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A role for the PhoP/Q regulon in inhibition of fusion between lysosomes and *Salmonella*-containing vacuoles in macrophages

Steven G. Garvis, Carmen R. Beuzón and David W. Holden*

Department of Infectious Diseases, Centre for Molecular Microbiology and Infection, Imperial College of Science, Technology and Medicine, The Flowers Building, Armstrong Road, London SW7 2AZ, UK.

Summary

After uptake by murine macrophages, *Salmonella typhimurium* is able to survive and replicate within specialized phagosomes called *Salmonella*-containing vacuoles (SCVs), which are segregated from the late endocytic pathway. The molecular basis of this process and the virulence factors required are not fully understood. In this study, we used confocal fluorescence microscopy to evaluate interactions between the endocytic pathway of the murine macrophage cell line RAW 264.7 and different *S. typhimurium* strains. The analysis was carried out using the fluid-phase marker Texas red–ovalbumin and antibodies against the lysosomal enzyme cathepsin D, the late endosomal lipid lysobisphosphatidic acid and the adaptor proteins AP-1 and AP-3. Less than 10% of wild-type SCVs were associated with these markers at 24 h after uptake by macrophages. A similar low level of association was observed for vacuoles containing mutant strains affected in the function of the *Salmonella* pathogenicity island (SPI)-2 type III secretion system or the virulence plasmid *spv* operon. However, at this time point, the proportion of vacuoles containing *phoP*$^-$ mutant bacteria that were associated with each of the markers ranged from 25% to 50%. These results show that the regulon controlled by the PhoP/Q two-component system makes a major contribution to trafficking of the SCV in macrophages. Segregation of SCVs from the endocytic pathway was also found to be dependent on bacterial proteins synthesized between 15 min and 4 h after uptake into macrophages. However, after this time, protein synthesis was not required to maintain the segregation of SCVs from late endosomes and lysosomes.

Received 5 April, 2001; revised 25 July, 2001; accepted 30 July, 2001. *For correspondence. E-mail d.holden@ic.ac.uk; Tel. (+44) 207 594 3073; Fax (+44) 207 594 3076.

Introduction

Phagocytosis of most microorganisms and particulate matter by macrophages leads to the formation of a phagosome, which matures over time into a phagolysosome, the contents of which are degraded (Aderem and Underhill, 1999). Maturation of the phagosome involves a complex series of interactions with the endocytic pathway (Desjardins *et al*., 1994). This process is usually accompanied by recycling of a subset of plasma membrane proteins and receptors, a drop in luminal pH and the acquisition of peptides, reactive oxygen intermediates and certain proteins that are characteristic of late endosomes and lysosomes, including lysosomal membrane glycoproteins (lgps) and hydrolases (Desjardins *et al*., 1994; Rohn *et al*., 2000; Tjelle *et al*., 2000; Garin *et al*., 2001).

Salmonella typhimurium is a facultative intracellular pathogen that can cause a lethal typhoid-like infection in mice. Systemic growth in mice is associated with the ability to survive and replicate within murine macrophages (Fields *et al*., 1986). After uptake by macrophages, *S. typhimurium* replicates in a membrane-bound compartment, referred to as the *Salmonella*-containing vacuole (SCV; Meresse *et al*., 1999a). Although two groups have concluded that, in macrophages, SCVs fuse with lysosomal compartments (Carrol *et al*., 1979; Oh *et al*., 1996), most studies have shown that the majority of SCVs are segregated from lysosomes and late endosomes (Ishibashi and Arai, 1990; Buchmeier and Heffron, 1991; Rathman *et al*., 1997; Uchiya *et al*., 1999; Hashim *et al*., 2000). A study using confocal immunofluorescence microscopy has shown that most SCVs do not acquire the mannose-6-phosphate receptor (MPR) or the lysosomal hydrolytic enzyme cathepsin L, which is normally delivered from the *trans*-Golgi network (TGN) to endocytic compartments via this receptor, but do acquire lgps such as LAMP-1 (Rathman *et al*., 1997). Furthermore, similar results have been obtained in epithelial cells, where the SCV acquires lgps, but does not acquire lysosomal markers delivered by the MPR (Garcia-del Portillo *et al*., 1993; Garcia-del Portillo and Finlay, 1995; Meresse *et al*., 1999b).

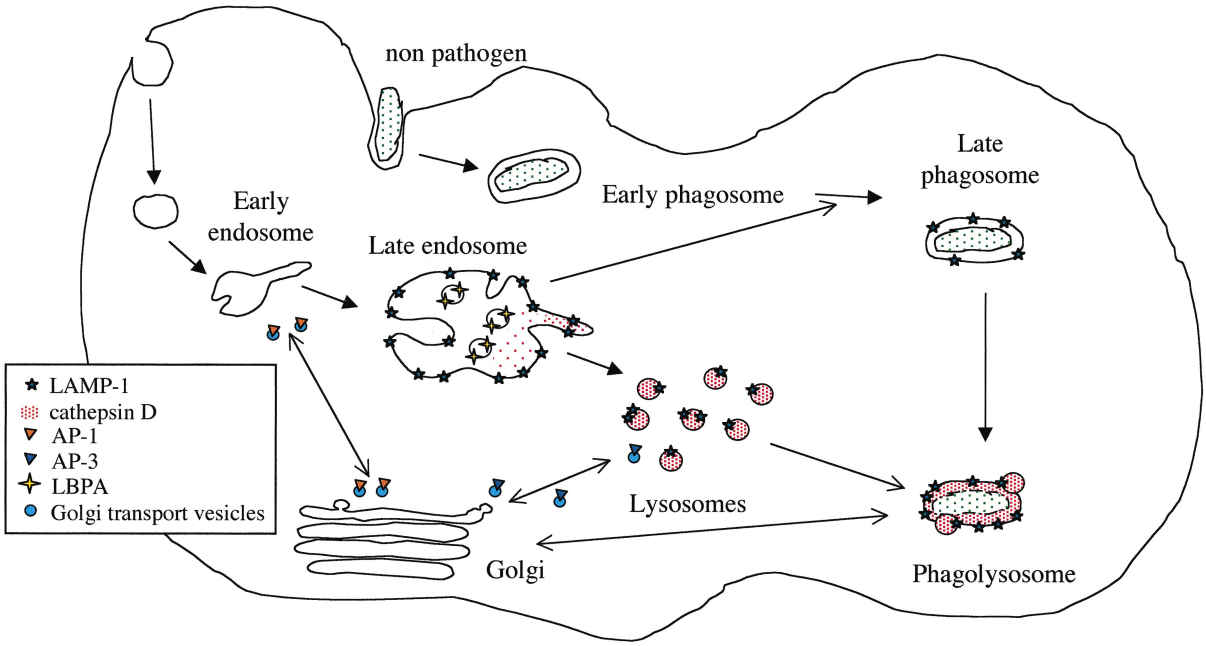


Fig. 1. Schematic representation of the subcellular localization and trafficking of the endocytic pathway markers used or discussed in this study in relation to maturation of a phagolysosome.

