Interaction of the Salmonella-containing Vacuole with the Endocytic Recycling System*§

Adam C. Smith‡§, Judith T. Cirulis**††, James E. Casanova§§, Marcia A. Seidmore¶¶, and John H. Brumell§§||

From the §Infection, Immunity, Injury, and Repair Program and the ||Cardiovascular Research Program, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada, the Departments of §Medical Genetics and Microbiology and ||Biochemistry, University of Toronto, Sick Kids, Toronto, Ontario M5S 1A1, Canada, the §§Department of Cell Biology, University of Virginia School of Medicine, Charlottesville, Virginia 22908, and the ¶¶Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

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Upon entry of the pathogen Salmonella enterica serovar Typhimurium into host cells, the majority of bacteria reside in a membrane-bound compartment called the Salmonella-containing vacuole (SCV). Previous studies have established that the SCV transiently interacts with early endosomes but only acquires a subset of late endosomal/lysosomal proteins. However, the complete set of interactions between the SCV and the endocytic machinery has yet to be characterized. In this study, we have shown that four characterized regulators of endocytic recycling were present on the SCV after invasion. Interaction kinetics were different for each of the regulators; ARF6 and Rab4 associated immediately, but their presence was diminished 60 min post-infection, whereas syntaxin13 and Rab11 association peaked at 60 min. Using a dominant negative approach, we determined that Rab11 regulates the recycling of CD44 from the vacuole but had no effect on major histocompatibility complex (MHC) class I recycling. In contrast, syntaxin13 regulated the recycling of MHC class I but not of CD44. We also determined that maturation of the SCV, measured by the acquisition of lysosomal associated membrane protein-1, slowed when recycling was impaired. These findings suggest that protein movement through the endocytic recycling system is regulated through at least two concurrent pathways and that efficient interaction with these pathways is necessary for maturation of the Salmonella-containing vacuole. We also demonstrate the utility of using Salmonella invasion as a model of endosomal recycling events.

Salmonella enterica serovar Typhimurium (Salmonella Typhimurium) is a facultative intracellular pathogen responsible for disease across species, ranging from gastroenteritis to enteric fever (1). Invasion into the intestinal epithelium of the host is modeled in vitro using epithelial cell cultures, which have provided a wealth of information regarding the pathogenesis of Salmonella Typhimurium (reviewed by Hurley and McCormick) (2). Uptake into epithelial cells is directed through the Salmonella pathogenicity island (SPI)1 1 type III secretion system, a needle-like device protruding from the bacterial cell wall that delivers bacterial virulence proteins into host cells (3). The virulence proteins, called effectors, are translocated from the bacteria into the host cytosol where they direct actin rearrangements leading to membrane ruffling, which results in uptake of the bacteria (4). Once inside, the bacteria reside in a membrane-bound compartment called the Salmonella-containing vacuole (SCV) and establish a replicative niche. Recently, an active SPI-1 system has been shown to be necessary for the intracellular survival and replication of Salmonella Typhimurium in epithelial cells (5).

Early in the infection of epithelial cells (up to ~15 min), trafficking of the SCV resembles that of model phagosomes (reviewed by Brumell and Grinstein) (6). Markers such as the early endosome antigen-1 (7) and the GTPase Rab5 (8) are transiently present on the SCV, indicative of interactions with early endosomes. These are rapidly removed, followed by the acquisition of several late endosome/lysosome markers, including Rab7 (9) and lysosomal membrane glycoproteins (10). Interestingly, acquisition of lysosomal associated membrane protein-1 (LAMP-1) is dependent on Rab7 activity (9); yet, despite the presence of active Rab7 on the early SCV (11), the bacteria avoid or delay fusion with late endosomes/lysosomes that carry LAMP-1. This is shown by the lack of mannose-6-phosphate receptor, cathepsin D, and lysobisphosphatidic acid on the SCV during the first 3 h post-infection (p.i.) (10, 12). Understanding the pathways behind these events may lead to an elucidation of the mechanisms Salmonella Typhimurium uses to direct its intracellular fate in host cells. To do this, it is necessary to characterize the interactions of the SCV with the host endocytic system from invasion onward.

