Role of 3-Phosphoinositides in the Maturation of Salmonella-containing Vacuoles within Host Cells*

Cameron C. Scott‡§§, Patricia Cuellar-Mata‡§, Tsuyoshi Matsuo**, Howard W. Davidson‡‡, and Sergio Grinstein§§ §§

From the ‡Division of Cell Biology, The Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada, the §Department of Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada, the **Division of Signal Transduction, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, Boston, Massachusetts 02115, and the ‡‡Wellcome Trust Center for Molecular Mechanisms of Disease, University of Cambridge, Addenbrookes Hospital, Cambridge CB2 2QQ, United Kingdom

Salmonella typhimurium invades mammalian cells and replicates within a vacuole that protects it from the host’s microbicidal weapons. The Salmonella-containing vacuole (SCV) undergoes a remodelling akin to that of the host cell’s endocytic pathway, but SCV progression is arrested prior to fusion with lysosomes. We studied the role of phosphatidylinositol 3-kinase (PI3-K) in SCV maturation within HeLa cells. Phosphatidylinositol 3-phosphate (P13P), monitored in situ using fluorescent conjugates of FYVE or PX domains, was found to accumulate transiently on the SCV. Wortmannin prevented P13P accumulation and the recruitment of EEA1 but did not affect the association of Rab5 with the SCV. Importantly, inhibition of PI3-K also impaired fusion of the SCV with vesicles containing LAMP-1. Rab7, which is thought to be required for association of LAMP-1 with the SCV, still associated with SCV in wortmannin-treated cells. We have therefore concluded that a 3-phosphoinositide-dependent step exists following recruitment of Rab7 to the SCV. The data also imply that 3-phosphoinositide-dependent effectors of Rab5 are not an absolute requirement for recruitment of Rab7. Despite failure to acquire LAMP-1, the SCV persists and allows effective replication of Salmonella within wortmannin-treated host cells. These findings imply that PI3-K is involved in the development of the SCV but is not essential for intracellular survival and proliferation of Salmonella.

The enteropathogenic bacterium Salmonella typhimurium is able to invade host cells, where it resides within a membrane-bound vacuole. By altering the normal traffic of intracellular membranes, the Salmonella vacuole remains isolated from the cellular lysosomes, thereby avoiding contact with their microbicidal contents. Although great progress has been made in elucidating the molecular mechanisms of invasion, the processes whereby the bacteria control the interaction of the vacuole with the endocytic pathway remain incompletely understood and often controversial (1–5).

Following attachment to the host cell surface, Salmonella uses a specialized type III secretion system to deliver bacterial effector proteins across the cell plasma membrane into the cytoplasm (6). These virulence factors include phosphatases (7), Rho-GTPase exchange factors (8), and other modulators of the host cell cytoskeleton (2, 9, 10). The net effect of these bacterial proteins is the induction of pronounced ruffling of the host cell membrane, leading to the internalization of the bacteria into a Salmonella-containing vacuole (SCV).1

Initially, the composition of the SCV resembles that of the host’s early endocytic compartment. Distinctive endosomal markers such as the early endosome autoantigen 1 (EEA1), the transferrin receptor, and Rab5 are detectable on the membrane of the early SCV (5, 11). At later stages, the SCV acquires selectively some, but not all, of the proteins known to be present in the host cell late endosomes. Thus, although the lysosome-associated membrane protein 1 (LAMP-1) (12), the vacuolar proton pump (11), and Rab7 (13) associate with the SCV, the mannose-6-phosphate receptor (M6PR) (14) is largely excluded. More importantly, unlike the late endosome, the SCV fails to merge with lysosomes. This diversion of the endocytic traffic is dictated by an additional set of bacterial proteins encoded by a separate pathogenicity island in the Salmonella genome. These proteins are also delivered via a type III system to the host cell cytosol, protecting the bacteria from exposure to the degradative environment of the lysosome (15).

The normal traffic of cellular endomembranes is directed by several types of regulatory proteins, including the Rab family of small GTPases (16). The fusion of early endosomes is directed by Rab5, in conjunction with several effector proteins that include EEA1 (17, 18). Both Rab5 and EEA1 have been detected on the early SCV (5, 11), but their precise roles in vacuolar maturation are not well defined. EEA1 attaches directly to Rab5, but this interaction needs to be stabilized by PI3P, a product of the type III phosphatidylinositol 3-kinase (PI3-K), VPS34. The latter is also thought to be a Rab5 effector (17).