Survival and replication of *S. typhimurium* within macrophages is a multifactorial process involving numerous bacterial genes. These include the *spv* operon, located on the large virulence plasmid (Gulig and Doyle, 1993; Libby *et al.*, 2000), and the *phoP/Q* regulon (Miller *et al.*, 1989a). The *phoP/Q* locus encodes a two-component regulatory system controlling the expression of at least 40 genes (Miller and Mekalanos, 1990), a proportion of which have been shown to be activated inside macrophages (Buchmeier and Heffron, 1990; Abshire and Neidhardt, 1993). A third locus involved in systemic growth and intramacrophage survival is *Salmonella* pathogenicity island 2 (SPI-2; Hensel *et al.*, 1995; 1998; Ochman *et al.*, 1996; Cirillo *et al.*, 1998). SPI-2 encodes a type III secretion system (TTSS), which is expressed after bacteria enter host cells (Valdivia and Falkow, 1997), and secretes effector proteins across the vacuolar membrane (Miao *et al.*, 1999; Uchiya *et al.*, 1999; Beuzón *et al.*, 2000; Miao and Miller, 2000). One such effector, SpiC, has been reported to be required for inhibition of SCV–lysosome fusion (Uchiya *et al.*, 1999).

In this paper, we have examined the involvement of the *spv*, *phoP* and SPI-2 loci in the specialized trafficking pathway of the SCV in macrophages, using both a fluid-phase endocytic tracer and several markers representing luminal and membrane components of the endocytic pathway. This work does not provide evidence for an involvement of the *spv* or SPI-2 loci in the inhibition of phagolysosomal fusion. Our results suggest that this phenomenon is a complex process involving several bacterial factors, at least one of which is controlled by the PhoP/Q regulatory system.

Results

Markers and bacterial strains used to study interactions between SCVs and the endocytic pathway

After uptake of *S. typhimurium* by RAW 264.7 macrophages, interactions between SCVs and the endocytic pathway were studied using confocal microscopic co-localization of fluorescent markers with green fluorescent protein (GFP)-expressing bacteria. Preliminary experiments using opsonized or non-opsonized wild-type bacteria, as well as an invasion-defective mutant (*prgH*$^{-}$), confirmed that mode of entry had no significant effect on SCV trafficking (data not shown; Buchmeier and Heffron, 1991; Rathman *et al.*, 1997). All further experiments were performed using bacteria opsonized with mouse serum. In one approach, macrophages were first pulse chased with Texas Red–Ovalbumin (TROv) to load lysosomal compartments before infection with GFP-*S. typhimurium*. In a second independent approach, macrophages infected with GFP-*S. typhimurium* were stained with antibodies that recognize different components of the late endocytic pathway and detected with secondary antibodies conjugated to Texas red–sulphonyl chloride (TRSC). A diagram outlining the interactions of these markers with the endocytic pathway and normal phagosomes is shown in Fig. 1. These methods were used to

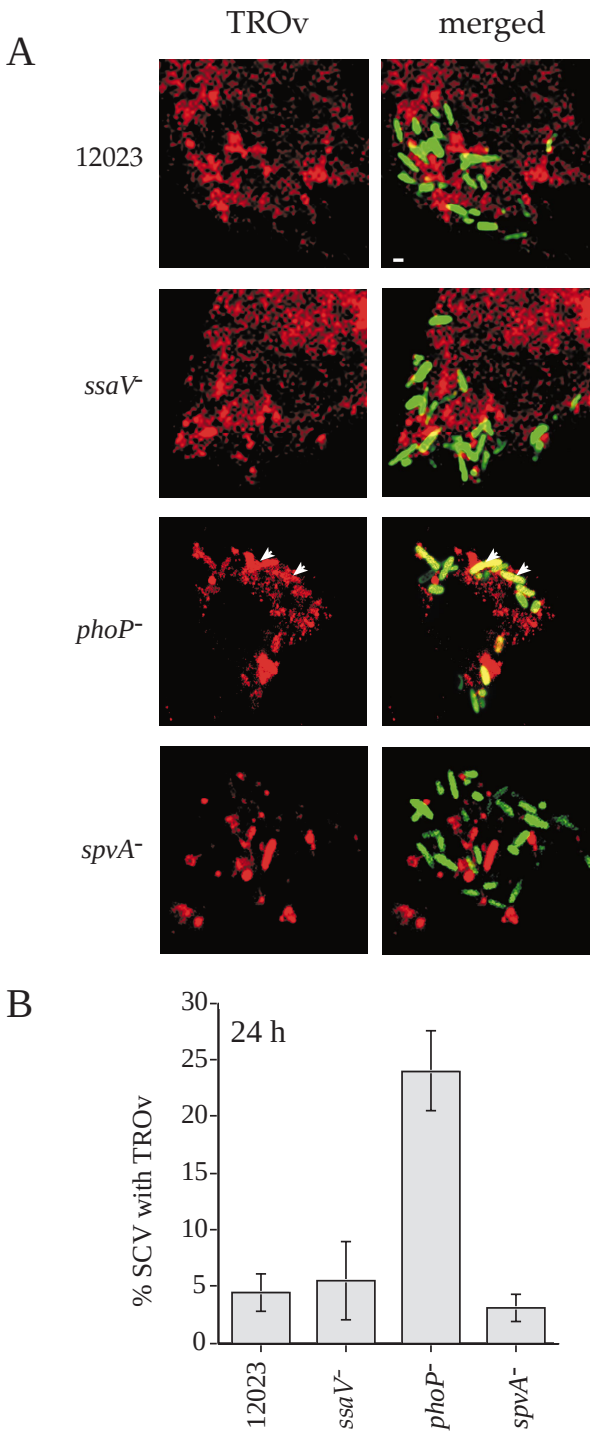


Fig. 2. Association of TROv with vacuoles containing different GFP-expressing *S. typhimurium* strains in RAW macrophages. Cells were pulse–chased with TROv, then infected and fixed 24 h after uptake.
A. Confocal fluorescence analysis of cells infected with wild-type strain 12023 or *ssaV*$^-$, *phoP*$^-$ or *spvA*$^-$ mutant strains. Arrowheads indicate typical SCVs that were considered as positive for association with TROv.
B. Results of three experiments in which SCVs from cells infected with each strain were scored for TROv association at 24 h after uptake. Values are given as mean percentage of SCVs associating with the marker ± standard error.

compare trafficking of wild-type bacteria with isogenic strains carrying mutations in the SPI-2, *spv* or *phoP/Q* loci. The SPI-2 mutant used in these studies contains a disruption of the *ssaV* gene. This gene is predicted to encode a component of the SPI-2 secreton and is essential for the secretion of SseB, a putative component of the SPI-2 translocon (Beuzón *et al.*, 1999). The *spv* mutant contains a transposon insertion in *spvA.* The virulence of this strain is strongly attenuated in mice (Shea *et al.*, 1999). The *phoP* mutant carries a transposon insertion in *phoP* (Miller *et al.*, 1989a), which encodes the response regulator element of the PhoP/Q regulatory system.

SCV association with the fluid-phase marker Texas Red–Ovalbumin

The soluble endocytic probe TROv has been used previously to observe interactions between lysosomes and bacterial phagosomes (Oh and Swanson, 1996; Swanson and Isberg, 1996). TROv distributes throughout the endocytic network after pinocytosis and can be chased into lysosomes to serve as a marker for these organelles (Kjeken *et al.*, 1995). In our experiments, macrophages were preloaded with TROv for 30 min, followed by incubation of cells with unlabelled medium for a further 2 h to allow the marker to localize to lysosomes. Cells were then infected with bacteria, fixed 24 h after uptake and examined by confocal microscopy. At this time point, only 4.4 ± 1.5% of wild-type, 5.5 ± 3.4% of *ssaV*$^-$ and 3.0 ± 1.2% of *spvA*$^-$ SCVs were associated with TROv. In contrast, 24.0 ± 3.5% of *phoP*$^-$ SCVs co-localized with this marker (Fig. 2A and B).

