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Salmonella induces the formation of filamentous structures containing lysosomal membrane glycoproteins in epithelial cells

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ABSTRACT **_Salmonella_ species invade and replicate within epithelial cells in membrane-bound vacuoles. In this report we show that upon infection of HeLa epithelial cells, _Salmonella typhimurium_ resides in vacuoles that contain lysosomal membrane glycoproteins (lgps). Four to six hours after invasion, intracellular bacteria induce the formation of stable filamentous structures containing lgps that are connected to the bacteria-containing vacuoles. Formation of these lgp-rich structures requires viable intracellular bacteria and is blocked by inhibitors of vacuolar acidification. These structures are not present in uninfected cells or in cells infected with another invasive bacteria, _Yersinia enterocolitica_. Tracers added to the extracellular medium are not delivered to the _Salmonella_-induced filaments, suggesting that these structures are different from previously described tubular lysosomes. Initiation of intracellular bacterial replication correlates with formation of these lgp-containing filaments. Certain avirulent _Salmonella_ mutants that are defective for intracellular replication fail to induce formation of these structures. These observations suggest that _Salmonella_-induced filaments containing lgps are linked to intracellular bacterial replication.**

Targeting of intracellular pathogens to lysosomal compartments within the host cell occurs with several parasites. *Toxoplasma gondii* prevents fusion to lysosomal membrane glycoprotein (lgp)-containing vacuoles, but this capacity is lost when the parasite is previously opsonized and enters the host cell via an Fc-mediated pathway (1). *Trypanosoma cruzi* invades eukaryotic cells, inducing the recruitment of lysosomes (i.e., lgp-containing vacuoles) to the site of entry of the parasite (2). *T. cruzi* and *Leishmania mexicana* reside within macrophages in a parasitophorous vacuole containing lgps (3, 4). Some examples of targeting of bacteria to and persistence of bacteria in lysosomes have also been reported for *Coxiella burnetii* (5), *Yersinia pestis* (6), *Mycobacterium lepraemurium* (7), and *Salmonella typhimurium* (8, 9).

Salmonella species can survive and replicate within vacuoles in epithelial cells (10–12). The nature of this bacteria-containing vacuole and its interaction with various organelles of the host cell has not been characterized in epithelial cells, although it has with phagocytic cells (8, 9, 13). One report showed that *S. typhimurium* prevented fusion of the bacteria-containing phagosome with lysosomes (13), while in another report it was shown that *S. typhimurium* fused with lysosomes soon after phagocytosis (9). In epithelial cells, intracellular replication of *Salmonella* within vacuoles is an essential virulence trait of this pathogen (14). To characterize the trafficking and interaction of *Salmonella*-containing vacuoles with lysosomes inside epithelial cells, we have examined the distribution of lgps in HeLa epithelial cells infected with *S. typhimurium*. These glycoproteins are major components of the lysosomal membrane (15–18).

We report here that upon infection of human epithelial cells, *Salmonella* resides and replicates in lgp-containing vacuoles and that intracellular bacterial replication involves the formation of stable filamentous structures, connected to the bacteria-containing vacuoles. This capacity appears specific for *Salmonella* species and requires viable intracellular bacteria.

MATERIALS AND METHODS

Bacterial Strains. *S. typhimurium* strains included SL1344 (19) and the intracellular-replication-defective mutants 3-11, 12-23, 17-21, and 22-11 (14). *Yersinia enterocolitica* strain 8081c was used. *S. typhimurium* strains were grown overnight in Luria–Bertani broth at 37°C without shaking, and *Y. enterocolitica* was grown at 30°C.

Epithelial Cells. HeLa epithelial cells (human epithelioid carcinoma) (ATCC CCL2) were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum and were maintained as described (10).