* This study was supported by the Canadian Institutes of Health Research (CIHR), the Arthritis Society of Canada, and the National Sanatorium Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†† Recipient of graduate studentships from the CIHR.

‡‡ Present address: Laboratorio de Bioquímica, Facultad de Química, Universidad de Guanajuato, Guanajuato, Mexico.

§§ CIHR Senior Scientist, International Scholar of the Howard Hughes Medical Inst., and the Pitblado Chair in Cell Biology at the Hospital for Sick Children. To whom correspondence should be addressed: Cell Biology Program, The Hospital for Sick Children, 555 University Ave., Toronto, Ontario M5G 1X8, Canada. Tel.: 416-813-5727; Fax: 416-813-5028; E-mail: sga@sickkids.ca.

1 The abbreviations used are: SCV, Salmonella-containing vacuole; EEA1, early endosome autoantigen 1; LAMP-1, lysosome-associated membrane protein 1; M6PR, mannose-6-phosphate receptor; PBP, phosphatase-buffered saline; PI3-K, phosphatidylinositol 3-kinase; PI3F, phosphatidylinositol 3-phosphate; p40phox, 40-kDa subunit of the NADPH oxidase; SLO, streptolysin-O; GFP, green fluorescent protein; LPS, lipopolysaccharide; FITC, fluorescein isothiocyanate; SIF, Salmonella-induced filament; MES,4-morpholineethanesulfonic acid .
Recently, PI3P was reported to be an essential factor in the normal maturation of endosomes (19) and also of phagosomes (20), which more closely resemble the SCV. It therefore appeared likely that this phosphoinositide could also play a role in SCV development and raised the possibility that alterations in the metabolism of PI3P may contribute to the maturation arrest of the vacuole. The purpose of the present experiments was therefore to analyze the metabolism of PI3P during the course of invasion of mammalian cells by Salmonella.

It recently became apparent that modular domains within certain proteins have the ability to interact with the head-groups of defined phosphoinositides. Two types of domains have been identified that recognize PI3P with great selectivity and considerable affinity: FYVE domains, such as the one present in EEA1 (21), and PX domains (22–24). In this report we have used chimeric constructs of GFP and the FYVE domain of EEA1 and of the PX domain of the 40-kDa subunit of the NADPH oxidase to monitor the distribution and dynamics of PI3P during the formation and progression of the SCV.

**EXPERIMENTAL PROCEDURES**

**Materials**—Murine monoclonal antibodies to giantin were generously provided by Dr. Hans-Peter Hauri, University of Basel. Goat polyclonal anti-human EEA1 (EEA1 N-19) was from Santa Cruz. Murine monoclonal anti-human LAMP-1 (H4A3) was from the Developmental Studies Hybridoma Bank. The rabbit polyclonal antibodies to S. typhimurium LPS (229483) were from Difco. FITC-conjugated donkey anti-rabbit IgG, Texas Red-conjugated donkey anti-rabbit IgG, Texas Red-conjugated donkey anti-mouse IgG, and Cy3-conjugated anti-goat IgG were from either Jackson ImmunoResearch or Molecular Probes. Tissue culture supplies were obtained from CellGro. Wortmannin and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)2000] were from Avanti Polar Lipids. Wortmannin and 0.1% sodium dodecyl sulfate in PBS. The L929 cells were diluted and seeded onto LB-agar plates, which were incubated overnight at 37°C. Finally, the number of bacterial colonies was counted.

**Immunostaining and Fluorescence Microscopy**—Cells were fixed with 4% paraformaldehyde in PBS for 1 h at room temperature and washed with 100 mM glycine in PBS for 10 min. The fixed cells were then permeabilized with 0.1% Triton X-100/0.5% powdered milk for 1 h at room temperature or overnight at 4°C. The primary antibodies, followed by the secondary antibodies, were added to the coverslips in PBS with 5% milk for 45 min at 37°C. The coverslips were washed three times with PBS after each antibody incubation. Coverslips were mounted on glass slides using Fluorescent Mounting Medium (Dako Corp.). Colocalization of intracellular bacteria with markers was determined using a Leica DMR microscope under a 100× oil immersion objective using standard FITC/CY3/Cy5 filters.