Targeting of cathepsin D to the SCV

Cathepsin D, an aspartyl protease involved in intracellular degradation of exogenous and endogenous proteins, is delivered to the lumen of late endosomes by the MPR (Kornfeld, 1986; Ludwig *et al.*, 1994; Munier-Lehmann *et al.*, 1996). Cathepsin D is one of the most abundant hydrolases accumulating in latex bead phagosomes as they mature (Garin *et al.*, 2001) and has been used previously as a marker for phagolysosomal fusion (Oh and Swanson, 1996).

We used an antibody against cathepsin D to study interactions between this enzyme and SCVs harbouring wild-type and mutant strains. At 24 h after uptake, cells were fixed, stained and examined by confocal microscopy. At this time point, less than 3% of wild-type, *spvA*$^-$ and *ssaV*$^-$ SCVs co-localized with cathepsin D, compared with 39.9 ± 9.7% of the *phoP*$^-$ SCVs (Fig. 3A and B). Over 75% of bacteria that had been heat killed before opsonization and uptake were associated with cathepsin

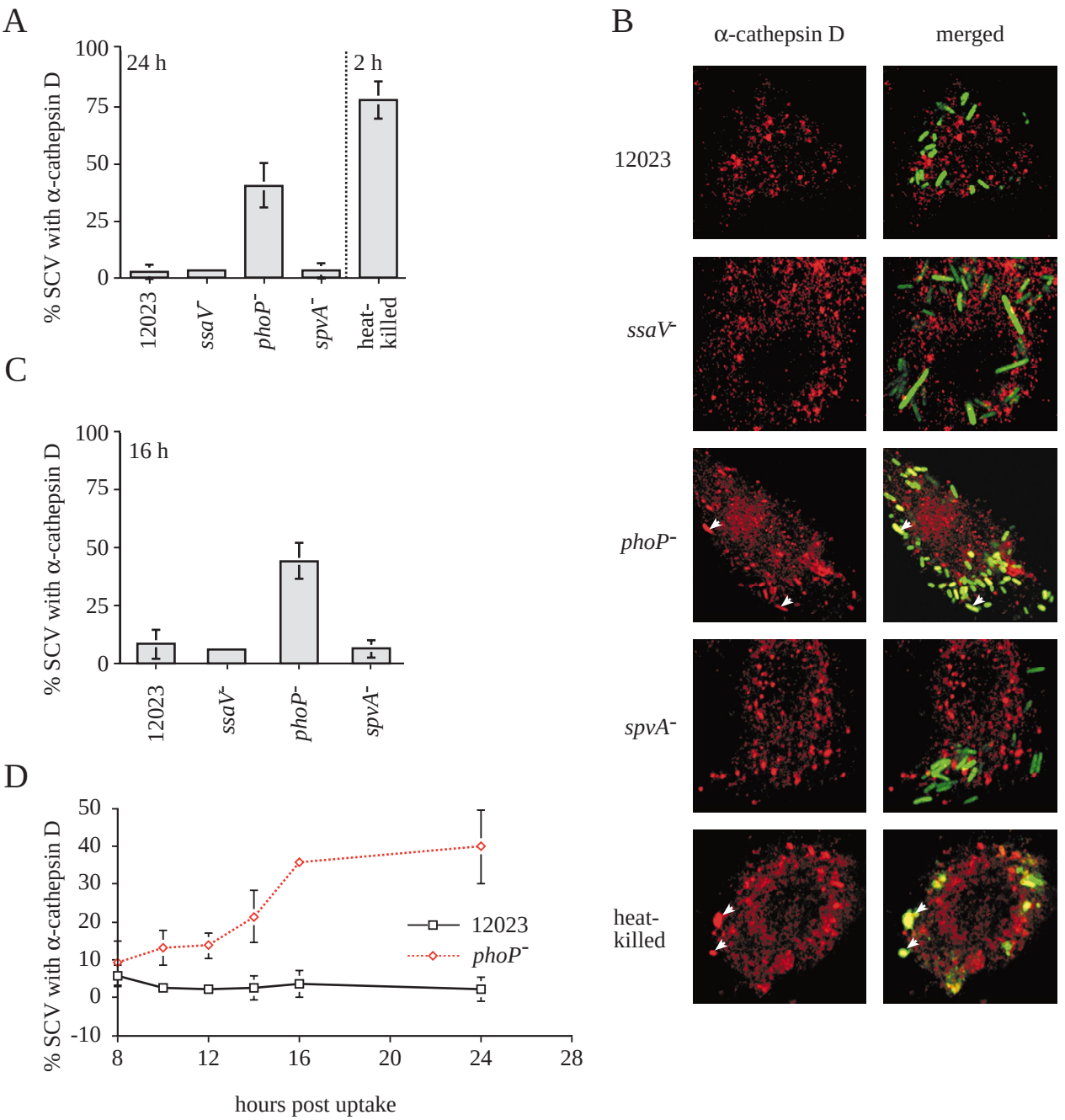


Fig. 3. Confocal immunofluorescence analysis of α-cathepsin D association with vacuoles containing different GFP-expressing *S. typhimurium* strains in RAW macrophages. Cells were fixed at specific times after uptake and stained with a rabbit α-cathepsin D antibody followed by TRSC-conjugated donkey α-rabbit antibody.
A. Percentage of SCVs co-localizing with α-cathepsin D antibody for each strain (at 24 h) and heat-killed bacteria (at 2 h).
B. Representative confocal images of macrophages containing wild-type, *ssaV*$^{-}$, *phoP*$^{-}$, *spvA*$^{-}$ and heat-killed bacteria. Heat-killed bacteria were detected with goat α-*Salmonella* and FITC-conjugated donkey α-goat antibodies. Arrowheads indicate typical SCVs that were considered as positive for co-localization with α-cathepsin D.
C. Percentage of SCV co-localization with α-cathepsin D antibody for each strain at 16 h.
D. Percentage of wild-type and *phoP*$^{-}$ SCV co-localization with α-cathepsin D over a 24 h time course. Macrophages were infected and pairs of coverslips were fixed and stained at the indicated times after uptake. Results shown in (A), (C) and (D) each represent three experiments in which SCVs from cells infected with each strain were scored for cathepsin D co-localization. Values are given as mean percentage of SCVs co-localizing with the marker ± standard error.

D by 2 h after uptake (Fig. 3A). It was not possible to assess the level of association between heat-killed bacteria and cathepsin D at 24 h, because the bacterial cells were extensively degraded [as shown by the absence of intact bacterial cells when stained with an α-*Salmonella* antibody and a secondary antibody conjugated to fluorescein isothiocyanate (FITC)]. Examination of the strains at 16 h after uptake produced similar results to those obtained at 24 h (Fig. 3C). To determine when the difference between *phoP*$^-$ and wild-type SCV co-localization with cathepsin D became apparent, wild-type and *phoP*$^-$ mutant strains were examined at 2 h intervals after bacterial uptake. The rise in cathepsin D association with *phoP*$^-$ mutant bacteria began at ≈ 10 h after uptake and continued to increase over the following 14 h (Fig. 3D). These results indicate that the PhoP/Q regulon is involved in the inhibition of interactions between SCVs and lysosomes or late endosomes.