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the internalization of plasma membrane to form the SCV as well as other endosomes. These endosomes can be empty or they may contain nearby Salmonella or inert “bystander” particles (13–15). Included on the SCV and surrounding endosomes are plasma membrane proteins that have been shown to aggregate at the site of invasion (16). Two such proteins are major histocompatibility complex (MHC) class I, involved in the presentation of peptides to T cells (17), and CD44, the receptor for hyaluronic acid involved in adhesion (18). The presence of these proteins on the surface of the SCV decreases over time (16), suggesting that active recycling is taking place. Endocytic recycling is necessary for phagosomal maturation (19, 20), but its role in SCV maturation remains unclear.

Endocytic recycling is a complex process involving multiple compartments and trafficking events (reviewed by Maxfield and McGraw) (21). Regulation of this process involves many protein families including Rab proteins, small GTPases involved in vesicle trafficking. These proteins mediate four key processes, namely vesicle budding, targeting, docking, and fusion, by recruiting cellular effector proteins to a specific membrane microdomain (reviewed by Zerial and McBride) (22). Rab4 localizes to early endosomes and regulates early sorting events from there (23, 24). Rab11 localizes to the perinuclear region (23, 25) and is involved in recycling to the plasma membrane or the trans-Golgi network (24, 26, 28). The ARF (ADP ribosylation factor) protein family, another group of small GTPases, also function as regulators of membrane traffic (reviewed by Chavrier and Goud) (29). ARF6 functions in the peripheral plasma membrane/endosomal system (30, 31) and is involved in clathrin-independent recycling and transport to regions of the plasma membrane undergoing reorganization (32–36). Syntaxins are soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs) involved in vesicular tethering and fusion (reviewed by Chen and Scheller) (37). Syntaxin13 localizes to the perinuclear region and is involved in the fusion events required for recycling (19, 38). Collins et al. (19) have shown recently that active syntaxin13 is necessary for proper maturation of the phagosome.

Studies of endocytic recycling compartments have shown that specific cargo is able to move through different kinetic pathways (23, 24, 39–41). However, it is often not clear which regulators are specific for which pathway or which cargo. Here we characterized the interaction of recycling proteins with the SCV. Moreover, we demonstrated that recycling from the SCV could occur through at least two independent, concurrent pathways that are influenced separately by syntaxin13 and Rab11. Finally, we showed that active recycling is required for efficient maturation of the SCV. These experiments revealed the utility of using Salmonella Typhimurium as a model for the study of membrane recycling events in mammalian cells.

MATERIALS AND METHODS

Cell Culture and Bacterial Strains—HeLa (human epithelial cell line) cells were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) Clone supplemented with 10% fetal bovine serum (Wisent) at 37 °C in 5% CO2 without antibiotics. Cultures were used between passage number 3–25. Wild type Salmonella Typhimurium SL 1344 (22) was used in this study.