Where required, extracellular bacteria were identified by sequential addition of PBS containing 1% bovine serum albumin, 2% donkey serum in PBS, antibodies against S. typhimurium LPS, and finally Alexa 350-conjugated goat anti-rabbit IgG on ice for 15 min each. The cells were then washed twice with ice-cold PBS before additions. The cells were then fixed and prepared for analysis as described above. Colocalization of intracellular bacteria with markers was quantified after identifying and disregarding the extracellular adherent bacteria. Fluorescence images were captured using a LSM 510 laser-scanning confocal microscope (Carl Zeiss, Inc.) with a 100× oil immersion objective. GFP/FITC and rhodamine/Cy3/Texas Red were examined using the conventional laser excitation and filter sets. Digital images were prepared using Adobe Photoshop 6.0 (Adobe Systems, Inc.) and CorelDraw® 8.0 (Corel Corp.).

**Intracellular Bacterial Proliferation**—Bacterial invasion was performed as described above except that 24-well plates without coverslips were used and the multiplicity of infection was ~10. Twenty minutes after infection, 50 μg/ml of gentamicin was added to eliminate extracellular bacteria. For prolonged experiments, the concentration of gentamicin was reduced to 5 μg/ml 2 h postinfection. At the appropriate time, cells were washed twice with ice-cold PBS and lysed using 1% Triton X-100, 0.1% sodium dodecyl sulfate in PBS. The lysate was then used to determine colony-forming units after plating serial dilutions on LB agar containing 100 μg/ml ampicillin. All data were normalized to the input number of bacteria. The remaining amount of bacteria was determined after 4 h of infection (15 min of the time course were used for the data in Fig. 3A), and the error bars represent the means ± S.E. of triplicate samples.

**REFERENCES AND DISCUSSION**

2-FYVE-GFP and p40PX-GFP Are Suitable Probes for PI3P in HeLa Cells—Two structurally distinct probes, based on the FYVE domain of EEA1 and the PX domain of p40phox, were used to monitor the distribution of PI3P. The efficiency and specificity of the probes were evaluated first. HeLa cells were transfected with either the FYVE-GFP construct (encoding a single FYVE domain) or the p40PX-GFP construct (encoding a tandem FYVE domains attached to GFP). The cellular distribution of the probes was compared with that of EEA1, a marker of early endosomes, which are the primary site of accumulation of cellular PI3P. The FYVE-GFP construct had a cytosolic distribution with no discernible association with cellular organelles (Fig. 1A). In contrast, the 2-FYVE-GFP probe accumulated in punctate structures that were also enriched in EEA1 (Fig. 1C and D). This localization is consistent with the single and tandem FYVE domain constructs in the single and tandem FYVE domain constructs is likely due to the increased avidity of the latter for PI3P-containing membranes. This is consistent with recent reports, which similarly found that the affinity of a single...
FYVE domain for PI3P is insufficient to promote detectable binding to endomembranes in vivo (28, 29). That the 2-FYVE-GFP probe associates with the membranes by interaction with 3-phosphoinositides was confirmed using wortmannin. Treatment of the cells with this PI3-K inhibitor displaced the bound 2-FYVE-GFP from the endosomes, yielding a cytosolic distribution (Fig. 1E) indistinguishable from that obtained with unconjugated GFP.

The specificity of the p40PX-GFP fusion was validated in Fig. 2. As in the case of 2-FYVE-GFP, the p40PX-GFP chimera accumulated preferentially in EEA1-positive organelles (Fig. 2, A and B). Importantly, a p40PX-GFP construct bearing the point mutation R57Q, which fails to bind PI3P in vitro (23), was found largely in the cytosol and did not concentrate in early endosomes (Fig. 2, A and B, insets). The selectivity of the p40PX-GFP probe was also tested using wortmannin. As illustrated in Fig. 2, C and D, (main panels), treatment with the inhibitor released p40PX-GFP from the endosomal membrane, yielding a uniform cytosolic distribution. Moreover, similar results were obtained with a chemically distinct PI3-K antagonist, namely LY294002 (Fig. 2, C and D, insets). Jointly, these results indicate that p40PX-GFP is a suitable probe for PI3P distribution in intact cells.

PI3P Accumulates in the Membrane of Salmonella-containing Vacuoles—HeLa cells transfected with 2-FYVE-GFP or p40PX-GFP were infected with S. typhimurium, and the distribution of the probes, considered hereafter as a reliable index of the presence of PI3P, was monitored by confocal fluorescence microscopy. As illustrated in Fig. 3 A, B, E, and F, both probes accumulated on the membrane of the SCV shortly after invasion. As described earlier (30), invasion by Salmonella is insensitive to wortmannin, which enabled us to test the involvement of PI3-K in the accumulation of 2-FYVE-GFP on the vacuole. As shown in Fig. 3C, the inhibitor precluded the accumulation of the probe on the SCV.