Bacterial Infection of Epithelial Cells. HeLa epithelial cells were grown overnight to 80% confluence and then infected at 37°C for 30 min with *S. typhimurium* SL1344 or for 60 min with the other *Salmonella* strains or *Y. enterocolitica*. The multiplicity of infection was 10, and the number of viable intracellular bacteria was calculated as described (14).

Antibodies and Immunofluorescence. Mouse monoclonal antibodies H5G11 [anti-human lysosomal membrane glycoprotein 1 (hlamp-1)] and H4B4 (anti-hlamp-2) were used (20). Cells grown on coverslips were fixed in 2% paraformaldehyde in phosphate-buffered saline for 10 min at 25°C and processed for indirect immunofluorescence (21). The secondary antibodies used included fluorescein isothiocyanate-conjugated goat anti-rabbit and goat anti-mouse, and rhodamine-conjugated goat anti-mouse immunoglobulins. For double immunofluorescence labeling, mouse monoclonal anti-lgp and rabbit polyclonal anti-*S. typhimurium* lipopolysaccharide were used. Percentages of infected epithelial cells containing lgp filaments were calculated as the ratio of infected cells containing the filamentous structures to the total number of infected cells that were screened.

Endocytic Probes and Lectins. Lucifer yellow (1 mg/ml) and rhodamine-conjugated ovalbumin (500 μg/ml) were used as fluid endocytic probes. Uptake was done for 2 hr to ensure labeling of lysosomes (21, 22). *Datura stramonium* lectin conjugated to biotin (100 μg/ml) was used to label lgps in live and fixed cells. This lectin binds to *N*-acetyllactosaminyl residues, which are present on lgps (18). Streptavidin–fluorescein isothiocyanate conjugate was used for fluorescence location of lgps which bound the lectin.

Inhibitors of Vacuolar Acidification. The following compounds were used to block acidification of the vacuolar system of the epithelial cell: (*i*) weak bases, including 20 mM

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Abbreviations: lgps, lysosomal membrane glycoproteins; hlamp-1 and hlamp-2, human lysosomal membrane glycoproteins 1 and 2.

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ammonium chloride, 5 mM methylamine, and 10 μM chloroquine (10); (*ii*) the ionophore monensin (20 μM) (10); and (*iii*) an inhibitor of the vacuolar-type H^+-ATPase, bafilomycin A_1 (1 μM) (23, 24).

RESULTS

***Salmonella* Induces the Formation of Filamentous Structures Containing lgps in Epithelial Cells.** To determine whether *S. typhimurium* is targeted to lysosomes upon infection of HeLa epithelial cells, we used indirect immunofluorescence to analyze colocalization of bacteria-containing vacuoles with vesicles containing the human lgps hlamp-1 and hlamp-2 (15–18, 20). Two hours after bacterial invasion, *S. typhimurium* SL1344 was found within vacuoles containing these lysosomal markers. Four to six hours after infection, long filamentous structures with periodic swellings were also labeled (Fig. 1 *A–C*). These structures were associated with intracellular bacteria-containing vacuoles, often linking different groups of intracellular bacteria within a single epithelial cell. Direct measurement in the confocal microscope of 54 different lgp-containing filaments gave an average diameter of 0.509 ± 0.106 μm. These lgp-rich structures were never observed in uninfected cells (Fig. 1 *D* and *E*) but were visible 6 hr after infection of HeLa epithelial cells with other virulent *Salmonella* species, including *S. choleraesuis*, *S. dublin*, and *S. enteriditis*, and in other cultured epithelial cell lines such as Henle-407 and Caco-2. Infection of HeLa cells with *Y. enterocolitica* 8081c, another invasive bacterium that remains within a vacuole but does not multiply (25), did not cause the formation of these filamentous lgp-containing structures, although all bacteria-containing vacuoles were labeled with anti-lgp antibodies (Fig. 1 *F* and *G*).