Plasmids and Transfection—The hemagglutinin (HA)-tagged ARF6 construct was described as previously prepared (43, 44). EGFP-Rab4 and EGFP-Rab11 constructs have been previously described (45). Cloning of a mutant version of the oligonucleotide (5’-GGTTTGGGAAATAATCTCTGGTCTGG-3’) and the mutant version of the oligonucleotide (5’-GAAAACGAGGATATCTTTCCAACTGATCTTGCA-3’) were performed using QuickChange (Stratagene) EGFP-Rab11S25N was constructed using QuikChange (Stratagene) and EGFP-Rab1 constructs have been previously described (45). Murine monoclonal anti-human HLA-ABC antibodies were obtained from Serotec (Raleigh, NC). Rabbit polyclonal antibodies to Salmonella Typhimurium O antiserum group B were obtained from Difco (Kansas City, MO). Murine monoclonal anti-HA antibodies were obtained from Covance (Princeton, NJ). Murine monoclonal anti-human FR2 antibodies were obtained from Biomol (Plymouth Meeting, PA). Murine monoclonal anti-human LAMP-1 antibodies (clone H4A3) developed by T. August were obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD, National Institutes of Health and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. The secondary antibodies used were Alexa 555-conjugated goat anti-rabbit IgG, Alexa 488-conjugated goat anti-rabbit IgG, Alexa 450-conjugated goat anti-mouse IgG, Alexa 488-conjugated goat anti-mouse IgG, and Alexa 568-conjugated goat anti-mouse IgG, all obtained from Molecular Probes (Burlington, Ontario, Canada).

Bacterial Infection of Cell Cultures—HeLa epithelial cells were seeded at 2.5 × 104 cells/well in 24-well tissue culture plates 40–45 h before use. Epithelial cells were transfected 16–20 h before use. Late log bacterial cultures were used for infecting HeLa cells (HeLa) and prepared using a method optimized for bacterial invasion (7). In brief, bacteria were grown for ~16 h at 37 °C with shaking and then sub-cultured (1:33) in Luria-Bertani broth for 3 h. Bacterial inocula were prepared by pelleting at 10,000 × g for 2 min, directly resuspended, diluted in PBS (pH 7.2), and added to cells at a multiplicity of infection of ~100:1 at 37 °C for 10 min. After infection, extracellular bacteria were removed by extensive washing with PBS and the addition of growth medium containing gentamycin (100 μg/ml). Following 75 min of bacterial infection, the gentamicin concentration was decreased to 0.05 μg/ml. Cells were fixed in 2.5% paraformaldehyde in PBS, pH 7.2, for 10–15 min at 37 °C.

Immunofluorescence—Fixed cells were washed twice with PBS and permeabilized/blocking with 1% goat serum (Calbiochem) in PBS containing 10% normal goat serum (SS-PBS) for 30–60 min. Primary and secondary antibodies were overlaid on coverslips in SS-PBS for 30 min to 1 h, followed by three washes with PBS. Coverslips were mounted onto 1-mm glass sides using fluorescent mounting medium (DakoCytomation, Mississauga, Ontario, Canada). Samples were analyzed using a Zeiss Axiosvert confocal microscope (63× objective). Confocal sections were imported into Adobe Photoshop in RGB format and assembled in Adobe Illustrator.

A Leica DMIRE2 fluorescence microscope was used to enumerate the association of different proteins with the SCV. The percentage of SCV was determined visually, with a distinct signal surrounding the bacteria considered positive. This was determined for at least 100 bacteria. The average ± S.D. for at least three experiments is presented.

For live cell imaging, HeLa cells were transfected with EGFP-Rab11 and then infected as described above with Salmonella Typhimurium expressing the DiRed protein from the plasmid pZ1590, generously provided by Dr. Francisco Ramos-Morales (Universidad de Sevilla, Sevilla, Spain) (46). Cells were then mounted on a Leica DMIRE2 fluorescence microscope with a heated stage. Images were acquired every 30 s through the duration of the experiment. All images were obtained with cells kept at 37 °C in RPMI media buffered to pH 7.2 with HEPES. For bacterial replication, a Leica DMIRE2 fluorescence microscope was used to enumerate the number of bacteria per cell. The number of intracellular bacteria was counted for at least 100 cells. The average ± S.D. for at least three experiments is presented.