Co-localization of lysobisphosphatidic acid with SCVs

To determine whether interactions between *phoP*$^-$ SCVs and the late endocytic pathway are restricted to luminal markers such as cathepsin D or TROv, we next examined the distribution of lysobisphosphatidic acid (LBPA), a component of the internal lamellar membranes of late endosomes (Kobayashi *et al*., 1998). LBPA is involved in regulating cholesterol transport (Kobayashi *et al*., 1999) and trafficking of MPR (Kobayashi *et al*., 1998; Reaves *et al*., 2000). To our knowledge, LBPA has not been shown previously to be present in phagosomes or phagolysosomes. Macrophages were infected for 24 h, then fixed and stained with an α-LBPA antibody. Whereas only 3.4 ± 3.0% of wild-type, 8.1 ± 4.1% of *ssaV*$^-$ and 2.1 ± 2.5% of *spvA*$^-$ SCVs were associated with LBPA, 30.7 ± 5.8% of *phoP*$^-$ SCVs were clearly associated with this lipid at this time point (Fig. 4A and B). The majority of heat-killed organisms examined at 2 h after uptake also associated with LBPA (data not shown). This indicates that a portion of the inner membrane network of late endosomes is delivered to and retained by phagosomes harbouring heat-killed and *phoP*$^-$ *S. typhimurium*, and that the majority of the vacuoles containing the *ssaV*$^-$, *spvA*$^-$ or wild-type strains do not acquire this lipid. The kinetics of accumulation of LBPA with wild-type and *phoP*$^-$ SCVs were examined at 2 h time intervals. As seen with cathepsin D, the rise in LBPA association with *phoP*$^-$ mutant bacteria began at ≈ 10 h after uptake and increased over the following 14 h (Fig. 4C).

The *pho-24* allele of *phoQ* leads to overexpression of *phoP*-activated genes (*pags*), which are normally expressed in macrophages (Miller and Mekalanos, 1990). The *pho-24* mutant strain displayed wild-type levels of association with LBPA (Fig. 4B). A *pagC* mutant also had levels of LBPA association similar to wild-type SCVs (Fig. 4B), suggesting that this gene is not involved in inhibition of interactions between SCVs and late endosomes.

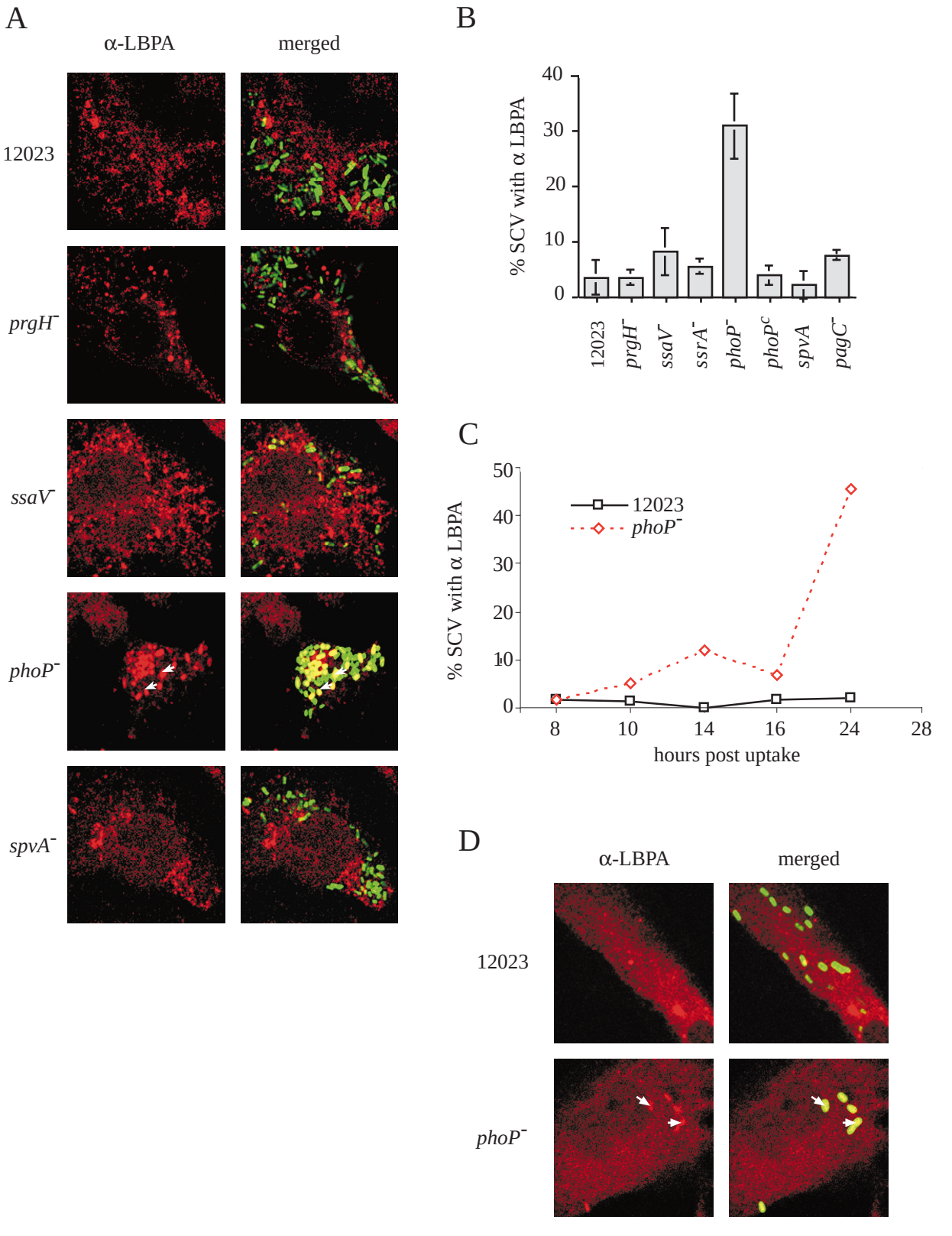
The associations between wild-type and *phoP*$^-$ SCVs and LBPA were also examined in mouse peritoneal macrophages. The survival and replication of *S. typhimurium* in peritoneal macrophages is significantly reduced compared with RAW macrophages (Buchmeier and Heffron, 1991; Hensel *et al*., 1998), making direct comparisons between these two cell types difficult. However, when infected peritoneal macrophages were examined at 14 h after uptake, significantly more *phoP*$^-$ SCVs were associated with the marker than wild-type SCVs. Although the overall level of *phoP*$^-$ SCV association with LBPA was lower than that seen in RAW macrophages, the difference between *phoP*$^-$ and wild-type strains was close to that seen in RAW cells (Fig. 4D).

Co-localization of AP-1 and AP-3 with SCVs

AP-1 and AP-3 are members of a family of adaptor proteins functioning in the traffic of transport vesicles between the TGN and the endocytic pathway (Dell'Angelica *et al*., 1997; Rohn *et al*., 2000; Rouille *et al*., 2000). AP-1 interacts with clathrin in the generation of vesicles budding from the TGN and mediates transport of MPRs and furin from the TGN to endosomes (Teuchert *et al*., 1999). AP-3 mediates lysosomal targeting of the lgps LAMP1, LAMP2 and CD63 from the TGN, but is not involved in MPR transport (Simpson *et al*., 1997; Le Borgne *et al*., 1998; Dell'Angelica *et al*., 1999). As SCVs in macrophages typically acquire lgps but not the MPR (Rathman *et al*., 1997), it was of interest to know whether AP-1 and/or AP-3 associated to any degree with SCVs. RAW macrophages were infected for 24 h and analysed using α-AP-1 λ-subunit and α-AP-3 δ-subunit antibodies. A significant proportion of *phoP*$^-$ SCVs were associated with AP-1 λ (48.4 ± 10.5%), whereas only a small proportion of wild-type (5.36 ± 2.7%), *ssaV*$^-$ (3.5 ± 4.9%) and *spvA*$^-$ (1.5 ± 0.21%) SCVs showed association (Fig. 5A and B). AP-3 also exhibited substantial co-localization with the *phoP*$^-$ SCVs (51.8 ± 10.0%), whereas wild-type (5.6 ± 3.2%), *ssaV*$^-$ (11.5 ± 5.6%) and *spvA*$^-$ (4.6 ± 4.0%) SCVs had little association (Fig. 6A and B). However, at 2 h after uptake, the level of co-localization between vacuoles harbouring heat-killed bacteria and either AP-1 or AP-3 was not significantly greater than that of the live wild-type strain (11.1 ± 4.5% and 15.7 ± 13.0% respectively).

