Microtubule motors control membrane dynamics of *Salmonella*-containing vacuoles

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Summary

Infection of host cells by *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) leads to the formation of specialised membrane-bound compartments called *Salmonella*-containing vacuoles (SCVs). Bacteria remain enclosed by the vacuolar membrane as they divide, and by translocating effector proteins across the vacuolar membrane through the SPI-2 type III secretion system, they interfere with host cell processes in ways that promote bacterial growth. One such effector is SifA, which is required to maintain the integrity of the vacuolar membrane and for the formation in epithelial cells of long tubular structures called Sifs that are connected to SCVs. Unknown effector(s) mediate the assembly of a meshwork of F-actin around SCVs. We report that intracellular bacteria also cause a dramatic accumulation of microtubules around *S. typhimurium* microcolonies in both epithelial cells and macrophages. Although this process appears to be independent of SPI-2-mediated F-actin assembly, it does require bacterial protein synthesis. In epithelial cells, microtubule accumulation is accompanied by the recruitment of both kinesin and dynein. Inhibition of the activity of either motor prevented both Sif formation and the loss of vacuolar membrane from *sifA* mutant bacteria. It also resulted in morphologically abnormal vacuoles enclosing wild-type bacteria, and impaired their replication. Our experiments indicate that recruitment of dynein to SCVs is dependent on Rab7 activity. We show that the recently described Rab7 effector RILP is also recruited to SCVs in a Rab7-dependent manner. However, overexpression of RILP did not restore dynein recruitment to SCVs in cells expressing dominant negative Rab7, suggesting that RILP requires a functional Rab7 to be activated at the SCV membrane, or that dynein recruitment is mediated by an effector other than RILP. Together, these experiments indicate that microtubule motors play important roles in regulating vacuolar membrane dynamics during intracellular replication of *S. typhimurium*.

Key words: Dynein, *Salmonella*, Microtubule, Vacuolar membrane, Rab7

Introduction

Salmonella enterica serovar Typhimurium (*S. typhimurium*) is a facultative intracellular bacterial pathogen responsible for gastroenteritis in humans and a typhoid-like illness in certain mouse strains. Invasion of epithelial cells by *S. typhimurium* is achieved by a type III secretion system (TTSS) encoded within *Salmonella* Pathogenicity Island-1 (SPI-1); this translocates effector proteins from the bacterial cytosol to the host cell plasma membrane and cytosol. These effectors cause actin polymerisation, membrane ruffling, and uptake of bacteria into a membrane-bound compartment known as the *Salmonella*-containing vacuole (SCV) (Galan, 2001). Intracellular bacterial replication occurs within vacuoles and requires the expression of a *Salmonella* Pathogenicity island-2 (SPI-2)-encoded TTSS, which translocates effector proteins into and across the vacuolar membrane. Several effectors have been identified in recent years. The functions of most have yet to be elucidated, but collectively they are required for intracellular replication of *S. typhimurium* in epithelial cells and macrophages, and virulence in mice (Beuzón et al., 2002; Cirillo et al., 1998; Hensel et al., 1995; Ochman et al., 1996; Paesold et al., 2002; Salcedo et al., 2001).

Following invasion of epithelial cells, the SCV rapidly acquires early endosomal markers such as EEA1 and the transferrin receptor (Méresse et al., 1999; Steele-Mortimer et al., 1999). These proteins are then gradually removed and replaced by others, which include the vacuolar proton ATPase and some lysosomal membrane glycoproteins (lgps), such as LAMP-1, LAMP-2, and LAMP-3/LIMP-1 (Beuzón et al., 2000; Garcia-del Portillo and Finlay, 1995). The process of lgp recruitment does not appear to involve direct interactions with lysosomes, since SCVs contain very low levels of the lysosomal enzyme cathepsin D, but is dependent on the small GTPase Rab7 (Méresse et al., 1999). By 6 hours post-invasion (p.i), infected cells are characterised by the aggregation of lgp-

Table 1. *S. typhimurium* strains and plasmids used in this study

Name	Designation	Source or Reference
Strains		
12023	Wild-type	NTCC (Colindale, UK)
P3H6	*sifA*⁻ in 12023	Beuzón et al., 2000
HH109	*ssaV*⁻ in 12023	Deiwick et al., 1999
HH130	*prgH*⁻ in 12023	Beuzón et al., 1999
HH193	*ssaV*⁻ *prgH*⁻ in 12023	Beuzón et al., 2001
Plasmids		
pUHD*myc*-Rab7T22N		Méresse et al., 1999
pRK5*myc*-p50/dynamitin		Etienne-Manneville and Hall, 2001
pGFP-KLC2–TPR		Rietdorf et al., 2001
PJK18 pCLXSN.notI-*gfp*-Rab7		This study
PJCK7 pCLXSN.notI-*gfp*-Rab7T22N		This study
pEGFP-RILP		This study
pEGFP-RILP-C33		This study
pRK5*myc* –FCγRIIA		Caron and Hall, 1998

enriched vesicles in the vicinity of bacterial microcolonies, and the formation of long tubular structures called *Salmonella* induced filaments (Sifs), which appear to extend from the SCV membrane (Garcia-del Portillo et al., 1993). Sif formation occurs along microtubules, and requires both the expression of the SPI-2 effector SifA and Rab7 activity (Brumell et al., 2002a; Brumell et al., 2001; Stein et al., 1996).

The *S. typhimurium sifA* mutant strain loses its vacuolar membrane and becomes exposed to the host cell cytosol (Beuzón et al., 2000). This phenotype and other evidence indicate that SifA and another SPI-2 effector, SseJ, have complementary activities in regulating SCV membrane dynamics (Beuzón et al., 2000; Ruiz-Albert et al., 2002). Additional unidentified effector(s) induce the polymerisation of an F-actin microfilament (MF) meshwork in the vicinity of the SCVs (Méresse et al., 2001). SseI and SspH2 are two further SPI-2 effectors that co-localise with these structures through interactions with the actin-binding protein filamin (Miao et al., 2003).

In view of the involvement of microtubules in Sif formation, and the finding that treatment of infected cells with nocodazole has an inhibitory effect on bacterial replication (Garcia-del Portillo et al., 1993), we undertook a more detailed investigation into the role of microtubules in the intracellular growth of *S. typhimurium*. We found that microtubules accumulate around *S. typhimurium* microcolonies in epithelial cells and macrophages. This process requires the synthesis of bacterial proteins but is apparently unconnected with SPI-2-dependent actin assembly. In epithelial cells, both microtubule-associated motors, dynein and kinesin, are recruited to *S. typhimurium* microcolonies. Inhibition of either motor prevented bacterial replication and resulted in morphologically abnormal vacuoles. Our experiments suggest that dynein recruitment to SCVs is dependent on Rab7 activity, and may involve the recently described Rab7 effector, RILP, which is recruited to SCVs in a Rab7-dependent manner. We conclude that microtubule motors play important roles during intracellular replication of *S. typhimurium*, probably by regulating dynamics of the vacuolar membrane.

Materials and Methods

Cells, bacterial strains and vectors

Human epithelial HeLa (clone HtTA1) and murine macrophage RAW 264.7 cells were obtained from the European Collection of Cell Cultures (ECACC). Human intestinal epithelial INT407 cells were a generous gift from A.L. Servin (U510 INSERM, France). Cells were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing glutamax and supplemented with 10% foetal calf serum (FCS). Cells were used at 50% confluency. Peritoneal macrophages were harvested 4 days after BALB/c mice were inoculated by intraperitoneal injection with 5 mM sodium periodate as described previously (De Groote et al., 1997). Cells were plated at a density of 5×10^5 cells per well in 24-well microtitre dishes and allowed to adhere for 2 hours. Non-adherent cells were flushed out with pre-warmed RPMI containing 10% FCS. The adherent macrophages were incubated for a further 48 hours before infection. The bacterial strains and vectors used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) medium supplemented with carbenicillin (50 μg/ml), kanamycin (50 μg/ml), tetracycline (25 μg/ml) or chloramphenicol (50 μg/ml) as appropriate.

Construction of vectors expressing Rab7 and RILP variants

For construction of GFP-Rab7 variants, the translational termination codon for GFP was removed by PCR using the primers 5′ CGG AAT TCC CGG GTC GAC GAG C-3′ and 5′-CCG CTA GCC TTG TAC AGC TCG TCC ATG C-3′ and pGreen Lantern (Gibco-BRL) as template. The ends of the GFP product were digested with *Eco*RI and *Nhe*I. The *rab7* genes were amplified using primers 5′-GCC GAT ATC AAT GAC CTC TAG GAA GAA AGT G-3′ and 5′-CCG GTC GAC TCA GCA ACT GCA GCT TTC CG-3′ and pGemR7 and pGemR7T22N as template DNA (Feng et al., 1995). The plasmid pET28a (Novagen) was digested with *Bam*HI, a Klenow fill-in reaction was performed to polish the 5′ ends, and then the DNA was digested with *Sal*I. The *rab7* PCR products were digested with *Eco*RV and *Sal*I and ligated into pET28a. The *rab7* genes were then removed from pET28a by digestion with *Nhe*I and *Not*I then ligated into the pGL-Rab5 plasmid backbone after the *rab5*-encoding fragment had been removed by digestion with *Nhe*I and *Not*I. The *rab7* inserts were sequenced using the primer 5′-ATC ACA CAT GGC ATG GAC-3′, which anneals near the 3′ end of *gfp* and extends across the fusion junction. Immunoblot analysis on transfected COS cells using polyclonal antibodies generated against C-terminal Rab7 peptides confirmed that a fusion product of the expected molecular mass was being produced by each construct. Each GFP-Rab fusion was then excised from the pGL vector using *Not*I. These inserts were then ligated into pCLXSN.*Not*I that had been similarly digested.