RESULTS

Salmonella Invasion Induces Transient Aggregation of Host Cell Surface Proteins in Epithelial Cells—Salmonella invasion of epithelial cells is unlike phagocytosis in that it is a bacterial driven process (reviewed by Brumell et al.) (47). Invasion has been described as being similar to macropinocytosis, because invasion of one bacteria causes a ruffling of the cell surface that drives the endocytosis of other bacteria into separate SCVs, as well as triggering the formation of multiple bacteria-free endo-
somewhere(13–15). Previous analysis has shown that the receptors MHC class I and CD44 co-localize with the SCV upon the invasion of epithelial cells (16). In addition, a large amount of each receptor is internalized during the invasion process and aggregates on endosomes near the site of bacterial entry (16). This is depicted in Fig. 1. HeLa cells were infected with wild type Salmonella Typhimurium SL1344, fixed 30 or 180 min p.i., and immunostained for bacteria for either MHC class I or CD44. An aggregation of MHC class I proteins was visible at the site of bacterial entry 30 min after infection (Fig. 1B) that was not present in uninfected cells (Fig. 1A). In addition, MHC class I noticeably co-localized with the SCV (Fig. 1B, Enlargement). Immunostaining for CD44 produced a similar result, with aggregation of receptors in the area of bacterial entry (Fig. 1E) and a direct co-localization with the SCV after 30 min (Fig. 1E, Enlargement). In both cases, receptor aggregation on endosomes was greatly reduced or no longer present after 180 min (Fig. 1C and F), and there was little co-localization with the SCV. This loss of cell surface proteins from the SCV and invasion-induced endosomes suggested active recycling events occur on these compartments.

Recruitment of Recycling Regulatory Proteins to the SCV—To address the mechanism by which CD44 and MHC class I are recycled from the SCV, we asked whether known recycling regulators were present on this compartment. EGFP-Rab4 associated transiently with the SCV (Fig. 2, A–C), with maximal association at 10 min p.i. and then diminishing until it was no longer present after 60 min (Fig. 2D). During bacterial invasion, ARF6 was noticeably present on membrane ruffles (data not shown).

Other recycling regulators include syntaxin13 and Rab11. To examine the SNARE syntaxin13, cells transiently transfected with syntaxin13-EGFP were infected, fixed, and analyzed microscopically. Prior to invasion, syntaxin13-EGFP was distributed in the perinuclear region and in vesicles throughout the cytoplasm (Fig. 2G), consistent with previous reports (19, 38). However, cells fixed 30 min p.i. showed that syntaxin13-EGFP clustered around intracellular bacteria (Fig. 2H) with clear co-localization with the SCV (Fig. 2H, inset, and M). After 180 min the aggregation had dispersed (Fig. 2I), and the majority of SCVs no longer co-localized with syntaxin13-EGFP (Fig. 2I, inset, and M). Similarly, Rab11 exhibited perinuclear and vesicular localization in HeLa cells immunostained with a monoclonal antibody (Fig. 2J), consistent with previous reports (23, 25). Cells fixed at 30 min post-invasion displayed an aggregation of Rab11 around the bacteria (Fig. 2K) as well as co-localization with the SCV (Fig. 2K, inset). After 180 min the aggregation was no longer present (Fig. 2L), and co-localization with the SCV had diminished (Fig. 2L, inset). The kinetics of Rab11 co-localization with the SCV showed maximum association (>50%) 60 min p.i. and declined to <35% by 180 min p.i. (Fig. 2M). Our data indicate multiple interactions of the SCV with recycling regulators, each having distinct kinetics of dynamic association with the SCV.

Both syntaxin13 and Rab11 displayed a rapid recruitment of protein away from the perinuclear region to the area around...
the SCV (Fig. 2, H and K). Fig. 3 shows still images taken from live imaging of EGFP-Rab11 transfected cells (supplemental movie 1, available in the on-line version of this article). The majority of EGFP-Rab11 was in the perinuclear region 15 min p.i. By 18 min p.i., EGFP-Rab11 began to relocate to the site of bacterial entry. After 30 min the majority of EGFP-Rab11 was aggregated around the bacteria, and this continued up to 60 min. Thus, our findings demonstrate that a massive reorganization of the endosomal system occurs during infection, characterized by localized recruitment of recycling factors to the SCV and surrounding endosomes at the site of bacterial entry.