Appearance of lgp-Containing Filaments Is Associated with *Salmonella* Intracellular Replication. The kinetics of appearance of the lgp-containing filaments was compared to the rate of *S. typhimurium* intracellular replication. Bacterial proliferation initiated 4 hr after infection (Fig. 2*A*). Thirty percent of infected cells had hlamp-2-containing filamentous structures at 4 hr, increasing to 75% by 6 hr (Fig. 2*B*). Yet prior to 4 hr, these structures were never observed. Similar results were obtained when anti-hlamp-1 antibody was used. These data suggest that there is a correlation between intracellular bacterial replication and formation of lgp-containing filamentous structures.

Tubular or filamentous lysosomes present in macrophages and cells treated with brefeldin A are supported by the microtubule network (21, 22, 26–30). We determined in *S. typhimurium*-infected cells the effect of the microtubule-depolymerizing drug nocodazole (10 μg/ml) on the formation of lgp-filaments and intracellular replication kinetics (Fig. 2). Less than 5% of infected cells contained lgp filaments when nocodazole was added at 2 or 3 hr after infection (Fig. 2*B*). When nocodazole was added 4 hr after infection, a decrease in the proportion of infected cells with lgp filaments was observed (15% at 6 hr postinfection versus 30% at 4 hr, Fig. 2*B*), indicating that microtubules are necessary for both the formation and the maintenance of the *Salmonella*-induced lgp-containing filaments. Fewer viable intracellular bacteria were also detected 6 hr after infection in nocodazole-treated cells (58–75% of the untreated-sample value) (Fig. 2*A*).

We also used *Salmonella* mutants that are completely avirulent in the mouse typhoid model and are unable to multiply inside epithelial cells (14, 31): *S. typhimurium* mutants 3-11, 12-23, and 17-21 are prototrophic (14) and 22-11 is a purine auxotroph (14); the *S. choleraesuis* mutant 96cB (31) is prototrophic. We have observed that *aroA* auxotrophic mutants of both *S. typhimurium* and *S. choleraesuis* (32) do not replicate intracellularly in epithelial cells. Cells infected with these mutants were tested for the presence of lgp-containing filamentous structures. The prototrophic mutants 3-11, 12-23, 17-21, and 96cB failed to induce the formation of lgp filaments at 4 hr, 6 hr, or later times, with intracellular bacteria located in large fused vacuoles containing lgps (Fig.