We monitored the accumulation of PI3P on the SCV as a function of time after invasion by acquiring images that were subsequently quantified by digital densitometry. As shown in Fig. 3G, PI3P accumulated only transiently on the SCV, although ~60% of the SCVs accumulated 2-FYVE-GFP 20 min after initiation of invasion, only ≤5% remained positive after 55 min. This pattern of transient accumulation resembles that reported in normal phagosomes, which proceed to become phagolysosomes (20). This suggests that the inability of the SCV to fuse with lysosomes and with a subpopulation of late endosomes is not attributable to a sustained accumulation of PI3P.

Acquisition of EEA1 but Not Rab5 Is Inhibited by Wortmannin Treatment—EEA1, which is present on the early SCV (11), possesses a FYVE domain capable of binding PI3P. The fact that EEA1 exists in dimeric form (31) suggests that interaction of the FYVE domains with PI3P could suffice to recruit this protein to the SCV. Indeed, the time course of EEA1 association with the SCV closely parallels the presence of PI3P on the vacuolar membrane (see Figs. 3G and 4I). To test this notion, cells were pretreated with wortmannin, and the association of EEA1 with the SCV was studied by immunostaining. The PI3-K inhibitor eliminated the binding of EEA1 to the vacuole almost completely (Fig. 4C). This implies that PI3P is strictly required for EEA1 recruitment or retention by the SCV and that the known interaction of Rab5 with EEA1 is insufficient to accumulate the latter on the vacuolar membrane.

Because EEA1 acquisition is dependent on the presence of PI3P on the SCV, we next determined whether the localization of Rab5, an EEA1 binding partner, to the SCV was affected by wortmannin. HeLa cells were transiently transfected with a construct encoding a Rab5-GFP fusion. When appropriate levels of the protein were expressed, the cells were infected with bacteria, and the presence of Rab5-GFP on the SCV was visualized and scored at different times. Rab5 could be readily detected on the membrane of early SCV (Fig. 4E). Like PI3P and EEA1, the association of Rab5 with the SCV was found to be transient, becoming virtually undetectable by 30 min (Fig. 4J). This finding is consistent with the report of Méresse et al. (13) but disagrees with the observations by Hashim et al. (5), who found that the maturation of the SCV is arrested at the Rab5 stage. Conceivably, this inconsistency could be the result of differential association of the three known Rab5 isoforms (32) with the SCV because our method tested only the fate of Rab5a, whereas the antibody used by Hashim et al. (5) may have reacted with Rab5b and Rab5c as well. Further experiments are required to resolve this discrepancy.

Wortmannin treatment was found to have little effect on Rab5 localization to the SCV (Fig. 4G). Therefore, neither PI3P

---

**FIG. 1.** Transient expression of FYVE-GFP and 2-FYVE-GFP in HeLa cells. HeLa cells were transfected with either a plasmid containing the FYVE-GFP construct (A and B) or the 2-FYVE-GFP construct (C–F), fixed with 4% paraformaldehyde, and immunostained with antibodies to EEA1, followed by a Texas Red-conjugated secondary antibody. Arrows point to 2-FYVE-GFP- and EEA1-positive structures. E and F, cells expressing 2-FYVE-GFP were treated with 100 nM wortmannin for 30 min prior to fixation and immunostaining. A, C, and E, green (GFP) fluorescence. B, D, and F, red (EEA1) fluorescence. Representative of three experiments. Size bar, 10 μm.

**FIG. 2.** Transient expression of p40PX-GFP in HeLa cells. HeLa cells were transfected with either a plasmid containing p40PX-GFP (A–D, main panels, and C and D, insets) or the R57Q mutant of p40PX-GFP (A and B, insets), fixed with 4% paraformaldehyde, and immunostained with antibodies to EEA1, followed by a Texas Red-conjugated secondary antibody. Arrows point to p40PX-GFP- and EEA1-positive structures. C and D, cells expressing wild-type p40PX-GFP were treated with either 100 nM wortmannin (main panels) or with 50 μM LY294002 for 60 min prior to fixation and immunostaining. A and C, green (GFP) fluorescence. B and D, red (EEA1) fluorescence. Representative of three experiments.
for recruitment of early endosomal proteins. The GTPase is for endosomes, whereby Rab5 is the primary factor responsible therein. These results are consistent with the model suggested with the SCV. Infected cells were scored as EEA1 or Rab5-GFP positive if at least one clearly stained SCV was present.