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**Syntaxin13 and Rab11 Regulate Recycling of Host Cell Surface Proteins from the SCV**—Because the presence of syntaxin13 and Rab11 on the SCV coincided with the time of CD44 and MHC class I recycling, we set out to determine whether these recycling factors played a role in the removal of cell surface proteins from the SCV. To test the role of syntaxin13 in receptor recycling, a dominant negative construct (syntaxin13ΔTM, a cytoplasmic portion of which is thought to form a stable complex with endogenous SNAREs, thereby competing off the wild-type SNARE) (19) was co-transfected with EGFP. Co-transfection with EGFP allows determination of transfected cells when one construct lacks a visualization tag. After invasion, cells were fixed and immunostained for bacteria and either MHC class I or CD44. Aggregation of MHC class I proteins in syntaxin13ΔTM-expressing cells continued up to 180 min p.i. (Fig. 4, B and C). However, unlike control cells (Fig. 4A), the aggregation of MHC class I proteins in syntaxin13ΔTM-expressing cells continued up to 180 min p.i. (Fig. 4B) in some cells. Thus, a dominant negative form of syntaxin13 leads to an inhibition of recycling, as shown by the continued aggregation of MHC class I proteins.

To quantify this inhibition, we took advantage of the large, well defined vacuole created by *Salmonella* Typhimurium. The
presence or absence of MHC class I on the SCV was a clear indicator of recycling from a specific vacuole. Quantification of association showed MHC class I co-localized equally (70%) with control cells after 30 min, but cells expressing the syntaxin13\textsuperscript{TM} construct had 15% more SCVs co-localized with MHC class I at 60 and 120 min (Fig. 6A). Co-localization was the same in control cells and in those expressing syntaxin13\textsuperscript{TM} at 180 min (Fig. 6A). These findings are consistent with the effect of this construct on the aggregation of MHC class I proteins near the SCV. A small change in the recycling rate would be expected to delay the removal of cell surface markers from the large amounts of endosomal membrane near the SCV, and detection of these markers in aggregates would persist. Therefore, syntaxin13 played a role in the recycling of MHC class I.

**Fig. 3.** EGFP-Rab11 aggregates around the SCV. HeLa cells transfected with EGFP-Rab11 (GFP-Rab11; top row) were infected with \textit{Salmonella} Typhimurium strain SL1344 expressing DsRed (WT1344; bottom row) and then mounted onto a Leica DMIRE2 fluorescence microscope with heated stage. Still images were taken of live cell imaging at the indicated times (minutes). Scale bar equals 10 μm.

**Fig. 4.** Syntaxin13 regulates recycling of MHC class I from endosomes near the SCV. HeLa cells transfected with EGFP (A) or co-transfected with syntaxin13\textsuperscript{TM} and EGFP (B) (top row) were infected with \textit{Salmonella} Typhimurium strain SL1344, fixed at the indicated times, and stained for bacteria (middle row) and MHC class I (bottom row). Arrowheads denote the location of the SCV. HeLa cells transfected with EGFP (C) or co-transfected with syntaxin13\textsuperscript{TM} and EGFP (D) (top panel) were infected with \textit{Salmonella} Typhimurium strain SL1344, fixed at the indicated times, and stained for bacteria (middle row) and CD44 (bottom row). Arrowheads denote the location of the SCV. Images represent confocal sections. Scale bar equals 10 μm.
The interaction of SCV with the endocytic recycling system.

Examination of CD44 showed that aggregation in the area of the SCV was present in both populations after 30 min and had dispersed by 180 min (Fig. 4, C and D). In addition, co-localization with the SCV showed no differences between control and syntaxin13TM-transfected cells (Fig. 6A). This suggests that syntaxin13 plays little or no role in the recycling of CD44.