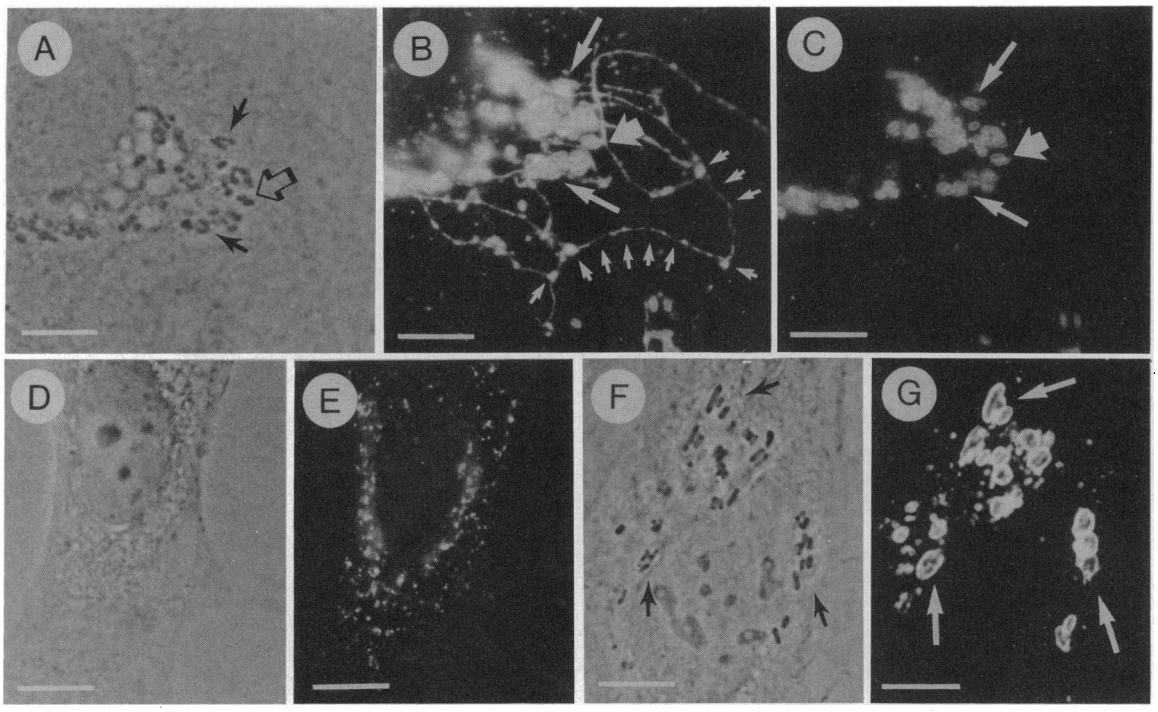

FIG. 1. Filamentous structures containing lgps in *Salmonella*-infected HeLa epithelial cells. (*A–C*) Phase-contrast image, hlamp-2 distribution, and intracellular bacteria in a *S. typhimurium*-infected cell 6 hr after infection. (*D* and *E*) Phase-contrast image and hlamp-2 distribution in an uninfected cell. (*F* and *G*) Phase-contrast image and hlamp-2 distribution in a *Y. enterocolitica*-infected cell 6 hr after infection. lgp-containing filamentous structures with periodic swellings are indicated by small arrows, and the location of some intracellular bacteria by large arrows. A wide arrow indicates a probable connection between a lgp filament and bacteria-containing vacuoles. (Bar = 10 μm.)

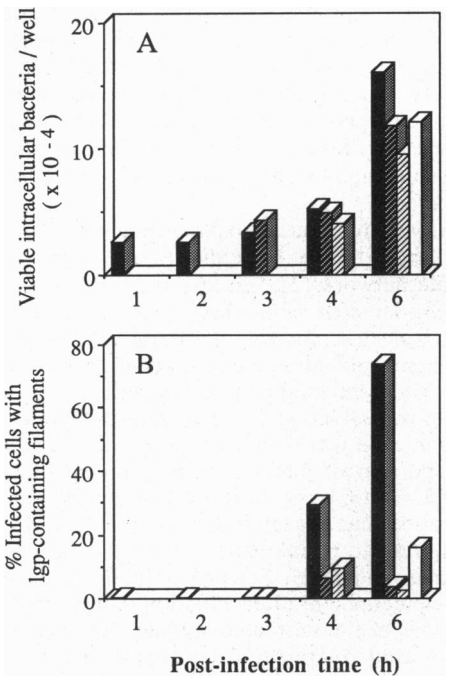

FIG. 2. Effect of nocodazole on lgp-filament formation and intracellular *Salmonella* replication in HeLa epithelial cells. (*A*) Viable intracellular bacteria at various times after infection. (*B*) Percentage of infected cells with filaments containing hlamp-2. Nocodazole (10 μg/ml) was added 2 hr (■), 3 hr (▨), or 4 hr (□) after infection. Control infected culture had no drug added (■). Data are representative of three experiments.

3 *A* and *B*). However, mutant 22-11 (purine auxotroph) and the two *aroA* mutants of *S. typhimurium* and *S. choleraesuis* induced formation of lgp-rich filamentous structures (Fig. 3 *C* and *D*). Specific bacterial loci involved in intracellular replication may direct the formation of the lgp-containing filaments.

