in increasing our comprehension in the eukaryotic factors that are involved in vesicular trafficking.

<u>SPECIFIC AIM 1</u>: Does SpiC inhibits docking or fusion step between phagosome and lysosome?

Rationale: In order to interfere with the host's vesicular trafficking, SpiC that is released to the cytosol needs to be associated with proteins that are involved in this pathway.

Since the inhibition of fusion by SpiC was determined by the increase in the number of phagosome that carry either early (30 min) or late (24hr) labeled compartment, it is not known whether SpiC inhibits docking or fusion. This experiment would further confine the specific step in the fusion process and thus further focus narrowly on the proteins potentially targeted by SpiC.

Method:

a. Morphological docking assay.

This method combines fusion and fluorescence assay (3). Phagosomes containing biotinylated wild type or spiC mutant *Salmonella* will be isolated using published protocol (8). Lysosome labeled with rhodamin will also be isolated. The labeled phagosome and lysosome will be incubated in the presence of purified SpiC in the cytosol from uninfected macrophage. Docking or fusion will be observed using confocal microscope. As a control, endosomes expressing a mutant α-SNAP (L294A) will be used, since this mutant protein prevents SNARE priming by inhibiting the stimulation of NSF ATPase activity (3). Therefore only docking but no fusion is exhibited by this mutant.

Because this method does not demonstrate biochemically the inhibition of the docking step, we will use a second approach using clostridial neurotoxin light chains.

b. Clostridium toxins

Two proteins that have been proposed to participate in vesicle docking are the SNAREs and the Rab proteins. The ternary complex of v-SNARE, t-SNARE, and SNAP-25 are the minimum requirements for vesicle docking, although this process occurs slowly in the absence of Rabs. Clostridial toxins TeNT, BoNT/C1, and BoNT A are known to degrade v-SNARE, t-SNARE or SNAP-25, respectively in their unbound state (7, 9). Once the ternary complex form during docking, they are resistant to the proteolysis effect of the toxins. Therefore the degradation of v-SNARE by the toxin in the presence of SpiC would indicate the absence of docking.

An *in vitro* system will be performed in the absence of NSF and α-SNAP, the proteins that are known to disasssemble the ternary complex (9). In this system, endosomes expressing v-SNARE and endosome expressing t-SNARE/SNAP25 will be incubated in the presence of purified SpiC. After incubation, botulinum toxin will be added to the reaction mixture. Then the SNARE proteins from the reaction mixture will be separated on the gel, and immunoblot will be performed using antibody against v-SNARE, t-SNARE, or SNAP-25 as probes to see if the complex is still intact.

Because arrest of *Salmonella*-containing phagosome with early endosome involves the efficient recruitment of Rab5, an important regulator of endosome-endosome fusion, and since Rab5 functions early in the docking process to regulate the formation of t-SNARE and v-SNARE/SNAP25 complex, it is very likely that SpiC is involved in this docking step. If docking is inhibited by SpiC, v- or t-SNARES, or SNAP-25 will be degraded by the clostridium toxin. However, this in vitro system does not show physiological condition during *Salmonella* infection, since it is lacking other proteins

involved in docking and fusion. Nonetheless, together with the results from the morphology assay, we should be able to determine whether or not docking takes place in the presence of SpiC.

SPECIFIC AIM 2: Is SpiC involved in the recruitment of cytosolic Rab5 and/or NSF?

Rationale: Spi C inhibitory action can be reversed upon addition of cytosol from *uninfected* macrophages to the *in-vitro* vesicle fusion, but rescue is eliminated by addition of trypsin to the cytosol (14). Therefore, SpiC might act by inactivating a factor normally present in the host cell cytosol or by competing with a cytosolic factor for a target in the endosomal membrane. Since phagosome containing live but not dead wild type *Salmonella* has been shown to recruit Rab5 and NSF, we would determine the function of SpiC by examining the effect of the recruitment of these proteins by phagosome containing SpiC mutant. Recruitment or lack of recruitment of Rab5 or NSF would further indicate whether SpiC acts early in inhibition of docking or late in fusion, respectively.

Method:

a. Immunogold labelling
   For localization of NSF or Rab5 in the phagosomes. phagosomes isolated from macrophages infected by wild type or *spiC* mutant will be incubated with mouse anti-NSF or rabbit anti-rab5 antibody. Subsequent incubation with secondary antibodies will be performed either with goat anti-mouse conjugated with 12 nm colloidal gold goat, or anti-rabbit IgG conjugated with 18 nm colloidal gold for anti-NSF and anti-rab5, respectively. The localization of the proteins will be observed with electron microscopy.