For construction of GFP-RILP variants, a 1600 bp *Sal*I-*Bam*HI fragment of RILP cDNA (Cantalupo et al., 2001) was ligated into pEGFPC1 plasmid that had been digested with *Sal*I and *Bgl*II. The

RILP-C33 *Eco*RI-*Sal*I 900 bp fragment (Cantalupo et al., 2001) was ligated into pEGFPC1 plasmid after digestion with *Eco*RI and *Sal*I.

Antibodies and reagents

The mouse monoclonal anti-β-tubulin antibody (clone E7) developed by Klymkowsky, and anti-LAMP-1 antibody (clone H4A3) developed by August and Hildreth, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, maintained by the University of Iowa (Department of Biological Sciences, Iowa, IA 52242) and were used at a dilution of 1:200. The mouse monoclonal antibody against BSA (clone 1520) was from Sigma. Mouse monoclonal antibodies against dynein (clone 1618) and kinesin (clone 1613) were from Chemicon International and used at a dilution of 1:100. Rabbit polyclonal RILP antibody was used at a dilution of 1:150 and has been described previously (Cantalupo et al., 2001). Goat anti-*S. typhimurium* polyclonal antibody CSA-1 was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD) and was used at a dilution of 1:200. Polyclonal rabbit anti-c-myc antibody was from Covance and anti-c-myc mouse monoclonal antibody (clone 9E10) was from Santa Cruz Biotechnology; both were used at a dilution of 1:100. Texas Red sulfonyl chloride (TRSC)-, cyanine 5 (Cy5)- and fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse, anti-rabbit and anti-goat antibodies were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA) and used at a dilution of 1:200.

Red-fluorescent Mitotracker CMXRos-H2 (Molecular Probes) was used to selectively label mitochondria. The culture medium was replaced with 100 μl of prewarmed 1 μM Mitrotracker diluted in DMEM. Cells were then incubated for a further 30 minutes at 37°C with 5% CO_2, washed three times with PBS and fixed for 4 minutes with cold methanol.

Nocodazole, cytochalasin D and aurintricarboxilic acid [ATA; described as component 11663 by Hopkins et al. (Hopkins et al., 2000)] were obtained from Sigma. Stock solutions were made in dimethyl sulfoxide (DMSO) and kept at –20°C. Drugs were added directly to the culture medium, with a pre-treatment of 3 hours prior to infection for ATA. The working concentrations were 1 μg/ml for cytochalasin D, 10 μM for nocodazole and 10 μM for ATA. None of these treatments caused host cell cytotoxicity as measured by lactate dehydrogenase release assay (cytotox-96 Promega), or affected bacterial viability.

Bacterial and latex bead uptake by HeLa cells

HeLa and INT407 cells were infected with *S. typhimurium* as described previously (Beuzón et al., 2000). Briefly, 24 hours before infection, cells were seeded onto glass coverslips (12 mm diameter) in 24-well plates at a density of 5×10^4 cells per well. Bacteria in log phase were added to the cells at a multiplicity of infection (m.o.i.) of ~100:1. The infection was allowed to proceed for 15 minutes at 37°C in 5% CO_2. Extracellular bacteria were killed by adding 100 μg/ml gentamicin for 1 hour, after which the gentamicin concentration was decreased to 15 μg/ml. Macrophages were infected with opsonized, stationary phase *S. typhimurium* as described previously (Garvis et al., 2001). In some experiments, tetracycline was used at a final concentration of 15 μg/ml. In control experiments, the addition of tetracycline at 15 minutes after uptake completely prevented the increase in the number of wild-type bacterial that would normally have occurred by 10 hours (data not shown).

Assays for FcγRIIA-mediated phagocytosis were essentially conducted as described previously (Caron and Hall, 1998). Latex beads (6.4 μm diameter; Sigma) were incubated overnight at 4°C in 10 mg/ml bovine serum albumin (BSA) in phosphate-buffered saline (PBS). After three washes in PBS at 4°C, beads were resuspended in a 1:100 dilution of rabbit anti-BSA IgG antibody (Sigma; clone 1520) for 1 hour at room temperature before washing and storing in PBS at 4°C. Opsonized latex beads were then used to infect FcγRIIA-transfected HeLa cells (10 beads/cell) as described above for bacteria. Latex beads were only internalised by transfected cells (data not shown).

Transient transfection

For transient transfection, cells were grown on coverslips to 50% confluency. Calcium-phosphate-precipitated plasmid DNA (2 μg) was added to the cells. After 16 hours, the cells were washed three times with PBS. 24 hours after transfection, cells were infected as described above. Under these conditions, 30-60% of cells were detected as transfected.

Immunofluorescence

For immunofluorescence, cells were fixed either in 3% paraformaldehyde in PBS pH 7.4 for 15 minutes at room temperature, or in cold methanol for 4 minutes at –20°C (for labelling tubulin, dynein or kinesin) then washed three times in PBS. Antibodies were diluted in 10% horse serum, 0.1% saponin in PBS. Coverslips were washed twice in PBS containing 0.1% saponin, incubated for 30 minutes with primary antibodies, washed twice with 0.1% saponin in PBS and incubated for 30 minutes with secondary antibodies. Coverslips were washed twice in 0.1% saponin in PBS, once in PBS and once in H_2O, and mounted in Mowiol. Samples were analysed using fluorescence (BX50; Olympus Optical Co., Ltd) or confocal laser scanning microscopy (LSM510, Zeiss). Images were processed using Adobe Photoshop 5.0.

Results

Accumulation of microtubules around intracellular *S. typhimurium*

To investigate microtubule remodelling by intracellular *S. typhimurium*, HeLa cells were infected with wild-type (WT) *S. typhimurium*, fixed 10 hours p.i., labelled with anti-β-tubulin and examined by confocal microscopy. Whereas microtubules extended throughout the cytoplasm of uninfected cells, a significant amount became concentrated around 63±12% of replicating WT bacterial microcolonies comprising four or more bacteria (Fig. 1A upper panel). The intensity of microtubule labelling was weaker in the periphery of infected cells, suggesting that tubulin accumulation around microcolonies results at least partly from redistribution of the pre-existing filament network. Microtubule accumulation increased with the size of the bacterial microcolony: the phenotype was readily apparent with microcolonies comprising at least 10 bacteria, but occasionally could be detected around colonies of three or four bacteria. Three-dimensional reconstructions of confocal Z-stacks of infected cells were generated. Re-slicing the reconstructions in an x/z plane revealed that microcolonies were surrounded by a uniform concentration of microtubules (Fig. 1B). In contrast to actin polymerisation (Méresse et al., 2001), microtubules were usually not detected between individual SCVs within a microcolony.

Analysis of microtubule accumulation around bacterial microcolonies over time showed that it was first detectable at 7 hours p.i. and increased in intensity and frequency thereafter (data not shown). Microtubules also accumulated around *S. typhimurium* in infected murine elicited peritoneal macrophages (Fig. 1A middle panel) and in the intestinal epithelial cell line INT407 (Fig. 1A lower panel), indicating that