nor EEA1 is required to recruit Rab5 to the SCV or to retain it therein. These results are consistent with the model suggested for endosomes, whereby Rab5 is the primary factor responsible for recruitment of early endosomal proteins. The GTPase is likely essential for the association of VPS34, the probable source of PI3P, with the SCV. The localized synthesis of the phosphoinositide then attracts and/or stabilizes the interaction of EEA1 with the early vacuole. Our observation that interaction with Rab5 alone is insufficient to localize EEA1 to the SCV is consistent with reports that both the FYVE domain and the Rab5-binding domains of EEA1 are required for its early endosomal localization (28).

LAMP-1 Acquisition by the SCV Is Inhibited by Wortmannin—We next sought to determine whether PI3P and EEA1 were required for the transition between the early and late stages of maturation of the SCV. Incorporation of LAMP-1 into the SCV was detected by immunolocalization in fixed and permeabilized cells. As shown in Fig. 5I, this late endosomal-lysosomal marker is acquired progressively over time, reaching a maximal level after ~60 min.

As shown in Fig. 5C, LAMP-1 acquisition by the SCV was greatly inhibited by treatment with wortmannin. Unlike the inhibition of EEA1 acquisition by wortmannin, which was virtually complete, LAMP-1 was present on a significant fraction of the SCV (~20%) after 30 min but persisted at this level over the period investigated (Fig. 5B). LAMP-1 is believed to reside primarily in late endosomes and lysosomes, but a smaller fraction of the protein is present in the plasma membrane and possibly also in early endosomes, en route from the biosynthetic pathway to the lysosomes. The latter population was shown recently to reach the SCV (33) and may account for the fraction of LAMP-1 that is rapidly acquired and insensitive to wortmannin.

Effect of Wortmannin on Rab7 Recruitment—Méresse et al. (13) reported that LAMP-1 is delivered to the SCV from a non-lysosomal compartment by a process that requires Rab7. The failure to acquire LAMP-1 in cells treated with wortmannin suggests that either the recruitment of Rab7 is itself a PI3K-dependent event or this GTPase requires 3-phosphoinositides to execute the fusion of the SCV with the LAMP-1-containing vesicles. These alternatives were explored by monitoring the effect of wortmannin on the acquisition of Rab7 by the vacuoles.

HeLa cells were transfected with a Rab7-GFP chimera, and when expression was sufficient for visualization (~18 h) they were infected with Salmonella. As illustrated in Fig. 5E, Rab7
FIG. 5. Effect of wortmannin on LAMP-1 and Rab7 acquisition by the SCV. HeLa cells were treated with 100 nm wortmannin in dimethyl sulfoxide (C, D, G, H) or with the solvent only (A, B, E, F) for 30 min prior to infection with S. typhimurium SL1344. The cells were then incubated for the indicated period of time and fixed with 4% paraformaldehyde for 1 h. A–D, cells incubated for 60 min were permeabilized and immunostained with antibodies to LAMP-1 (A and C) and to Salmonella (B and D). E–H, the cells were transfected with Rab7-GFP 18 h prior to incubation with Salmonella for 30 min. E and G, Rab7-GFP fluorescence. F and H, immunolocalization of Salmonella. Arrows indicate the location of intracellular bacteria. I, time course of the association of LAMP-1 with the SCV, quantified at the times after invasion indicated on the abscissa. Ordinate, percentage of SCV that contained clearly detectable LAMP-1. J, time course of the association of Rab7 with the SCV. Infected cells were scored as LAMP-1- or Rab7-GFP-positive if at least one clearly stained SCV was present. Solid symbols, control cells. Open symbols, wortmannin-treated cells. Results shown are the means ± S.E. of three separate experiments each with at least 25 individual vacuoles counted.