Kinetics of SCV fusion with late endocytic compartments

It is known that inhibition of phagolysosomal fusion by *S.*


A
α-LBPA
merged
12023
prgH-
ssaV-
phoP-
spvA-
B
% SCV with α LBPA
40
30
20
10
0
12023
prgH-
ssaV-
ssrA-
phoP-
phoPc
spvA
pagC-
C
% SCV with α LBPA
50
40
30
20
10
0
12023
phoP-
8
10
14
16
24
28
hours post uptake
D
α-LBPA
merged
12023
phoP-

typhimurium requires live bacteria and bacterial protein synthesis (Ishibashi and Arai, 1990; Buchmeier and Heffron, 1991; Rathman *et al*., 1997). To investigate the kinetics of this requirement, macrophages were infected with wild-type *S. typhimurium* that had either been treated with tetracycline before uptake or exposed to the antibiotic at different times after bacterial internalization. The infections were allowed to proceed for either 2 h or 24 h, at which point the cells were fixed and stained with antibodies against cathepsin D and LBPA and analysed by confocal microscopy. For bacteria exposed to the antibiotic 15 min after uptake and fixed 2 h after uptake, the degree of association between SCVs and the two markers was 63.0 ± 3.0% for cathepsin D and 63.0 ± 4.0% for LBPA. Similar results were obtained for bacteria treated with tetracycline before uptake and fixed 2 h after uptake (data not shown). These results are similar to the association observed between these markers and phagosomes containing heat-killed organisms fixed at 2 h (71.6 ± 9.0% for cathepsin D and 67.3 ± 11.3% for LBPA). If the infection progressed for 2 h before the addition of tetracycline, only 33.0 ± 5.0% of the SCVs were associated with cathepsin D and 34.0 ± 6.9% with LBPA at the 24 h time point. However, delaying exposure to tetracycline until 4 h after uptake reduced the co-localization at 24 h to levels (17.0 ± 6.4% for cathepsin D and 16.3 ± 7.7% for LBPA) close to those observed for wild-type bacteria in the absence of antibiotic (6.2 ± 5.5% for cathepsin D and 9.1 ± 8.6% for LBPA) (Fig. 7). Infected macrophages exposed to tetracycline 2 h after uptake and examined 2 h later also showed little SCV association with cathepsin D (data not shown). These results confirm that the segregation of the SCV from the endocytic pathway is a consequence of bacterial protein synthesis. Furthermore, they indicate that segregation is established between 15 min and 4 h after uptake in this cell line and that, once segregation of SCVs from the late endocytic pathway has been established, protein synthesis is no longer required for its maintenance.

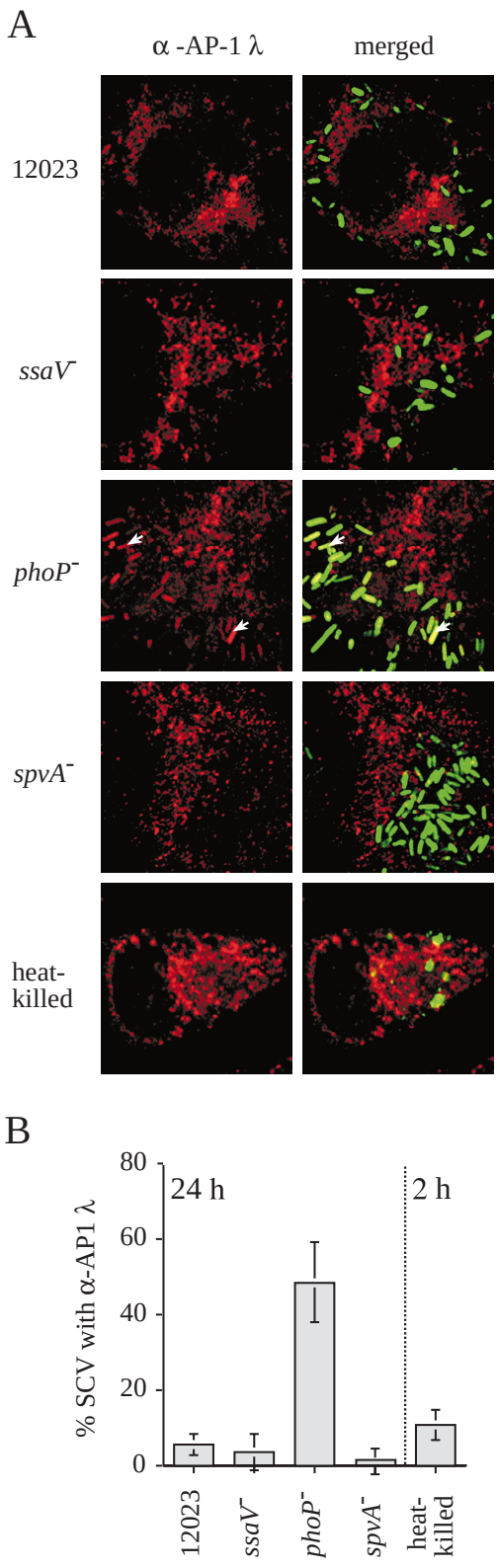
Discussion

The main goal of this study was to examine the involvement of three independent virulence loci of *S. typhimurium* (*spv*, *phoP/Q and* SPI-2) in the inhibition of interactions between the SCV and late endocytic compartments of macrophages. Our results with wild-type *S. typhimurium* are in agreement with previous studies of *S. typhimurium* trafficking in primary and immortalized murine macrophages, which have shown that most SCVs do not fuse with lysosomes (Ishibashi and Arai, 1990; Buchmeier and Heffron, 1991; Rathman *et al*., 1997; Uchiya *et al*., 1999; Hashim *et al*., 2000). Previous work has shown that neither the mode of entry into the host cell (Rathman *et al*., 1997) nor lipopolysaccharide (LPS) structure (Ishibashi and Arai, 1990; Buchmeier and Heffron, 1991) seem to have significant effects on the ability of *S. typhimurium* to evade phagolysosomal fusion. However, bacterial protein synthesis is known to be required for fusion inhibition (Ishibashi and Arai, 1990). As the *spv* operon, SPI-2 genes and some genes under the control of PhoP/Q become activated in macrophages, and all three loci have important but poorly understood roles in intracellular bacterial survival and replication, examining their role in SCV trafficking was of particular interest.