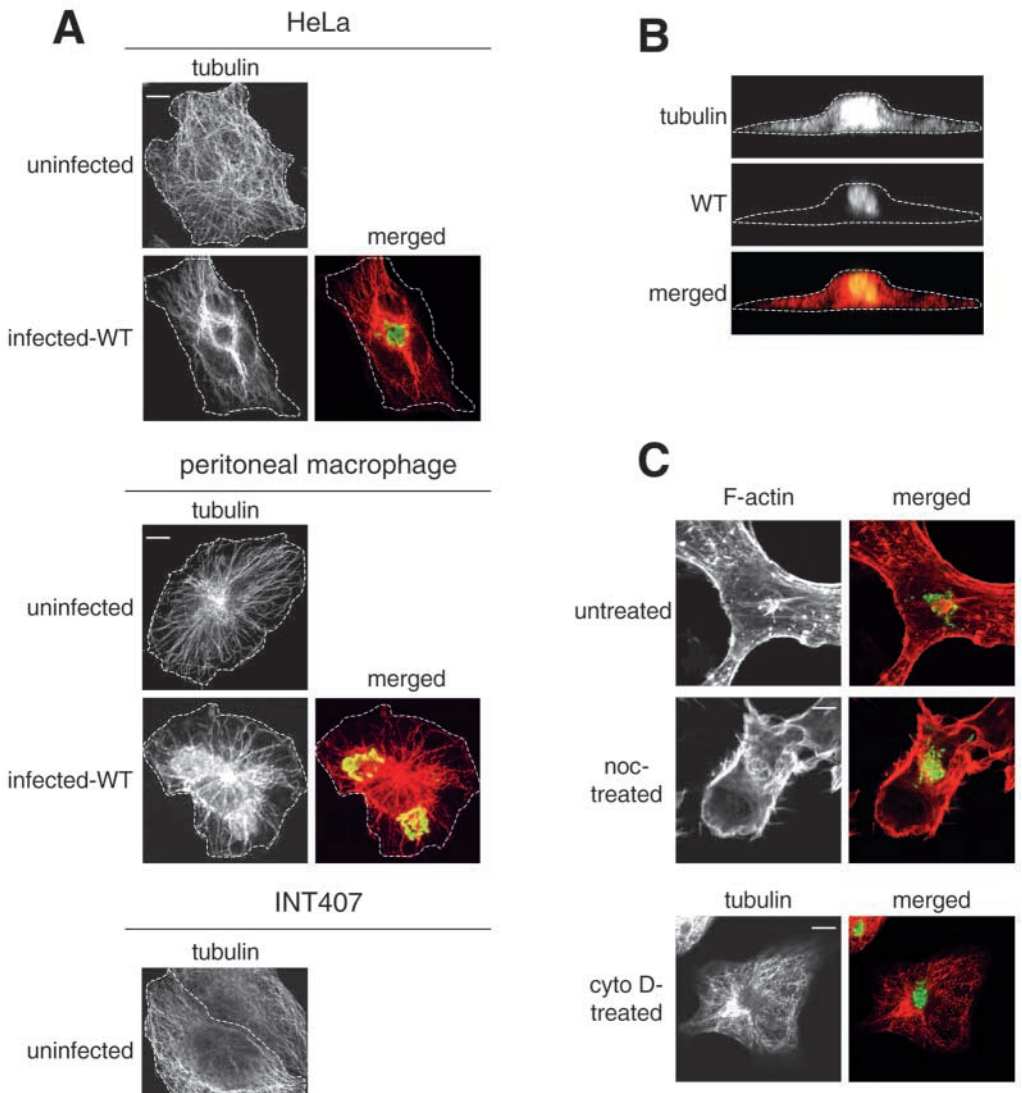

Fig. 1. Remodelling of the microtubule network by intracellular *S. typhimurium*. Cells were infected for 10 hours with *S. typhimurium* and fixed with cold methanol. Bacteria (WT, green in merged images) were detected with an anti-LPS antibody and cytoskeletal proteins (red in merged images) were detected using either an antibody against β-tubulin, or TRITC-phalloidin to visualize F-actin. (A) Confocal *x/y* plane micrographs showing tubulin accumulation around intracellular bacteria in HeLa cells (upper panel), murine elicited peritoneal macrophages (middle panel) and INT407 cells (lower panel). (B) *x/z* section generated from a confocal *z*-stack of an infected HeLa cell. (C) Confocal micrographs of representative cells treated with cytochalasin D (cyto D) (lower panel) or nocodazole (noc) (upper panel) showing F-actin and tubulin (red in merged images) around *S. typhimurium* (green in merged images). Scale bars: 5 μm. Dotted lines indicate cell perimeters.

this phenomenon is probably a general feature of intracellular *S. typhimurium*. Because microtubule rearrangements were most easily detectable in HeLa cells, and many studies on intracellular *S. typhimurium* have been performed using this cell line (Garcia-del Portillo and Finlay, 1995; Garcia-del Portillo et al., 1993; Méresse et al., 1999), further analysis of this phenomenon was carried out in HeLa cells.

Bacterial recruitment of F-actin and microtubules are independent of each other

As the microtubule network and actin cytoskeleton are interconnected physically and functionally (Krendel et al., 2002), we next investigated whether the rearrangement of one network is an indirect consequence of alteration of the other. To do this, actin or microtubule filaments were depolymerised chemically and the appearance of the other network at 10 hours p.i. was examined by confocal microscopy. When infected cells were incubated with cytochalasin D at 2 hours p.i., microfilaments were completely depolymerised and SCV-associated actin polymerisation was completely prevented, as shown previously (Méresse et al., 2001). However, 59±14.1% of *S. typhimurium* microcolonies were associated with microtubules in cytochalasin D-treated cells (Fig. 1C lower panel), compared to 65±2.8% in untreated cells (Fig. 1A). Conversely, when microtubules were disrupted with nocodazole, actin filaments were still detected around 75±11.3% of bacterial microcolonies in nocodazole-treated cells compared to 73.5±7.8% in untreated cells (Fig. 1C upper panel). These results show that accumulation of microtubules and F-actin around *S. typhimurium* microcolonies are independent processes.

Microtubule rearrangements require bacterial protein synthesis

It is possible that the recruitment of microtubules around

bacterial microcolonies represents a non-specific response of the infected cell to the presence of a large volume of foreign material. To determine if this is the case, we examined whether internalisation of latex beads of a diameter similar to that of a typical bacterial microcolony at 10 hours p.i. (between 6 and 10 μm) would cause similar changes to the microtubule network. As HeLa cells are not naturally phagocytic, they were first transfected with a vector encoding the human Fc gamma receptor IIA (FcγRIIA), then incubated with IgG-opsonized latex beads. As expected (Caron and Hall, 1998), the expression of FcγRIIA induced the uptake of beads; however, no accumulation of microtubules occurred around these beads at any time point observed (Fig. 2A).

To determine if bacterial proteins are required for microtubule rearrangements, infected cells were exposed to tetracycline at 7 hours p.i. to block bacterial protein synthesis. 5 hours later (at 12 hours p.i.), cells were fixed and examined by confocal microscopy. Only 29±14% of microcolonies were associated with microtubules in tetracycline-treated cells compared to 66±6.5% of microcolonies in untreated cells (Fig. 2B left panel). Moreover, in the majority of tetracycline-treated cells, *S. typhimurium* microcolonies were found in the perinuclear region and tubulin was distributed normally (Fig. 2B right panel). These results indicate that bacterial protein synthesis is required for microtubule accumulation around bacterial microcolonies.

In an attempt to identify bacterial genes involved in this phenomenon, we investigated the capacity of different *S. typhimurium* mutant strains affected in functions related to the SPI-1 and SPI-2 TTSSs, for their ability to promote microtubule rearrangements. Inactivation of the SPI-1 secretion system causes a significant invasion defect in epithelial cells (Galan, 2001), whereas SPI-2 null mutants are proficient for invasion (Ochman et al., 1996) but have an intracellular replication defect (Cirillo et al., 1998; Ochman et al., 1996). Since Sifs form along microtubules and require the action of the SPI-2 effector SifA, a *sifA* mutant strain was first examined for microtubule rearrangements. However, SifA does not appear to be directly involved in this process because the subpopulation of *sifA*$^-$ mutant bacteria enclosed by a vacuolar membrane at 8 hours p.i. were still capable of inducing changes to the microtubule network (Fig. 2C). Furthermore, both a SPI-2 null mutant (*ssaV*$^-$) and SPI-1 null mutant (*prgH*$^-$) were capable of inducing tubulin rearrangements in HeLa cells (Fig. 2C). A SPI-1, SPI-2 double mutant strain (*prgH*$^-$, *ssaV*$^-$) is strongly impaired both in invasion and replication. As a result, intracellular bacterial clusters of sufficient size to investigate tubulin remodelling were rarely observed. However, in cases where microcolonies of three or four bacteria had formed, no accumulation of microtubules was ever observed (Fig. 2C). These results suggest that the SPI-1 and SPI-2 TTSSs are involved in microtubule remodelling and that the effector responsible can be translocated through either machinery, or that microtubule rearrangements involve more than one effector and both secretion systems are needed.