was clearly identifiable in late SCV. A detailed time course of the acquisition of Rab7 following invasion is summarized in Fig. 5, panel J. The rate and extent of Rab7 recruitment by the SCV were also studied in cells treated with wortmannin (Fig. 5G). The kinase inhibitor delayed but did not prevent the acquisition of Rab7 by the SCV. This unexpected result suggests that PI3P-dependent effectors of Rab5, such as EEA1 and Rabenosyn-5, are not absolutely required for the recruitment of Rab7. Other effectors of Rab5 may be involved in this process, or the recruitment of Rab7 to the SCV occurs, at least in part, independently of Rab5. Our findings also suggest that Rab7 itself employs products of PI3-kinase to accomplish the fusion of the SCV with the LAMP-1-containing compartment. There is precedent for a role of PI3-K activity in late endosome trafficking, because inhibition of this enzyme impaired M6PR recycling to the trans-Golgi network and the redistribution of LAMP-1 and LAMP-2 to a M6PR-negative compartment (34, 35). Finally, these results imply that Rab7 is recruited to the SCV, at least partly, from a compartment devoid of LAMP-1. This may occur through fusion with a Rab7-positive, LAMP-1-negative organelle or more likely by recruitment of soluble Rab7 upon activation of a membrane-associated exchange factor/anchor.

Intracellular Proliferation of Salmonella Is Not Affected by Wortmannin—By secreting the virulence factors encoded by the pathogenicity island 2, Salmonella modulates the maturation of the vacuole, ostensibly to facilitate its own survival and proliferation. Indeed, Salmonella proliferation has been reported to be dependent on the changes in the intravacuolar environment that accompany maturation (36, 37). It was therefore of interest to determine whether interference with the normal course of SCV development, as observed in wortmannin-treated cells, would reduce the ability of the bacteria to proliferate within the host cells. This premise was tested by measuring the intracellular growth of Salmonella inside HeLa cells, using the conventional gentamicin exclusion assay (Fig. 6). In five experiments, the proliferation rate of Salmonella was similar or slightly greater in cells treated with wortmannin than in the corresponding untreated control cells for up to 6 h after invasion. These data suggest that changes in the composition of the luminal environment of the SCV that are critical for optimal bacterial proliferation occur before or independently of the maturation step blocked by wortmannin. Clearly, neither PI3P, EEA1, nor LAMP-1 acquisition by the vacuole is essential for successful bacterial replication. We speculate that the presence of these molecules in the SCV is the result of the normal endosomal maturation process of the host cell and is not directed by Salmonella nor is it essential for proliferation of the parasite. This conclusion is consistent with a recent report demonstrating that, although expression of a constitutively active mutant of Rab5 significantly altered SCV maturation in epithelial cells, bacterial proliferation was not inhibited (33). Additionally, bacterial proliferation within dendritic cells proceeds in SCV devoid of LAMP-1 (38), further indicating that this lysosomal glycoprotein is not essential for growth.

Salmonella Remains within Vacuoles in Wortmannin-treated Cells—In some cell types, including HeLa, the SCV develops into filamentous structures termed “Sifs” (Salmonella-induced filaments) (12). Formation of these filamentous vacuoles is dictated by the bacterial gene product known as SifA (39). It was recently reported that deletion of the SifA gene not only precludes formation of Sifs but also results in escape of the bacteria from the vacuole into the host cell cytosol (4). Because inhibition of PI3-K also interferes with the development of the vacuole, it was of interest to determine whether Salmonella remains within a modified vacuole in wortmannin-treated cells or whether it escapes into the cytosol. To evaluate the integrity of the SCV, we used SLO to selectively permeabilize the plasma membrane to gain access to the cytosol. This approach, similar to the one employed by Beuzón et al. (4), uses...
were added to SLO-permeabilized (Fig. 7). Permeabilization the plasmalemma was shown using antibodies but will fail to do so if the bacteria are enclosed within the SCV.

Labeling (Fig. 7) to cells permeabilized with Triton X-100 yielded strong positive fraction of labeled bacteria appeared to be within necrotic cells failed to label when antibodies were added (Fig. 7). When cells were permeabilized with SLO, the vast majority of bacteria cells (Fig. 7). The small activity of wortmannin-sensitive isoforms of PI3-K. This implies that either Rab5 promotes the recruitment of Rab7 by PI3P-independent means or, more intriguingly, that Rab5 activation may not be necessary for the association of (at least a fraction of) Rab7 with the SCV. Second, fusion of the SCV with the compartment(s) bearing LAMP-1 depends on one or more products of PI3-K. Rab7 was reported earlier to be essential for incorporation of LAMP-1 into the SCV (13). Thus, it is likely that, as in the case of Rab5, one or more of the effectors of Rab7 may require PI3P or another 3-phosphoinositide to be recruited and/or to become functional. Alternatively, it is conceivable that wortmannin acts upstream, preventing the delivery of LAMP-1 to the Rab7-sensitive compartment. We consider this possibility less likely, given the comparatively short duration of the wortmannin treatment in our experiments. Last, we conclude that normal bacterial proliferation can occur in a compartment that is largely devoid of LAMP-1. The nature of this compartment remains elusive, but our evidence suggests that at least in the early stages it is not the cytosol, because the bacteria were inaccessible to antibodies added to SLO-permeabilized cells 4 h after infection. It remains to be defined whether at later times filamentous structures like the Sifs, yet devoid of LAMP-1, are formed in wortmannin-treated cells or whether the bacteria break out into the cytosol.