The *spv* mutant strain used in this study contains a transposon insertion in *spvA*, the first gene of a four-gene operon. SpvA is not essential for virulence in mice (Roudier *et al*., 1992); therefore, the high attenuation of the *spvA* mutant (Shea *et al*., 1999) must result from a polar effect on *spvB*, the only gene in the operon shown to have a role in virulence (Roudier *et al*., 1992). SpvB has been shown recently to ADP-ribosylate actin (Lesnick *et al*., 2001; Tezcan-Merdol *et al*., 2001). Although the significance of this activity in terms of intracellular bacterial replication is not yet known, the similar trafficking of the *spvA*$^-$ mutant and the wild-type strain suggests that SpvB is not necessary for inhibition of SCV–lysosome fusion.

The failure to detect a difference between *ssaV*$^-$ and wild-type strains in terms of their association with any of the endocytic pathway markers used was unexpected. SsaV is proposed to be a component of the SPI-2 secretion apparatus (Hensel *et al*., 1997). *ssaV* is required for systemic virulence in mice (Shea *et al*., 1999), replication in macrophages (Hensel *et al*., 1998) and secretion of SseB, a predicted component of the SPI-2 translocon (Beuzón *et al*., 1999). Hence, the *ssaV*$^-$

Fig. 4. Confocal immunofluorescence analysis of α-LBPA co-localization with vacuoles containing different GFP-expressing *S. typhimurium* strains in RAW (A–C) or peritoneal macrophages (D). Cells were infected, fixed 24 h after uptake and stained with mouse α-LBPA and TRSC-conjugated donkey α-mouse antibodies.
A. Confocal images showing wild-type, *ssaV*$^-$, *phoP*$^-$ and *spvA*$^-$ SCVs in α-LBPA-stained cells. Arrowheads indicate typical SCVs that were scored as positive for α-LBPA.
B. Results of three experiments in which SCVs from cells infected with each strain were scored for LBPA co-localization. Values are given as mean percentage of SCVs co-localizing with the marker ± standard error.
C. Percentage of wild-type and *phoP*$^-$ SCV co-localization with α-LBPA over a 24 h time course. Macrophages were infected and pairs of coverslips were fixed and stained at the indicated times after uptake.
D. Confocal images showing wild-type and *phoP*$^-$ SCVs in α-LBPA-stained peritoneal macrophages at 14 h after uptake. Arrowheads indicate examples of SCVs co-localizing with α-LBPA.

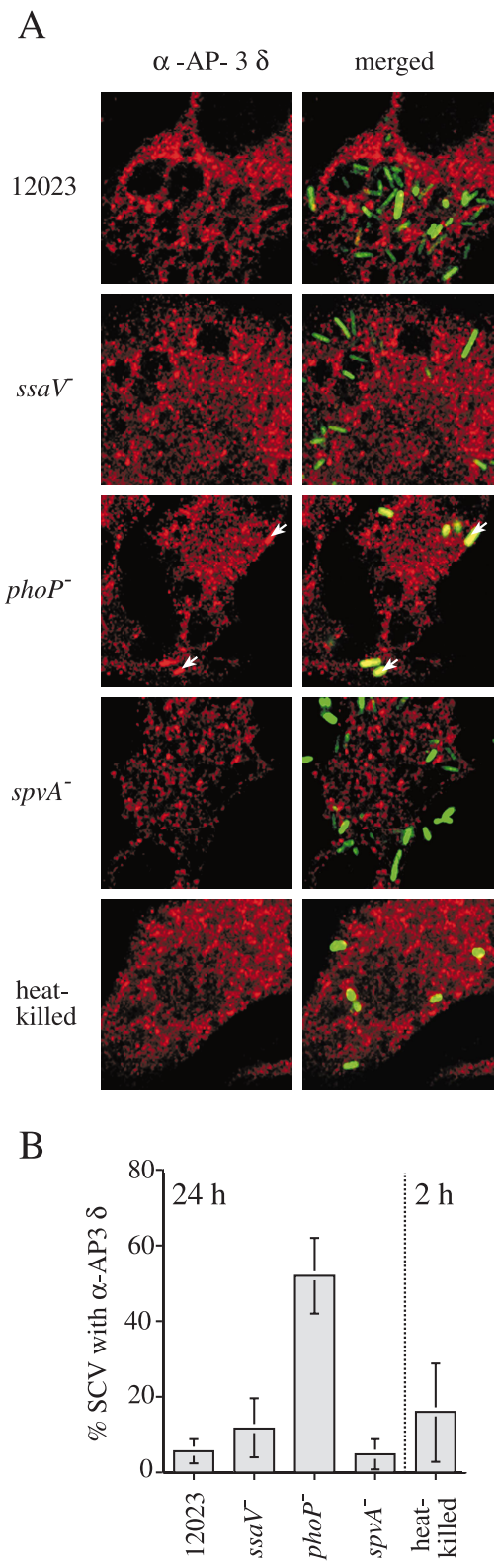


mutant is likely to be defective for secretion of all SPI-2 effectors. SpiC was reported by Uchiya *et al.* (1999) to be a SPI-2-secreted effector involved in inhibition of SCV–lysosome and SCV–endosome fusion. Our results appear to be inconsistent with this observation. However, the intracellular fate of a strain lacking one effector may be different from one lacking all effectors. Indeed, a mutant strain defective only for SifA, a SPI-2 effector, is released into the cytosol of macrophages, whereas an *ssaV*⁻ mutant remains within the SCV (Beuzón *et al.*, 2000). An analogous situation might also apply to the trafficking of vacuoles containing *ssaV*⁻ or *spiC*⁻ mutant strains.

In contrast to *ssaV*⁻ and *spvA*⁻ SCVs, a significant proportion of vacuoles carrying *phoP*⁻ bacteria associated with all the markers tested, indicating that these vacuoles were interacting with late endocytic and lysosomal compartments. We conclude from this that PhoP-regulated gene(s) play a central role in SCV trafficking. It is also interesting to note that the acquisition of cathepsin D, LBPA and TROv by *phoP*⁻ SCVs was delayed and never as high as for phagosomes containing heat-killed bacteria: this implies that there must be additional *S. typhimurium* genes with a role in preventing SCV–lysosome fusion. Furthermore, the retention of significant levels of AP-1 λ-subunits and AP-3 δ-subunits on SCVs containing *phoP*⁻ bacteria does not seem to be typical of phagolysosomes, as the proteins were observed only on a small number of phagosomes containing heat-killed bacteria at 2 h after uptake. Presumably, other bacterial genes that influence SCV trafficking remain functional in the *phoP*⁻ mutant and may help to prevent maturation of the *phoP*⁻ SCV into a typical phagolysosome.

The finding that the *phoP* locus inhibits interactions between SCVs and late endocytic compartments provides an explanation for the observation that *phoP* influences processing and presentation of antigens in murine macrophages (Wick *et al.*, 1995). Subcompartments of the endosomal/lysosomal system are important sites for the generation of MHC class II complexes. They receive antigenic fragments from endosomes and lysosomes and load them onto class II molecules for transport to the

Fig. 5. Confocal immunofluorescence analysis of α-AP-1 λ co-localization with vacuoles containing different GFP-expressing *S. typhimurium* strains in RAW macrophages. Cells were infected, fixed 24 h after uptake and stained with mouse α-AP-1 λ and TRSC-conjugated donkey α-mouse antibodies.
A. Confocal images showing vacuoles containing wild-type, *ssaV*⁻, *phoP*⁻, *spvA*⁻ and heat-killed bacteria. Heat-killed bacteria were fixed at 2 h after uptake and detected with goat α-*Salmonella* and FITC-conjugated donkey α-goat antibodies. Arrowheads indicate typical SCVs that were scored as positive for α-AP-1 λ.
B. Results of three experiments in which SCVs from cells infected with each strain were scored for AP-1 λ co-localization. Values are given as mean percentage of SCVs co-localizing with the marker ± standard error.



plasma membrane, where they can trigger T-cell activation (Benaroch *et al*., 1995; Pieters, 1997). Wick *et al*. (1995) studied *phoP*⁻ *S. typhimurium* strains expressing defined class II T-cell epitopes as fusion proteins and found significantly greater MHC class II presentation from the mutant compared with wild-type control strains. The population of *phoP*⁻ bacteria that we found associated with late endosomal and lysosomal markers is therefore a likely source of antigen for class II presentation.