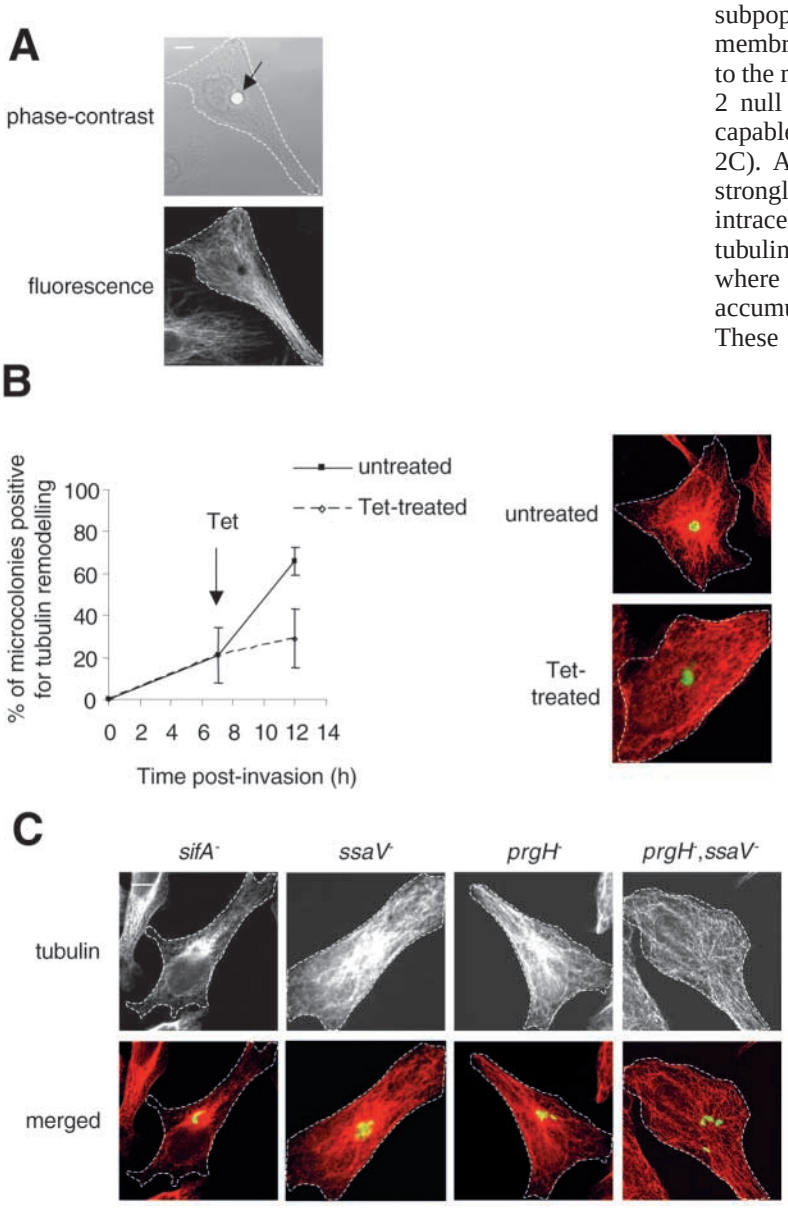

Fig. 2. *S. typhimurium* mediates microtubule remodelling. (A) HeLa cells were transfected with a vector expressing FcγRIIA, exposed to IgG-opsonized latex beads, then fixed 10 hours later and immunolabelled for β–tubulin. Arrow indicates position of a latex bead. Scale bar corresponds to 10 μm. (B) Bacterial protein synthesis was blocked by adding tetracycline (Tet) at 7 hours p.i. Cells were fixed at 12 hours p.i, and examined for tubulin distribution. Results in left panel show the means ± s.d. of three independent experiments in which at least 70 *S. typhimurium* microcolonies were examined. Confocal images in right panel show tubulin distribution (red) around an *S. typhimurium* microcolony (green) in untreated and tetracycline-treated HeLa cells. (C) Representative confocal micrographs showing tubulin distribution in cells infected with *S. typhimurium* mutant strains. HeLa cells were infected for 8 hours with a *sifA* mutant or 10 hours for SPI-2 (*ssaV*$^-$), SPI-1 (*prgH*$^-$) or SPI-1/SPI-2 (*prgH*$^-$, *ssaV*$^-$) null mutant strains (green in merged images). Tubulin distribution (red in merged images) was examined by confocal microscopy. More than 50 *S. typhimurium* microcolonies containing at least three bacteria were examined for each strain. Scale bar: 5 μm. Dotted lines indicate cell perimeters.

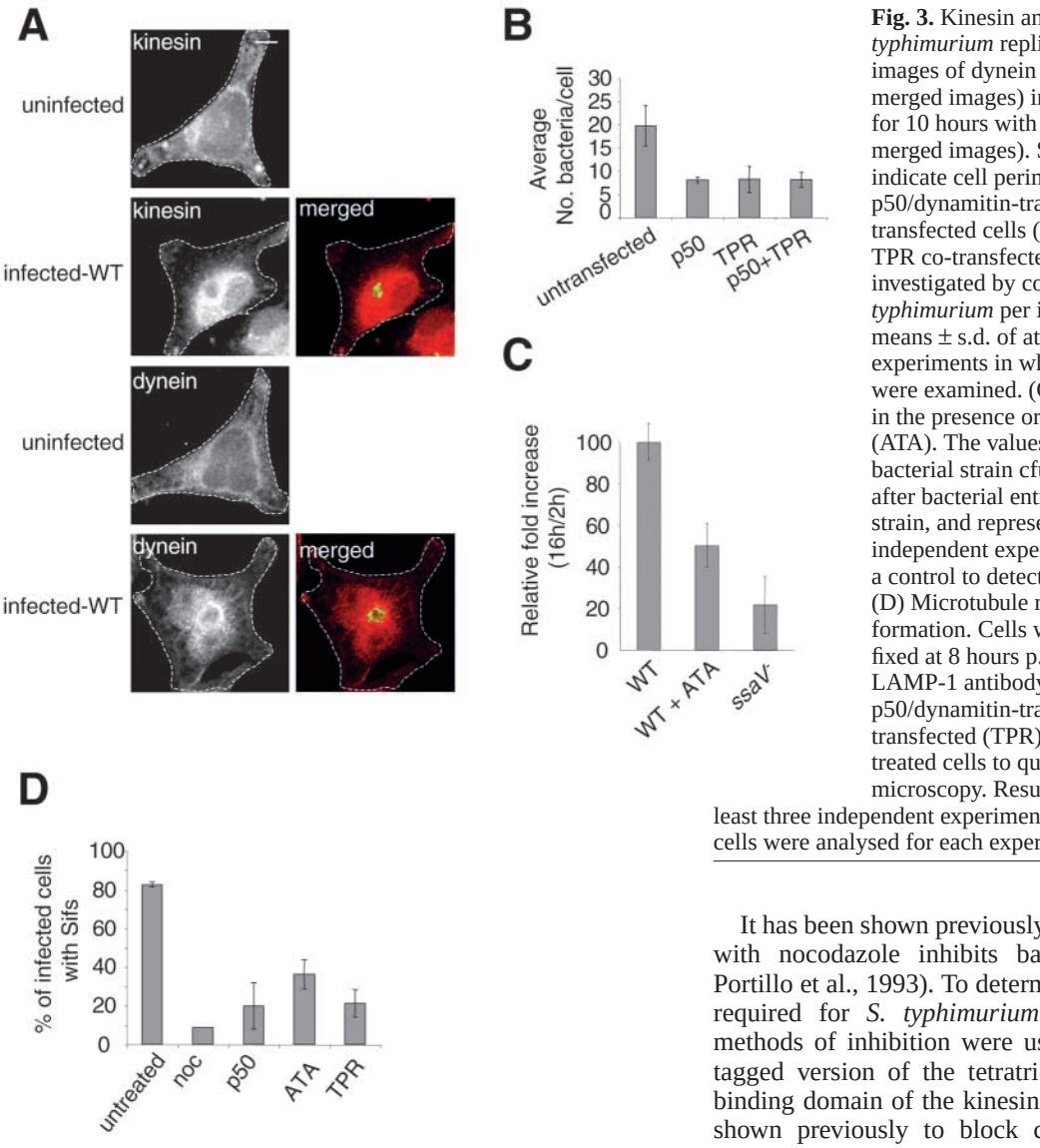

Fig. 3. Kinesin and dynein are required for *S. typhimurium* replication in HeLa cells. (A) Confocal images of dynein and kinesin distribution (red in merged images) in uninfected cells and cells infected for 10 hours with *S. typhimurium* (WT, green in merged images). Scale bar: 5 μm. Broken lines indicate cell perimeters. (B) Bacterial replication in p50/dynamitin-transfected cells (p50), KLC2-TPR transfected cells (TPR) or in p50/dynamitin, KLC2-TPR co-transfected cells (p50+TPR), was investigated by counting the number of *S. typhimurium* per infected cell. Results shown are the means ± s.d. of at least three independent experiments in which a total of at least 100 bacteria were examined. (C) Replication of *S. typhimurium* in the presence or absence of aurintricarboxilic acid (ATA). The values indicate the fold increase in bacterial strain cfu's between 2 hours and 16 hours after bacterial entry, relative to that of the wild-type strain, and represent the means ± s.d. of at least three independent experiments. The *ssaV* mutant serves as a control to detect impaired replication of bacteria. (D) Microtubule motors are required for Sif formation. Cells were infected with *S. typhimurium* fixed at 8 hours p.i. and labelled with an anti-LAMP-1 antibody in nocodazole-treated (noc), p50/dynamitin-transfected (p50), KLC2-TPR-transfected (TPR), or aurintricarboxilic acid (ATA)-treated cells to quantify Sif formation by confocal microscopy. Results shown are the mean ± s.d. of at least three independent experiments in which more than 100 infected cells were analysed for each experiment.