**Acknowledgments**—We thank Dr. M. Yaffe for providing the p40PX-GFP constructs, Drs. B. B. Finlay and J. Brumell (University of British Columbia) for advice and for providing bacterial strains, Jonathan Plumb for preparation of the pool-3 fraction of polyethyleneimine used for transfections, and Dr. M. Zerial for providing the Rab7 cDNA.

**REFERENCES**

1. Ohman, H., Sancin, F. C., Solomon, F., and Groisman, E. A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 7800–7804
2. Hayward, R. D., and Koronakis, V. (1999) *EMBO J.* **18**, 4926–4934
3. Uchiya, K., Barbieri, M. A., Funato, K., Shah, A. H., Stahl, P. D., and Groisman, E. A. (1999) *EMBO J.* **18**, 3924–3933
4. Beuzon, C. R., Meroses, S., Unsworth, K. E., Ruiz-Albert, J., Garvis, S., Waterman, S. R., Ryder, T. A., Bourcet, E., and Holdon, D. W. (2000) *EMBO J.* **19**, 3235–3249
5. Hashim, S., Mukherjee, K., Raje, M., Basu, S. K., and Mukhopadhyay, A. (2000) *J. Biol. Chem.* **275**, 16281–16288
6. Kubori, T., Matushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., Galan, J. E., and Aizawa, S. I. (1998) *Science* **280**, 602–605
7. Norris, P. A., Wilson, M. P., Wallis, T. S., Galyov, E. E., and Majerus, P. W. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 14057–14059
8. Aragon, A., Bremnes, B., Stenmark, H., and Arrigo, A., S. (2001) *Nat. Cell. Biol.* **5**, 3610–3616
9. Meroses, S., Steele-Mortimer, O., Meresse, S., Gervel, J. P., Toh, B. H., and Finlay, B. B. (1999) *Cell Microbiol.* **1**, 33–49
10. Garcia-del Portillo, F., and Finlay, B. B. (1995) *Cell Biol. 129*, 81–97
11. Hensel, M. (2000) *Mol. Microbiol.* **36**, 1015–1023
12. Bock, J. B., Matern, H. T., Peden, A. A., and Scheller, R. H. (2001) *Nature* **409**, 849–841
13. Christoforidis, S., Miazynska, M., Ashman, K., Wilm, M., Zhao, L., Yip, S. C., Waterfield, M. D., Backer, J. M., and Zerial, M. (1999) *Nat. Cell. Biol.* **1**, 249–252
14. Simonson, A., Lippe, R., Christoforidis, S., Gaujard, J. M., Brech, A., Callaghan, J., Toh, B. H., Murphy, C. Z., and Stenmark, H. (1998) *Nature* **394**, 494–498
15. Cheever, M. L., Stelzer, E. H., and Cullin, A. (1999) *Nat. Cell. Biol.* **1**, 231–237
16. Xu, Y., Hortsman, H., Seet, L., Wong, S. H., and Hong, W. (2001) *Nat. Cell. Biol.* **3**, 658–666
17. Bieber, T., and Elsasser, H. P. (2001) *BioTechniques* **30**, 74–77, 80–71
18. Bragonzi, A., Boletta, A., Biffi, A., Muggia, A., Sersale, G., Cheng, S. H., Bordin, C., Assael, B. M., and Conese, M. (1999) *Gene Ther.* **6**, 12775.