Using this method, we will be able to see not only the recruitment of either rab5 or NSF, but also the distribution of these proteins in the phagosome after infection. We will also perform an in vitro assay to show the ability of NSF- or Rab5-stripped phagosomes to recruit cytosolic NSF or rab5, respectively.

b. In vitro recruitment assay and western blot analysis.
   Endogenous NSF on the phagosome containing wild type or SpiC mutant will be removed by treatment with 0.5M KCl (8). These phagosomes will be incubated in the presence of purified NSF in the fusion buffer containing cytosol from uninfected macrophages. In parallel, the phagosomes will also be treated with Rab-GDI that removes endogenous rab5 as well as NSF, and then incubated in the presence of Rab5-GST. The presence of Rab5 or NSF in the phagosome will be visualized using anti-rab5 or anti-NSF antibodies by Western blot.

If SpiC affects the recruitment of Rab5, it would strongly suggest the role of SpiC during docking, while the effect on NSF recruitment would suggest its involvement in the fusion step. The result from these experiments would determine whether to study the *direct binding* of Rab5 or of NSF in the next step.

SPECIFIC AIM 3: Does SpiC interact with Rab5 or NSF during recruitment?

Rationale:

The presence of live *Salmonella* in the phagosome correlates with the efficient recruitment of rab5 in GTP-bound state on the phagosomal membrane by SopE, another protein released by type III secretion machinery in *Salmonella* (8). Moreover, SpiC inhibits endosome-endosome in vitro to the same extent as GTPase mutant Rab5. In a similar mechanism is employed by the pathogen *Mycobacterium tuberculosis*, arrest of phagosome-lysosome fusion during infection was shown to be associated with the retaining of Rab5 and the exclusion of Rab7 on the *Mycobacterium*-containing phagosome, although the Htif involved in this process is still unknown (10). In addition, because SpiC also affects normal recycling of transferrin to the plasma membrane, which involves Rab4, it is possible that SpiC binds to several different rab proteins. This possibility needs to be investigated. Rab proteins are the regulator of endosomal fusion, and recruited early in the fusion process than other proteins such as NSF, SNARE, and α-SNAP. These data suggest a role of rab proteins in the control of fusion during phagosome maturation and therefore these proteins are very likely to be the target of SpiC. However, if the experiments proposed above gives indication that SpiC affects fusion rather than docking process, the interaction with NSF needs to be examined.

Method:

a. Immunofluorescence microscopy
   The macrophage cells from wild type or SpiC mutant of *Salmonella* will be grown on microscope slips and permeabilized with triton-X. The cells will be stained with anti-SpiC and either anti-Rab5 or anti-NSF primary antibodies followed by FITC-conjugated or rhodamin-conjugated secondary antibodies (5). Then the images will be visualized with fluorescence microscope. Computer modification would indicate colocalization if the two fluorescence colors overlap.

This experiment would show if SpiC colocalizes with either Rab5 or NSF, but it will not tell if the two proteins bind. Binding will be verified using immunoblotting.

b. Immunoblotting
   GST-Rab5, GST-Rab 7, GST-Rab4, GST-NSF or GST alone will be immobilized with glutathione beads and incubated in the presence of purified SpiC proteins or SopE proteins (as a control). The proteins will be separated by SDS-PAGE and Western blot of the proteins bound to the beads will be performed using anti-SpiC or anti-SopE antibodies. Proteins will be visualized using appropriate HRP-labeled secondary antibodies. SopE will be used as a control since it will bind to Rab5 but not Rab4 or Rab7 (8).

Discussion

Because very little is known about the mechanism of how SpiC functions, the results from the proposed experiments would greatly enhance our understanding of this

protein. Although only Rab5 and NSF will be tested, other proteins are also likely to be involved. For example, SpiC may block docking by associating with t-SNARE, such as shown by pallid protein which interacts with syntaxin13, a t-SNARE protein (5). Similarly, interaction with α-SNAP is also possible. Nevertheless, interaction with Rab5 and/or NSF will help us narrow down these possibilities. Once interaction is demonstrated, further questions need to be addressed, for example, if SpiC binds to Rab5 does it bind to GDP- or GTP- form? Does it affect GTPase activity of Rabs? Moreover, experiments need to be performed to distinguish if the inhibition of phagosome maturation by SpiC is actually caused by avoiding loss of Rab5 or by blocking the recruitment of other proteins involved in docking or fusion with lysosome (12). Finally, knowledge of the mechanism of interference in host vesicular trafficking by SpiC in *Salmonella* would be immensely helpful for the intervention of *Salmonella* infection in humans.

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Figure 1. Possible mechanisms of SpiC in inhibiting the maturation of *Salmonella*-containing phagosome (adapted from 6, 11 and 14).