Dynein and kinesin activities are required for intracellular bacterial replication

A major function of microtubules is to serve as tracks for motor protein-driven movement of intracellular cargo and organelles. Conventional kinesin and cytoplasmic dynein are the major plus-end directed and minus-end directed motors, respectively. The distribution of these proteins in infected cells was therefore examined using antibodies against kinesin heavy chain and dynein. Microscopic analysis revealed, as expected, punctate patterns of labelling throughout uninfected HeLa cells. By contrast, in cells that had been infected for 10 hours with *S. typhimurium*, intense rings of kinesin and dynein were observed in the immediate vicinity of 46±11.5% and 62±9.6% of bacterial microcolonies, respectively. In cases where labelling around bacteria was particularly strong, very little kinesin or dynein was detected at the cell periphery (Fig. 3A). In the immediate vicinity of bacterial microcolonies, dynein and kinesin co-localised with LAMP-1, an lgp that is also recruited to the SCV membrane (Beuzón et al., 2002) (data not shown).

It has been shown previously that disruption of microtubules with nocodazole inhibits bacterial replication (Garcia-del Portillo et al., 1993). To determine if kinesin motor function is required for *S. typhimurium* replication, two independent methods of inhibition were used. Overexpression of a GFP-tagged version of the tetratricopeptide repeat (TPR) cargo-binding domain of the kinesin light chain 2 (KLC2) has been shown previously to block conventional kinesin-dependent movement (Rietdorf et al., 2001). pGFP-KLC2-TPR transfected cells were infected with *S. typhimurium* for 10 hours and numbers of bacteria in transfected and untransfected cells were counted with the aid of a microscope. In cells expressing GFP-KLC2-TPR, a significant inhibition of replication was observed (mean of 8.1±1.6 bacteria/cell in GFP-KLC2-TPR-transfected cells, compared to 19.7±4.3 bacteria/cell in untransfected cells, Fig. 3B). The second method involved the use of aurintricarboxylic acid (ATA), a potent inhibitor of kinesin (Hopkins et al., 2000). To verify that ATA was effective in inhibiting kinesin-dependent movement when added to cells, mitochondria were stained with CMXRos-H2 tracker. In untreated cells, mitochondria were evenly distributed throughout the cytoplasm. In cells incubated with ATA, they were clustered mainly in the perinuclear area (data not shown), consistent with the known effects on the distribution of mitochondria caused by the inhibition of kinesin (Vale and Fletterick, 1997). In infected cells incubated with ATA, bacterial replication was reduced to approximately 50% of that in untreated cells, similar to the effect of GFP-KLC2-TPR overexpression (Fig. 3C).

To investigate the requirement of dynein motor function in

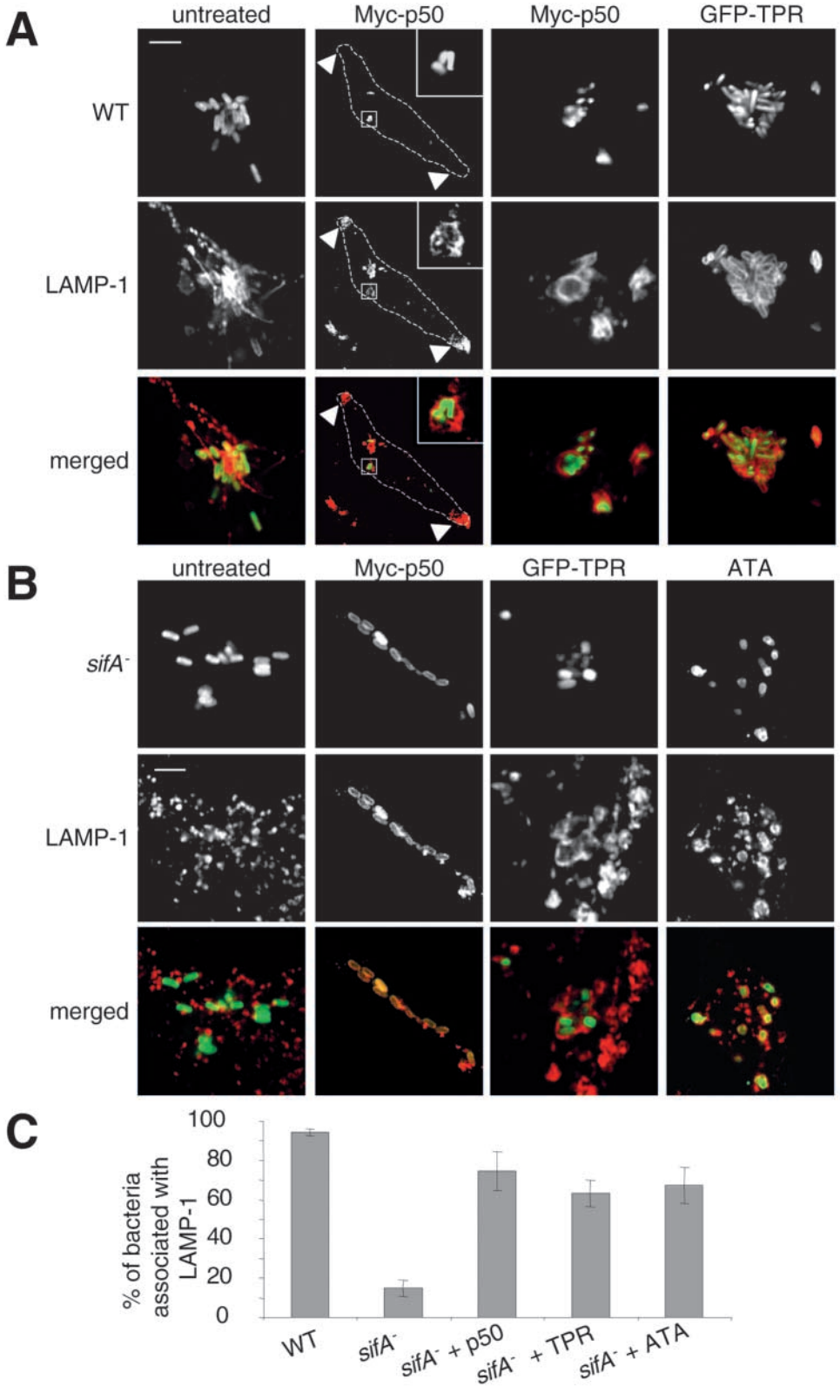

Fig. 4. Effect of inhibiting kinesin or dynein on SCVs. HeLa cells were treated with aurintricarboxilic acid (ATA) 3 hours prior to infection, or transfected with vectors expressing p50/dynamitin (p50) or GFP-KLC2-TPR (TPR) 16 hours prior to infection with either wild-type (WT) *S. typhimurium* or a *sifA* mutant strain (green in merged images). Cells were fixed 8 hours p.i. and labelled with an anti-LAMP-1 antibody (red in merged images). (A) Representative confocal micrographs showing LAMP-1 distribution in cells infected with GFP-WT bacteria. Left panel of p50-expressing cells is at lower magnification to show redistribution of LAMP-1 to cell periphery (indicated by arrowheads); boxed area is at higher magnification. Right panel of p50-expressing cells shows a second example of SCVs at higher magnification. Scale bar: 2 μm. (B) Representative confocal micrographs showing LAMP-1 distribution (red in merged images) in cells infected with GFP-*sifA* mutant (green in merged images). (C) Association of bacteria with LAMP-1 in untreated cells or cells expressing p50 or TPR, or which had been incubated with ATA. Association was evaluated by confocal microscopy. Results show the mean ± s.d. of at least three independent experiments in which more than 100 bacteria were analysed for each experiment.

intracellular *S. typhimurium* replication, HeLa cells were transfected with a vector expressing C-myc-p50/dynamitin, which uncouples the dynactin complex and disrupts dynein function (Echeverri et al., 1996). Microscopic analysis of untransfected cells using an antibody against LAMP-1 revealed characteristic punctate labelling, mainly in the perinuclear region. In cells expressing C-myc-p50/dynamitin, LAMP-1 was redistributed to the cell periphery (data not shown), in keeping with the known requirement for dynein motor function in retention of lysosomes and late endosomes in the perinuclear region (Burkhardt et al., 1997). Although the majority of LAMP-1 was also redistributed to the cell periphery in infected C-myc-p50/dynamitin-expressing cells, almost all intracellular bacteria were associated with LAMP-1 (Fig. 4A). However, there were fewer bacteria present in these cells at 10 hours p.i. compared to the numbers in untransfected cells (8±0.6 bacteria/cell in C-myc-p50/dynamitin-transfected cells compared to 19.7±4.3 bacteria/cell in untransfected cells; Fig. 3B). Simultaneous inhibition of both microtubule motors by co-

transfection of vectors expressing C-myc-p50/dynamitin and KLC2-TPR did not result in an additive effect on inhibition of bacterial replication (Fig. 3B). Together, these results show that both kinesin and dynein are recruited to bacterial microcolonies and are required for replication of intracellular *S. typhimurium*.