Next, we examined the role of Rab11 in the recycling of cell surface proteins from the SCV. We compared HeLa cells transfected with EGFP to those transfected with EGFP-Rab11S25N (a GDP-bound dominant negative form) (28). MHC class I aggregation in EGFP-Rab11S25N-transfected cells (Fig. 5B) was unaltered compared with control cells (Fig. 5A). The aggregation of MHC class I proteins at the site of bacterial invasion was present after 30 min and had dissipated by 180 min. Similarly, association of MHC class I with the SCV was found to be unaffected by the expression of EGFP-Rab11S25N (Fig. 6B). However, when studying CD44 in EGFP-Rab11S25N-expressing cells, we found aggregation around the SCV present at both 30 and 180 min after infection (Fig. 5D). This was in marked contrast to control cells, where aggregation of CD44 was lost by 180 min (Fig. 5C). Co-localization of CD44 with the SCV (Fig. 6B) was higher 30 min p.i. in Rab11 dominant negative-transfected cells than in control cells. The difference (10–15%) was maintained throughout the 180-min time course.

Therefore, Rab11 showed a role in CD44, but not MHC class I, recycling. In total, these findings indicate that multiple recycling pathways concurrently regulate the recycling of cell surface proteins from the SCV.

**Syntaxin13 and Rab11 Are Required for Efficient Maturation of the SCV**—To determine whether recycling pathways play a role in maturation of the SCV, we used a dominant negative approach and examined the accumulation of LAMP-1 on the SCV. LAMP-1 accumulation has been widely used as a marker of SCV maturation (9, 10, 48). Transiently transfected cells were infected with *Salmonella* Typhimurium, and SCVs were analyzed for the presence or absence of LAMP-1. LAMP-1 was not present on the SCV 15 min after invasion (consistent with published results) (9) in control cells transfected with EGFP (Fig. 7A) or any of the dominant negative constructs (data not shown). However, LAMP-1 was rapidly acquired on most SCVs in control cells during a 1-h period p.i. (Fig. 7B). Expression of EGFP-Rab11S25N or syntaxin13TM decreased the rate of LAMP-1 accumulation compared with EGFP-transfected control cells (Fig. 7D). Co-transfection of both dominant negative constructs had an additive effect, lowering the rate of LAMP-1 accumulation to approximately half the level of controls in cells fixed at 60 min (Fig. 7, C and D). To ensure that our transfection protocol or the expression of dominant negative constructs did not increase the number of bacteria in the cytosol, thus reducing LAMP-1 positive bacteria, we performed a 60-min infection and immunostained for FK2, a marker of cytosolic bacteria (49). We did not find an increase of bacteria in the
monella Typhimurium bacteria per cell over an 8-h time course. We saw no difference in the number of Salmonella Typhimurium bacteria per cell throughout the experiment (data not shown). Therefore, inhibition of Rab11 and syntaxin13 function does not affect the replication of Salmonella Typhimurium in HeLa cells.

**DISCUSSION**

It is now well established that Salmonella Typhimurium can modify maturation of the SCV to generate a replicative niche in cells of its host. However, the complete set of interactions that exist between the SCV and the host cell endosomal system is currently unknown. Here, we have demonstrated the association of endocytic recycling regulators with the SCV in HeLa cells, detailing multiple recycling pathways and the role of recycling in SCV maturation.