The PhoP/PhoQ regulatory system modulates the expression of at least 40 proteins, a subset of which (encoded by *pag* genes) are activated within macrophages (Miller *et al*., 1989a). However, the importance of individual *pag* genes in virulence is unclear (Gunn *et al*., 1995; 1998). We failed to reveal a difference between trafficking of a *pagC*⁻ mutant and the wild-type strain using the endocytic markers described in this work. Hence, other gene(s) controlled by PhoP/Q are likely to be responsible for inhibition of interactions between the SCV and the endocytic pathway.

In addition to showing that PhoP contributes significantly to inhibition of SCV–lysosomal fusion, we have also found that this process requires bacterial protein synthesis between 15 min and 4 h after uptake. Previous work has shown that peak induction of *Salmonella* gene expression inside macrophages takes place between 30 min and 3 h after uptake (Buchmeier and Heffron, 1990). The timing of SCV segregation from the endocytic pathway is therefore consistent with this. We have also shown that, once segregation of the SCV from the endocytic pathway has been established, protein synthesis is no longer required for the maintenance of segregation. This suggests that the SCV becomes permanently altered or is in some other way no longer recognized by late endosomal or lysosomal compartments as a target for fusion.

Overall, our results show that the inhibition of phagolysosomal fusion by *S. typhimurium* is a complex, multifactorial process. It is already known that, through the action of the SPI-2 TTSS, the SCV avoids exposure to the respiratory burst (Vazquez-Torres *et al*., 2000), maintains

Fig. 6. Confocal immunofluorescence analysis of α-AP-3 δ co-localization with vacuoles containing different GFP-expressing *S. typhimurium* strains in RAW macrophages. Cells were infected, fixed 24 h after uptake and stained with mouse α-AP-3 δ and TRSC-conjugated donkey α-mouse antibodies.
A. Confocal images showing vacuoles containing wild-type, *ssaV*⁻, *phoP*⁻, *spvA*⁻ and heat-killed bacteria. Heat-killed bacteria were fixed at 2 h after uptake and detected with goat α-*Salmonella* and FITC-conjugated donkey α-goat antibodies. Arrowheads indicate typical SCVs that were scored as positive for α-AP-3 δ.
B. Results of three experiments in which SCVs from cells infected with each strain were scored for AP-3 δ co-localization. Values are given as mean percentage of SCVs co-localizing with the marker ± standard error.

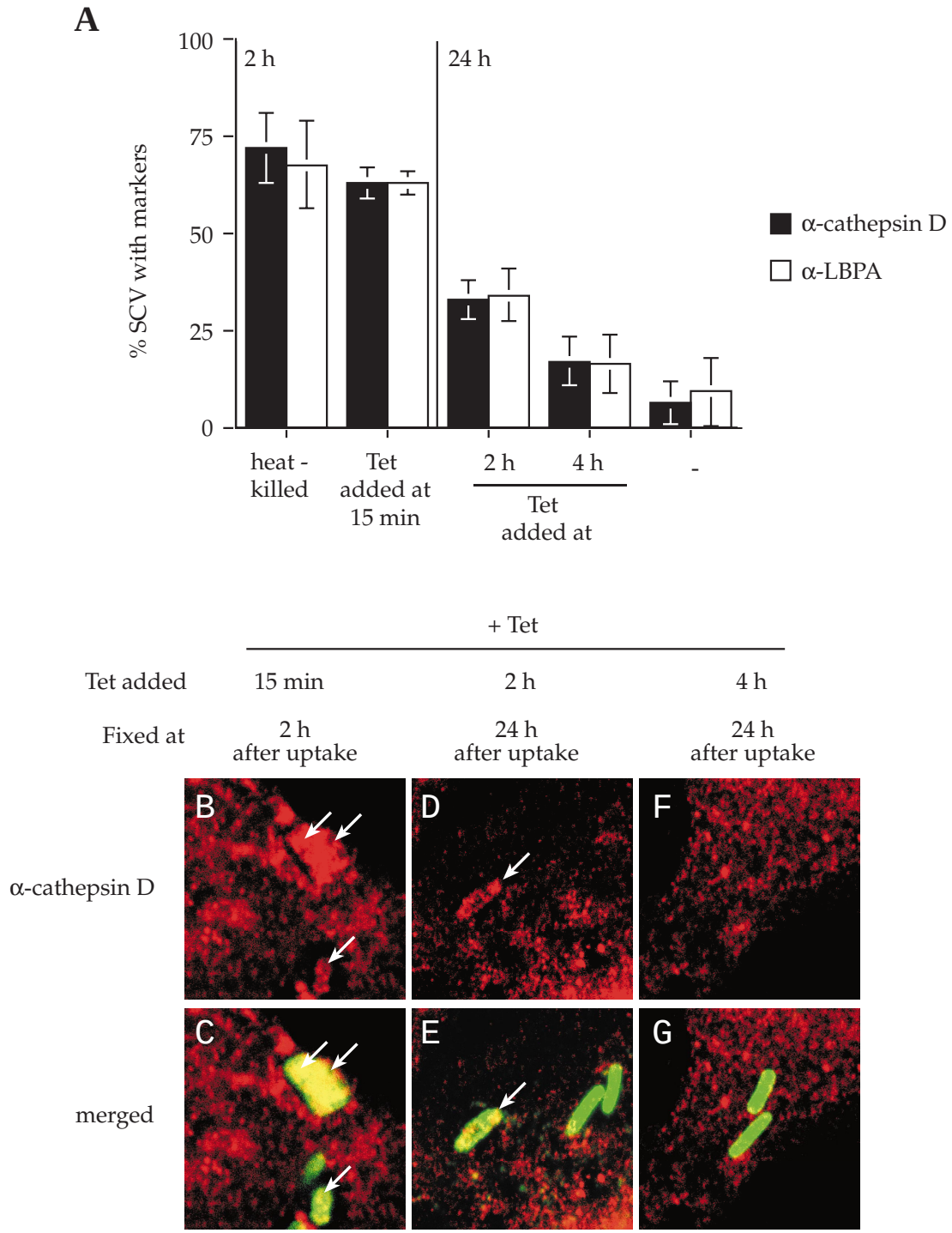


Fig. 7. Kinetics of SCV fusion with late endocytic compartments in RAW macrophages. Confocal immunofluorescence analysis of α-cathepsin D and α-LBPA co-localization with vacuoles containing wild-type *S. typhimurium*, exposed to tetracycline at different time points after uptake. Cells were incubated for different periods of time with either heat-killed or live bacteria, fixed and stained with rabbit α-cathepsin D and TRSC-conjugated donkey α-rabbit or mouse α-LBPA and TRSC-conjugated donkey α-mouse antibodies.
A. Results of experiments in which vacuoles containing heat-killed or live bacteria were scored for cathepsin D or LBPA co-localization. Cells were treated with tetracycline 15 min after uptake and fixed at 2 h, or treated with tetracycline 2 h after uptake, 4 h after uptake or left untreated, and fixed at 24 h. Values are given as mean percentage of SCVs co-localizing with the markers ± standard error.
B–G. Confocal images showing SCV co-localization with α-cathepsin D in cells infected with wild-type bacteria, then treated with tetracycline at the indicated times and fixed at 2 or 24 h. Bacteria were detected with goat α-*Salmonella* and FITC-conjugated donkey α-goat antibodies. Arrowheads indicate typical SCVs that were scored as positive for cathepsin D.

the integrity of its vacuolar membrane (Beuzón *et al*., 2000) and, via SpiC, inhibits some aspects of intracellular trafficking (Uchiya *et al*., 1999). We have now shown that PhoP/Q is also involved in the trafficking of this complex organelle. Identification of the PhoP-regulated gene(s) that are responsible for the inhibition of fusion between the SCV and late endocytic compartments will help us to understand the molecular mechanisms underlying this process.