Inhibition of dynein and kinesin affects SCV morphology

In the course of these experiments, we observed that only 20.1±11.8% and 25.5±3.3% of infected cells expressing C-myc-p50/dynamitin or KLC2-TPR respectively, contained Sifs, compared to 82.8±1.2% in untransfected cells (Fig. 3D). Since Sifs are thought to represent tubular extensions of the SCV membrane, these results suggested that microtubule motors might have a role in regulating vacuolar membrane dynamics. In HeLa cells, bacteria are frequently located in distinct vacuoles, as revealed by LAMP-1 labelling, which normally follows the outline of individual bacteria (Fig. 4A). In infected p50/dynamitin-expressing cells almost all intracellular bacteria were associated with LAMP-1. However, although the labelling was very intense around the periphery of microcolonies, it was absent or weak between bacteria within microcolonies, indicative of enlarged vacuoles containing several bacteria (Fig. 4A). These morphologically abnormal vacuoles suggest that dynein is involved in partitioning of the SCV membrane that accompanies intracellular bacterial replication. By contrast, in KLC2-TPR-expressing (Fig. 4A), or in ATA-treated cells (data not shown), the SCV membrane, as revealed by LAMP-1 labelling, appeared even more closely associated with individual bacteria than in untransfected cells (Fig. 4A).

By 8 hours p.i., less than 20% of *sifA* mutant bacteria remain associated with LAMP-1, compared to over 80% of WT bacteria, reflecting a loss of vacuolar membrane around the mutant strain (Beuzón et al., 2000). Therefore this mutant provides an alternative tool to study SCV membrane dynamics. In p50/dynamitin, KLC2-TPR-expressing or ATA-treated cells, the percentage of the *sifA* mutant bacteria that were associated with LAMP-1 was 74.4±9.8%, 63.1±6.8% and 67.4±9.2%, respectively (Fig. 4B,C). These results show that the loss of the vacuolar membrane around the *sifA* mutant requires the action of both dynein and kinesin. Taken together, the requirement of dynein and kinesin for Sif formation and the effects of their inhibition on vacuoles containing WT and *sifA* mutant strains implicate both motors in SCV membrane dynamics.

Rab7 controls recruitment of dynein and RILP to intracellular *S. typhimurium*

The Rab7 GTPase is a key regulator of

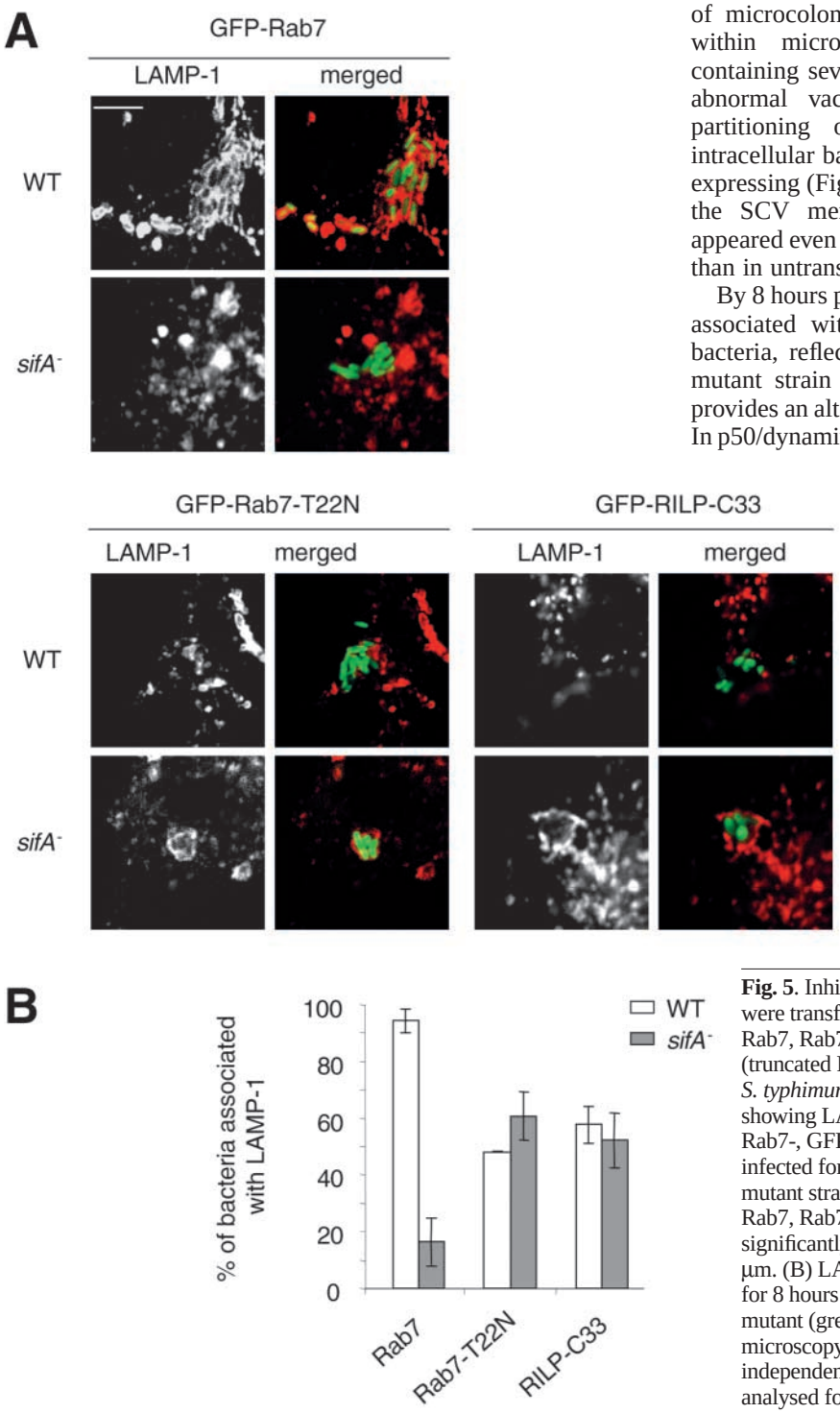

Fig. 5. Inhibition of Rab7 affects the SCV membrane. HeLa cells were transfected with vectors encoding GFP-tagged wild-type Rab7, Rab7-T22N (dominant negative variant), or RILP-C33 (truncated Rab7-binding variant of RILP) prior to infection with *S. typhimurium*. (A) Representative confocal micrographs showing LAMP-1 distribution (red in merged images) in GFP-Rab7-, GFP-Rab7-T22N-, and GFP-RILP-C33-expressing cells infected for 8 hours by GFP-*S. typhimurium* (WT) or GFP-*sifA* mutant strains (bacteria are green in merged images, GFP-tagged Rab7, Rab7-T22N and RILP are not visible because of significantly lower overall fluorescence intensity). Scale bar: 5 μm. (B) LAMP-1 association with bacteria in HeLa cells infected for 8 hours with GFP-*S. typhimurium* (white bars) or the GFP-*sifA* mutant (grey bars). LAMP-1 association was assessed by confocal microscopy. Results shown are the mean ± s.d. of at least three independent experiments in which more than 100 bacteria were analysed for each experiment.

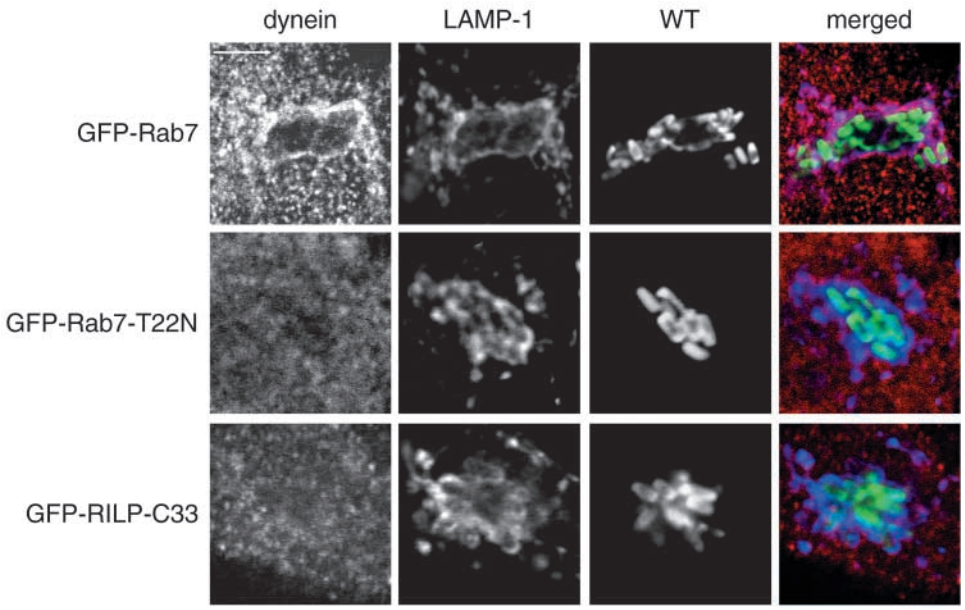

Fig. 6. Inhibition of Rab7 prevents dynein recruitment to SCVs. Representative confocal micrographs showing dynein distribution (red in merged images) in GFP-Rab7- (upper panel), GFP-Rab7-T22N- (middle panel), or GFP-RILP-C33-expressing cells (lower panel) infected with *S. typhimurium* (WT, green in merged images) for 10 hours. Vacuolar membrane integrity was assessed by labelling LAMP-1 (blue in merged images). Scale bar: 5 μm.

vesicular transport in the late endocytic pathway and during phagosome biogenesis (Desjardins et al., 1994; Méresse et al., 1995; Vitelli et al., 1997). Rab7 is also present on the SCV membrane and is required for SCV maturation and Sif formation (Brumell et al., 2001; Méresse et al., 1999). Rab7 has recently been shown to recruit functional dynein-dynactin motor complexes to late endosomes and lysosomes through its effector RILP (Jordens et al., 2001). We therefore studied the involvement of Rab7 in recruitment of dynein and RILP to SCVs.