The association of recycling regulators with the SCV is reminiscent of their association with endosomes/phagosomes. Rab4 has been shown to interact with early endosomes and regulate rapid recycling to the plasma membrane (23, 24). Our data show an interaction of Rab4 with the SCV early in infection (10 min), followed by a loss from the membrane. These data coincide with maturation of the SCV as characterized by loss of early endosome antigen 1 (7). ARF6, also present on the SCV early in infection, is involved in regulating recycling to areas of membrane reorganization (35, 36). The interaction of ARF6 with the SCV may be a response to the membrane rearrangements occurring because of internalization of Salmonella Typhimurium. Indeed, the presence of ARF6 on membrane ruffles coincides with a previous report detailing the role of ARF6 in a signal transduction pathway initiated by Salmonella (55). A report has described a role for ARF6 in the recycling of MHC class I through a pathway separate from the perinuclear recycling complex (33). Because the level of ARF6 on the membrane of the SCV during the time of MHC class I recycling was low, we suspect that it does not play a significant role in the recycling of MHC class I from the SCV in HeLa cells. At this point it is not clear what Rab4 and ARF6 control in terms of recycling from the SCV.

Syntaxin13 and Rab11 have both been shown to regulate recycling through the perinuclear recycling complex (19, 24, 26–28, 38). In our study, both proteins displayed significant interaction with the SCV 30 min p.i., which corresponds with a previous report showing Rab11 interaction with endosomes containing transferrin 30 min after internalization (28). However, this contradicts a study demonstrating syntaxin13 interaction with nascent phagosomes 5–10 min after internalization (19). This difference in kinetics may be due to differences in cell type (HeLa versus RAW) and/or mechanism of entry (bacterial invasion versus phagocytosis).

We have shown that MHC class I and CD44 were recycled in a syntaxin13- and Rab11-dependent manner, respectively. Previously, three distinct kinetic pathways for recycling of cargo from endosomes have been described, namely recycling directly from the sorting endosome mediated by Rab4 (23, 24), a rapid recycling pathway through the perinuclear recycling compartment (41), and a "slow pathway" through the perinuclear recycling compartment (39, 40). Movement through the slow pathway has a t½ of ~30 min (39), corresponding to the timing of both MHC class I and CD44 recycling from the SCV (this study). Therefore, our data suggest that the slow pathway encompasses two distinct pathways of recycling occurring simultaneously and regulated, at least in part, by syntaxin13 and Rab11.

The association of the SCV with MHC class I may suggest a role for syntaxin13 in antigen presentation. A proteasome- and TAP (transporter associated with antigen processing)-independent MHC class I loading pathway involving processing of...
peptides in an endosomal/phagosomal compartment has been reported (56–61). Peptides can be exchanged within endosomes (62, 63), and MHC class I can recycle back to the cell surface (64). Based on the internalization of MHC class I upon infection with Salmonella Typhimurium, it is possible that bacterial antigens are being loaded as MHC class I is interacting with the SCV and are then recycled to the plasma membrane for presentation. Our study demonstrates that syntaxin13 plays a role in MHC class I recycling to the cell surface.

The accumulation of LAMP-1 has been used to mark the maturation of the SCV to a late endosome-like compartment (9, 10, 48). Our findings showed dominant negative recycling constructs that interfere with proper recycling inhibit maturation of the SCV, as characterized by a delay in LAMP-1 accumulation. This is consistent with previous results demonstrating the inhibitory effects of dominant negative syntaxin13 on maturation of latex bead-containing phagosomes in macrophages (19). Although there is an effect on maturation, it is clearly not one of complete inhibition. Throughout the study, the inhibitory effects of dominant negative constructs caused only a partial reduction in recycling or maturation. This suggests the presence of redundant recycling pathways or regulators. When we examined the replication of Salmonella Typhimurium in inhibited cells, we saw no change in replication (data not shown), implying that redundant pathways are able to complete the necessary recycling in order for replication to take place.

In summary, our study demonstrated that the SCV interacts with multiple recycling regulators. Importantly, we showed that Rab11 and syntaxin13 regulate specific, concurrent pathways. We have also shown that recycling and maturation are linked. Together with the aggregation of cell surface markers upon invasion, we believe that early trafficking of the SCV can be used as a good model of endocytic recycling. There are still many questions to be answered regarding the trafficking of the SCV, and a broad study to determine a more complete set of interactions with the cellular machinery is needed to provide these answers.

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