Experimental procedures

Cell culture

RAW 264.7 murine macrophage cells were obtained from ECACC (ECACC 91062702). Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in 5% CO_2. Peritoneal macrophages were obtained from BALB/c mice after elicitation with 5 mM sodium periodate as described previously (De Groote *et al*., 1997). Cells were plated at a density of 5.0×10^5 cells per well in 24-well microtitre dishes and allowed to adhere for 2 h. Non-adherent cells were removed by washing, and the adherent macrophages were incubated for a further 48 h before infection.

Bacterial strains and growth conditions

The *S. enterica* serovar Typhimurium strains used in this study were: 12023 (wild type; Wray and Sojka, 1978); HH109 (*ssaV::aphT*; Deiwick *et al*., 1998); P5D10 (*spvA*::mTn*5*; Hensel *et al*., 1995); HH114 (*phoP-102*::Tn*10d*Cm; Miller *et al*., 1989a); P3F4 (*ssrA*::mTn*5*; Hensel *et al*., 1995); HH124 (*prgH*020::Tn*5lacZY*; Bajaj *et al*., 1996); CS014 (*pagC1*::Tn*phoA*; Miller *et al*., 1989b); and TA2367 (*phoP*^{c}; *pho*-24; Kier *et al*., 1979). All strains are derivatives by P22 transduction of wild-type *S. typhimurium* strain 12023 except for TA2367 (*pho-24*), which is a derivative of wild-type strain SL1344. For clarity, these strains are referred to as wild type, *ssaV*$^-$, *spvA*$^-$, *phoP*$^-$, *ssrA*$^-$, *prgH*$^-$, *pagC*$^-$ *and phoP*^{c} respectively. Plasmid pFPV25.1, carrying *gfpmut3a* under the control of a constitutive promoter, was introduced into the bacteria for green fluorescence visualization (Valdivia and Falkow, 1997). Bacteria were grown at 37°C in Luria–Bertani (LB) media supplemented with kanamycin (50 μg ml^{-1}), ampicillin (50 μg ml^{-1}) or chloramphenicol (50 μg ml^{-1}) when appropriate.

Bacterial infection of macrophages

Macrophages were seeded onto glass coverslips in 24-well tissue culture plates 24 h before use, at a density of 1–5×10^5 cells per well. Bacteria were cultured at 37°C overnight with shaking, diluted 1:4 and opsonized in DMEM–FCS and 10% normal mouse serum for 20 min before uptake. Bacteria were added to either peritoneal or RAW 264.7 macrophages at a multiplicity of infection (MOI) of ≈100:1, centrifuged at 170 *g* for 5 min at room temperature and then incubated for a further 25 min at 37°C in 5% CO_2. The macrophages were washed once with DMEM–FCS and 100 μg ml^{-1} gentamicin and incubated in this medium for 1 h. The medium was replaced with DMEM–FCS and 16 μg ml^{-1} gentamicin for the remainder of the experiment. Under these conditions, the *ssaV*$^-$ and *phoP*$^-$ strains displayed a five- to 10-fold replication defect in RAW cells after 24 h compared with the wild-type strain, consistent with previous reports (Miller *et al*., 1989a; Hensel *et al*., 1998). Tetracycline was used at a final concentration of 15 μg ml^{-1} where indicated. In control experiments, the addition of tetracycline at 15 min or 2 or 4 h after uptake completely prevented the increase in wild-type bacterial numbers that would normally have occurred by 24 h (data not shown).

Confocal microscopy

Coverslips were fixed at the indicated time points in 3.7% paraformaldehyde (PFA) in PBS (pH 7.4) for 15 min and then washed three times in PBS. Antibodies were diluted in 10% horse serum, 1% bovine serum albumin (BSA) and 0.1% saponin in PBS. Coverslips were washed once in PBS containing 0.1% saponin, incubated with the primary antibody for 30 min, washed twice in PBS containing 0.1% saponin and incubated for 30 min with the secondary antibody. Coverslips were then washed twice in PBS with 0.1% saponin, once in PBS and once in H_2O and then mounted in antifade mounting medium (Molecular Probes). Samples were analysed using a confocal laser scanning microscope (LSM510; Zeiss). An SCV was considered positive for a marker if it fulfilled three criteria: (i) the marker was detected throughout the area occupied by the bacterium, as visualized by green fluorescence; (ii) the marker was concentrated in this area, compared with the immediate surroundings; (iii) a significant proportion of the staining exhibited pixel to pixel co-localization. To determine the percentage of bacteria that co-localized with each marker, a minimum of 50 intracellular bacteria was analysed for each strain in each experiment, counting 10 or fewer bacteria per cell. Results were calculated from three independent experiments and are presented as mean percentage of association ± standard error. To investigate whether the process of infection resulted in significant loss of macrophages from coverslips, macrophages in randomly chosen microscopic fields were counted at 24 h after uptake and compared with the same number of fields of uninfected cells. No significant differences in numbers were observed.

SCV fusion with TROv-loaded lysosomes

RAW 264.7 macrophages seeded on glass coverslips were labelled by pulsing with 50 μg of TROv (Molecular Probes) ml^{-1} DMEM–FCS for 30 min at 37°C in 5% CO_2. To allow the TROv to accumulate in lysosomes, the labelling media was removed, and the cells were washed three times with DMEM–FCS and incubated for 2 h at 37°C in 5% CO_2. After the chase period, the cell monolayers were infected as described previously. At 24 h after uptake, the cells were fixed as described previously (McLean and Nakane, 1974; Swanson and Isberg, 1996) in PLPS buffer (2 mM MES, 7 mM NaCl, 0.5 mM KCl, 0.5 mM $MgCl_2$, 7 mM lysine,

0.2 mM EGTA, 13 mM sucrose, 1 mM $NaIO_4$, 3.7% PFA) for 20 min, rinsed twice in PBS, once in H_2O and mounted for analysis.

Antibodies and probes

S. Kornfeld (Washington University) provided rabbit α-mouse cathepsin D antibody, which was used at a dilution of 1:600. Mouse α-LBPA antibody was provided by J. Gruenberg (University of Geneva) and was used at a dilution of 1:200. Rabbit α-AP-1 λ and AP-3 δ antibodies were gifts from M. S. Robinson (University of Cambridge), and both were used at a dilution of 1:200. Goat α-*Salmonella* antibody was purchased from Kirkegaard and Perry Laboratories and used at a dilution of 1:200. FITC-conjugated donkey α-goat, TRSC-labelled donkey α-mouse and donkey α-rabbit antibodies were purchased from Jackson Immunoresearch Laboratories and all used at a dilution of 1:200.

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