HeLa cells were transfected with vectors expressing a GFP fusion of either wild-type Rab7 or a dominant-negative form (Rab7-T22N), then infected with WT or *sifA* mutant bacteria for 8 h. In Rab7-expressing cells (Fig. 5A), the levels of LAMP-1 association for WT and *sifA* mutant bacteria were 94.3±4.1% and 16.3±8.4%, respectively, similar to those found in untransfected cells (Ruiz-Albert et al., 2002) (Fig. 4). However, in Rab7-T22N-expressing cells, significantly fewer WT bacteria were associated with LAMP-1 (48.1±0.21%), reflecting a loss of vacuolar membrane, as found by Brumell et al. (Brumell et al., 2002b). In contrast, the majority of *sifA* mutant bacteria (60.8±8.6%) remained positive for this marker (Fig. 5A,B), reflecting the presence of an intact vacuolar membrane. RILP-C33, a truncated form of RILP lacking the N-terminal region, is non-functional but retains its ability to bind Rab7 (Cantalupo et al., 2001). Consistent with the results obtained with Rab7-T22N, only 57.7±6.6% of WT and up to 52.2±9.6% of *sifA* mutant bacteria, were associated with LAMP-1 in RILP-C33-expressing cells (Fig. 5A,B). Therefore, inhibition of Rab7 activation and function causes a significant loss of vacuolar membrane around WT bacteria, and inhibition of dynein activity results in morphologically abnormal vacuoles. In contrast, inhibition of either Rab7 or dynein activity prevents loss of the vacuolar membrane surrounding *sifA* mutant bacteria.

No dynein was associated with those bacteria that were not associated with LAMP-1 in Rab7-T22N- or RILP-C33-expressing cells (data not shown). It was not possible to determine whether SCVs in these cells were associated with dynein prior to vacuolar membrane loss, because dynein recruitment was only readily apparent from 10 hours p.i., whereas vacuolar membrane loss occurred between 5 and 10 hours p.i. Nevertheless, when LAMP-1-positive SCVs were examined at 10 hours p.i., dynein was associated with only 15±4.1% and 11.9±6.8% of microcolonies in Rab7-T22N- or RILP-C33-expressing cells, respectively, compared to 63±9.5% in Rab7-expressing cells (Fig. 6). However, Rab7-T22N or RILP-C33 did not affect the recruitment of microtubules to LAMP-1 associated microcolonies (data not shown). These results strongly suggest that dynein recruitment to *S. typhimurium* microcolonies is dependent on the activity of Rab7.

As expected (Cantalupo et al., 2001), overexpressed RILP aggregated in the perinuclear region (Fig. 7A upper panel) where it colocalized with LAMP-1-positive late endosomal and lysosomal compartments (Cantalupo et al., 2001) (data not shown). In infected cells, *S. typhimurium* microcolonies were surrounded by overexpressed RILP (Fig. 7A lower panel). As an independent test of RILP recruitment to SCVs, HeLa cells were infected for 8 hours, then fixed and labelled for endogenous RILP and LAMP-1. In this experiment, 94.8±4.2% of vacuoles containing WT *S. typhimurium* were associated with RILP, which co-localised with LAMP-1 present on the vacuolar membrane (Fig. 7B upper panel). In HeLa cells expressing Rab7-T22N, only 6.4±3.4% of LAMP-1-positive SCVs were associated with RILP, indicating that RILP recruitment to the SCV is Rab7 dependent (Fig. 7B lower panel).

Overexpression of RILP has been shown to counteract the effects of dominant negative Rab7 on organization and transport of late endosomal compartments (Cantalupo et al., 2001), indicating that RILP is the downstream effector of Rab7 mediating these effects. In cells transfected with a vector expressing full-length RILP, 90.5±2.5% of the WT strain and 13.6±10.2% of the *sifA* mutant were found associated with LAMP-1 at 8 hours p.i. (Fig. 7C). However, in cells co-transfected with vectors encoding RILP and Rab7-T22N, 40.2±7.8% of WT and 51±9.8% of *sifA* mutant bacteria were found associated with LAMP-1, similar to the results obtained

with Rab7-T22N alone (Fig. 5B). In addition, whereas 70.1±13.2% of SCVs recruited dynein in cells expressing only RILP, only 1.1±0.3% of SCVs were associated with dynein in cells co-expressing RILP and Rab7-T22N (data not shown). Therefore, overexpression of RILP is not sufficient to overcome the effect of Rab7-T22N on SCV membrane integrity or dynein recruitment.

Discussion

In this paper we show that microtubules and the microtubule-associated motors dynein and kinesin, accumulate around *S. typhimurium* as bacteria begin to multiply inside host cells. Survival and multiplication of *Salmonella* in host cells involves a large number of virulence proteins, some of which are delivered into the host cell by the SPI-1 and SPI-2 TTSSs. Although microtubule accumulation requires intracellular bacterial protein synthesis, it is not dependent solely on the SPI-2 TTSS. Most studies on the SPI-1 TTSS have focussed on its role in bacterial invasion of host cells, but recent work has shown that this TTSS is also necessary for vacuole biogenesis and intracellular proliferation in epithelial cells (Steele-Mortimer et al., 2002). However, the few SPI-1 single mutants that entered HeLa cells were also indistinguishable from the WT strain in terms of recruitment of microtubules. The SPI-1, SPI-2 double mutant strain produced very few microcolonies of sufficient size to be able to determine if they were defective for microtubule accumulation. Nonetheless, in the few cases where microcolonies of three to four bacteria were observed, there were no microtubule rearrangements. Therefore, it is possible that the bacterial effector(s) responsible for these changes might be able to use either secretion system to gain access to the host cell cytosol. Indeed, two such effectors have been described (Miao and Miller, 2000; Miao et al., 1999), and further analysis of strains carrying mutations in genes for these and other effectors might identify the proteins mediating this activity.

Microtubules are known to be exploited in the invasion of host cells by several bacteria, including *Campylobacter*, *Citrobacter* and *Shigella* (Oelschlaeger and Kopecko, 2000; Yoshida and Sasakawa, 2003). Microtubules are also required for the intracellular movement and cell-to-cell spread of *Actinobacillus actinomycetemcomitans* (Meyer et al., 1999), and both microtubules and dynein have previously been found to localise around *Chlamydia*