**Role of PI3P in Salmonella Invasion**

**FIG. 7. Subcellular localization of Salmonella within wortmannin-treated cells.** A and B, HeLa cells were either left intact (A) or were permeabilized using SLO as described under “Experimental Procedures” (B). The cells were then incubated in the cold with antibodies to giantin, followed by fluorescent secondary antibodies. C and D, HeLa cells were treated with 100 ng/well wortmannin for 30 min, infected with *S. typhimurium* SL1344, and incubated at 37 °C for 4 h. Outside bacteria were labeled with antibodies to *S. typhimurium* LPS, followed by a FITC-conjugated secondary antibody (C). The cells were then permeabilized with SLO (D), exposed again to an antibody raised against *S. typhimurium* LPS, followed by a Cy3-conjugated secondary antibody, and finally fixed with 4% paraformaldehyde. E and F, HeLa cells were treated with 100 ng/well wortmannin and infected as in C and D. E, the external bacteria were labeled as in C. The cells were next fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and immunostained with *S. typhimurium* LPS antibodies, followed by Cy3-conjugated secondary antibodies (F). FITC staining corresponding to adherent extracellular bacteria is shown on the left and Cy3 staining, corresponding to bacteria accessible after permeabilization, is shown on the right.

accessibility to antibodies to determine whether *Salmonella* is surrounded by a vacuolar membrane or is free in the cytosol. In the latter case, antibodies that are allowed to enter the cytosol via SLO will react with *Salmonella* if the bacteria are cytosolic but will fail to do so if the bacteria are enclosed within the SCV. Typical results are illustrated in Fig. 7. That SLO successfully permeabilized the plasmalemma was shown using antibodies to giantin, a protein of the Golgi complex that exposes epitopes to the cytosol. Giantin became clearly labeled when antibodies were added to SLO-permeabilized (Fig. 7B) but not to intact cells (Fig. 7A). By contrast, when cells infected with *Salmonella* were permeabilized with SLO, the vast majority of bacteria failed to label when antibodies were added (Fig. 1D). The small fraction of labeled bacteria appeared to be within necrotic cells in which SCV integrity had been lost. Addition of the antibody to cells permeabilized with Triton X-100 yielded strong positive labeling (Fig. 7F), indicating that both the epitope and the antibody were present but unable to interact when the SCV was intact. In two separate experiments, the fraction of the bacteria that were encapsulated by an endomembrane vacuole was ~90% in both control and wortmannin-treated cells 4 h after invasion. Because the majority of the bacteria was inaccessible to the anti-*Salmonella* antibody in wortmannin-treated cells, we conclude that SCV integrity persisted in the absence of functional PI3-K, at least during the early stages of the invasion process.

**Concluding Remarks**—Three main conclusions can be derived from our results. First, acquisition of Rab7 by the SCV is not stringently dependent on the presence of PI3P nor on the activity of wortmannin-sensitive isoforms of PI3-K. This im-
Role of PI3P in Salmonella Invasion

1995–2004
28. Lawe, D. C., Patki, V., Heller-Harrison, R., Lambright, D., and Corvera, S. (2000) J. Biol. Chem. 275, 3699–3705
29. Gillooly, D. J., Morrow, I. C., Lindsay, M., Gould, R., Bryant, N. J., Gaullier, J. M., Parton, R. G., and Stenmark, H. (2000) EMBO J. 19, 4577–4588
30. Mesas, J., Raupach, B., and Falkow, S. (1998) Mol. Microbiol. 28, 1269–1281
31. Callaghan, J., Simonsen, A., Gaullier, J. M., Toh, B. H., and Stenmark, H. (1999) Biochem. J. 338, 539–543
32. Bucci, C., Lutcke, A., Steele-Mortimer, O., Olkkonen, V. M., Dupree, P., Chiarriello, M., Bruni, C. B., Simons, K., and Zerial, M. (1995) FEMS Lett. 366, 65–71
33. Baldeon, M. E., Ceresa, B. P., and Casanova, J. E. (2001) Cell Microbiol. 3, 473–486
34. Row, P. E., Reaves, B. J., Demin, J., Luzio, J. P., and Davidson, H. W. (2001) Biochem. J. 355, 655–661
35. Reaves, B. J., Bright, N. A., Mullock, B. M., and Luzio, J. P. (1996) J. Cell Sci. 109, 749–752
36. Deiwick, J., Nikolaus, T., Erdogan, S., and Hensel, M. (1999) Mol. Microbiol. 31, 1759–1773
37. Rathman, M., Sjaastad, M. D., and Falkow, S. (1996) Infect. Immun. 64, 2765–2773
38. Garcia-Del Portillo, F., Jungnitz, H., Rohde, M., and Guzman, C. A. (2000) Infect. Immun. 68, 2985–2991
39. Stein, M. A., Leung, K. Y., Zwick, M., Garcia-del Portillo, F., and Finlay, B. B. (1996) Mol. Microbiol. 20, 151–164