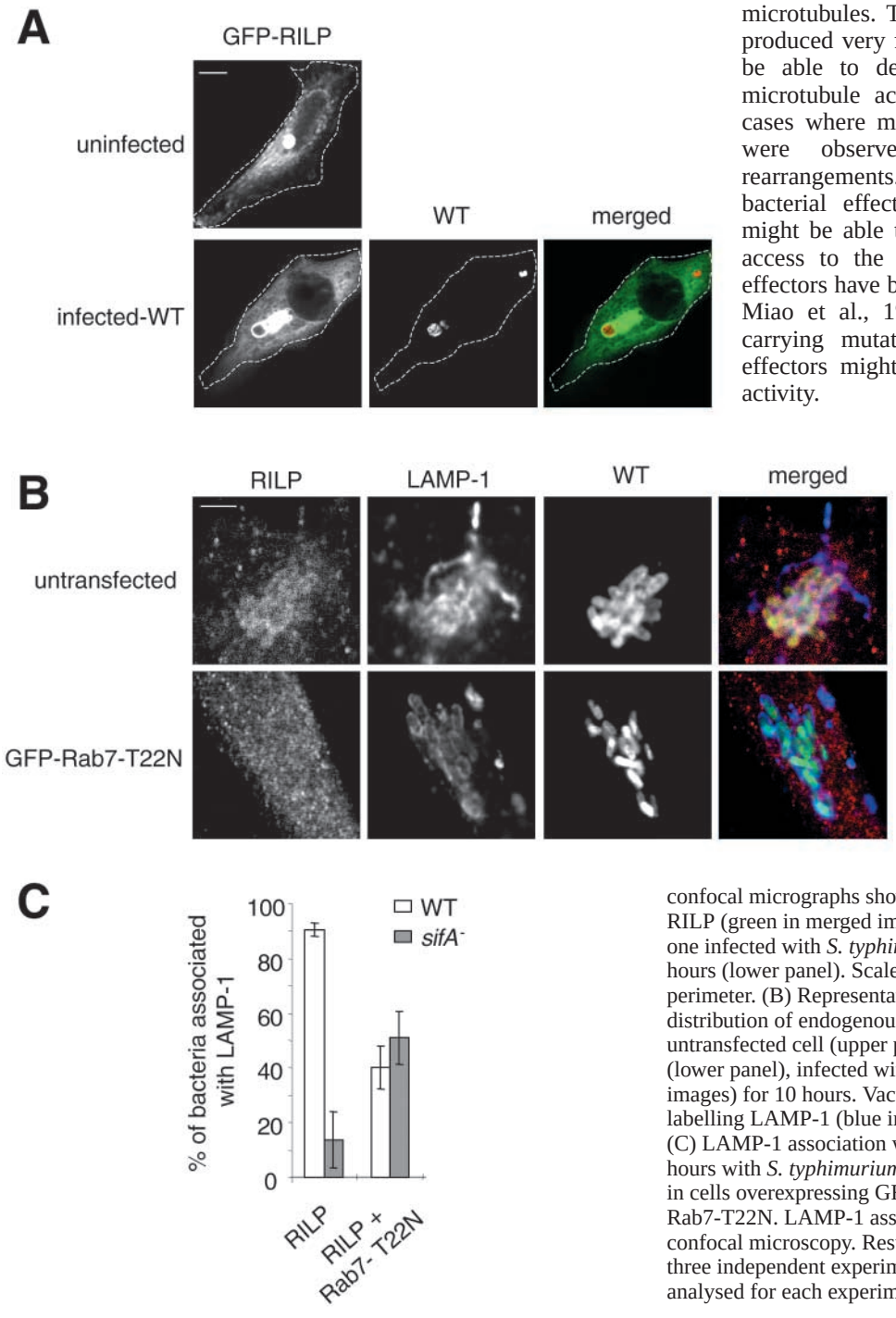

Fig. 7. RILP recruitment to SCVs is controlled by Rab7. (A) Representative confocal micrographs showing distribution of overexpressed GFP-RILP (green in merged image) in an uninfected cell (upper panel) or one infected with *S. typhimurium* (WT, red in merged image) for 10 hours (lower panel). Scale bar: 5 μm. Dotted lines indicate cell perimeter. (B) Representative confocal micrographs showing distribution of endogenous RILP (red in merged images) in an untransfected cell (upper panel) and a Rab7-T22N-expressing cell (lower panel), infected with *S. typhimurium* (green in merged images) for 10 hours. Vacuolar membrane integrity was assessed by labelling LAMP-1 (blue in merged images). Scale bar: 2 μm. (C) LAMP-1 association with bacteria in HeLa cells infected for 8 hours with *S. typhimurium* (white bars) or the *sifA* mutant (grey bars) in cells overexpressing GFP-RILP or GFP-RILP together with myc-Rab7-T22N. LAMP-1 association with bacteria was assessed by confocal microscopy. Results shown are the mean ± s.d. of at least three independent experiments in which more than 100 bacteria were analysed for each experiment.

trachomatis-containing vacuoles several hours after infection of epithelial cells (Clausen et al., 1997). Dynein activity is required for migration of *C. trachomatis* to the peri-Golgi region, although this appears to be independent of p50/dynamitin (Grieshaber et al., 2003). The recent finding from our laboratory that *Salmonella* microcolonies also develop in close association with the Golgi (Salcedo and Holden, 2003) provides further evidence of similarities in the behaviour of these two intracellular pathogens. Kinesin motor activity is involved in the intracellular development of *Chlamydia psittaci*, probably by transporting mitochondria to bacterial inclusion bodies (Escalante-Ochoa et al., 1999). By contrast, we did not observe any accumulation of mitochondria around SCVs, when infected cells were labelled with CMXRos-H2 to reveal mitochondria (data not shown). Nevertheless, microtubules are known to provide a scaffold for Sifs (Brumell et al., 2002a), and it has been shown that treatment of infected HeLa cells with nocodazole has an inhibitory effect on bacterial replication (Garcia-del Portillo et al., 1993).

We have previously shown that intracellular *S. typhimurium* induces the formation of an actin meshwork around SCVs in a variety of host cell types (Méresse et al., 2001). In infected macrophages, depolymerisation of F-actin with latrunculin B or cytochalasin D caused the selective loss of vacuolar membrane around WT bacteria, suggesting that the physiological role of SCV-associated F-actin is connected with recruitment or stabilisation of the vacuolar membrane (Méresse et al., 2001). Actin assembly around SCVs requires a functional SPI-2 TTSS but the bacterial effectors mediating the process have not yet been identified (Miao et al., 2003; Yu et al., 2002). Although the results described here show that recruitment of microtubules around bacterial microcolonies is independent of actin polymerisation and does not solely require the SPI-2 TTSS, the evidence indicates that SCV-associated microtubules and motors are also involved in vacuolar membrane dynamics. Inhibition of either motor in cells infected with WT bacteria resulted in morphologically abnormal vacuoles. Dynein inhibition resulted in formation of misshapen vacuoles that frequently contained more than one bacterial cell. In contrast, when kinesin activity was inhibited the vacuolar membrane became more sharply defined around individual bacteria. Further evidence for the involvement of these motors in vacuolar membrane dynamics comes from their requirement for Sif formation and for the loss of vacuolar membrane that normally occurs several hours after uptake of *sifA* mutant bacteria.

The effect on the SCV of inhibition of kinesin does not provide an obvious clue as to its role in intracellular bacterial growth, although recent work showing that kinesin can mediate membrane tubulation (Roux et al., 2002) could provide an explanation for its requirement for Sif formation. However, the effect of dynein inhibition is interesting in relation to recent evidence implicating it in nuclear envelope breakdown during mitosis in higher eukaryotes. It has been shown that dynein is concentrated on the cytoplasmic side of the nuclear envelope and can induce membrane rupture by pulling the membrane along microtubules (Beaudouin et al., 2002; Salina et al., 2002). Replication of *S. typhimurium* in host cells is accompanied by a controlled expansion of the vacuolar membrane to accommodate new bacterial cells. This is thought to occur by recruitment to the SCV membrane and fusion of a specific class of lgp-containing vesicles, probably mediated by the SPI-2 effector SifA (Beuzón et al., 2000; Ruiz-Albert et al., 2002). This process must be balanced by other activities to ensure the correct partitioning of the SCV membrane that accompanies bacterial cell division and the segregation of individual SCVs in the infected cell. The effect of dynein inhibition on the morphology of SCVs suggests that the function of SCV-associated dynein might be to drive the process of membrane detachment that occurs during the segregation of vacuoles. In the case of *sifA* mutant bacteria, this would result in the breakdown of their vacuolar membranes. However, it is also possible that the redistribution of late endosomal compartments to the cell periphery, caused by dynein inhibition, makes them less accessible as a source of membrane for the developing bacterial microcolony, which could lead to morphological abnormalities.

Our evidence indicates that dynein recruitment to SCVs is controlled by Rab7 activity. However, the functions of Rab7 in relation to vacuolar membrane dynamics appear to extend beyond its involvement in dynein recruitment, because inhibition of Rab7 caused membrane loss from vacuoles containing WT bacteria. Rab7 mediates the recruitment of dynein to late endosomes through its effector RILP (Cantalupo et al., 2001; Jordens et al., 2001). Although we found that RILP is also recruited to SCVs in a Rab7-dependent manner, it is not clear if SCV-associated RILP is required for dynein recruitment, because the expression of full-length RILP was not sufficient to overcome the effects of dominant-negative Rab7 on the SCV membrane. It is possible that dynein recruitment by Rab7 is mediated by an effector other than RILP; alternatively RILP might require a functional Rab7 to be recruited to and/or activated at the SCV membrane.

Rab7 is known to play an important role in controlling the maturation of the SCV in terms of its selective interactions with the endocytic pathway during the first hour following bacterial uptake into epithelial cells (Méresse et al., 1999). The results described in this paper and elsewhere (Brumell et al., 2002b) indicate that it also has an important role at a later stage, when intracellular bacteria begin to replicate. The inhibition of Rab7 phenocopies the effect of a *sifA* mutation, in that it blocks Sif formation (Brumell et al., 2001) and leads to significant loss of vacuolar membrane (Brumell et al., 2002b). This suggests a functional link between these proteins. Although SifA and Rab7 are not required for microtubule accumulation around SCVs, translocation of SifA might be necessary for Rab7 activation and recruitment of dynein to the vacuolar membrane, possibly through the action of RILP.

Very little is known about the control of vacuolar membrane dynamics for intravacuolar pathogens. However, the recent discovery that *S. typhimurium* proteins such as SifA and SseJ (Beuzón et al., 2000; Ruiz-Albert et al., 2002) and host proteins such as Rab7 (Brumell et al., 2002b) and dynein (this work) are involved in this process, provide tools for future studies to gain a more detailed understanding of this critical aspect of intracellular pathogenesis.

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