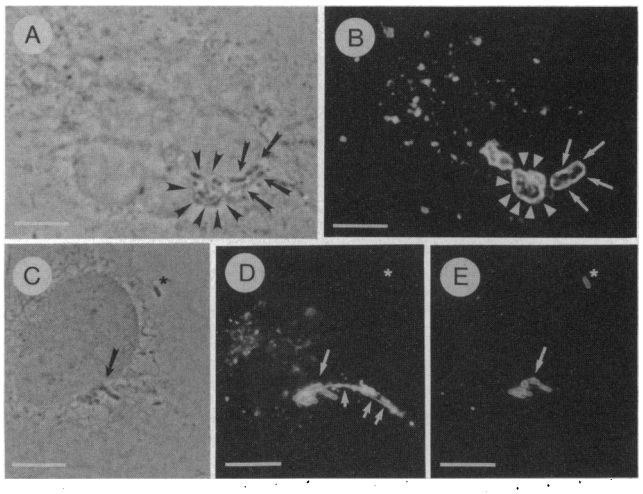

FIG. 3. Failure of induction of lgp filaments by certain *S. typhimurium rep*$^-$ mutants. Infected HeLa epithelial cells were fixed 6 hr after infection. (*A* and *B*) Phase contrast and hlamp-2 distribution in a cell infected with the prototrophic *rep*$^-$ mutant 3-11. Arrowheads and large arrows show two large fused vacuoles containing several intracellular bacteria. (*C–E*) Phase contrast, hlamp-2 distribution, and localization of intracellular bacteria in a cell infected with the purine auxotrophic mutant 22-11. Large arrows show intracellular bacteria and small arrows a lgp-containing filament. An asterisk shows extracellular bacteria not labeled with anti-hlamp-2 antibody. The same phenotype as 3-11 was observed for the two other prototrophic *rep*$^-$ mutants, 12-23 and 17-21, and the same as 22-11 for the *aroA* mutant. (Bar = 10 μm.)

Formation of lgp-Containing Filaments Requires Viable Intracellular *Salmonella* and Vacuolar Acidification. Cells infected with *S. typhimurium* SL1344 were treated with tetracycline (15 μg/ml) or rifampicin (16 μg/ml) at various times after infection to block protein or RNA synthesis in intracellular bacteria. Antibiotic addition prevented the formation of lgp filaments when added before 3 hr, but not at 3 hr or later times (Table 1). Percentages of intracellular bacteria that remained viable 6 hr after infection were similar when antibiotics were added at 2, 3, or 4 hr (20–40%, Table 1), although an inhibitory effect on lgp-filament formation was observed only when drugs were added at 3 hr or earlier. These data indicate that the formation of lgp-rich filaments in infected cells requires metabolically active bacteria during the first 3 hr after infection, but once the lgp-rich filaments are formed (4 hr), their maintenance and stability do not require viable bacteria.

To test whether vacuolar acidification was necessary to induce the formation of lgp-rich filamentous structures, we used various compounds that are known to increase the intravacuolar pH (10). Fig. 4*A* shows that ammonium chloride, methylamine, chloroquine, monensin, and bafilomycin A_1 blocked the formation of lgp-containing filaments when added 3 hr after infection and maintained in the medium until 7 hr after infection. When the compounds were added only from 3 to 5 hr after infection, a partial recovery was observed in the capacity of intracellular bacteria to induce the formation of lgp-containing filaments between 5 and 7 hr (Fig. 4*A*), probably due to the reversible acidification of the bacteria-containing vacuoles. The only exception was bafilomycin A_1 treatment, since no lgp filaments were observed irrespective of incubation time. When intracellular replication rates were determined after these treatments, bafilomycin A_1, monensin, and chloroquine partially blocked bacterial proliferation, but no such effect was observed with ammonium chloride or methylamine (Fig. 4 *B* and *C*). Although lgp-filament formation may have a strict requirement for an acidic intravacuolar pH, intracellular replication can continue if certain conditions favor bacterial proliferation (i.e., a general increase in

Table 1. Formation of lgp filaments requires viable *S. typhimurium*

Treatment	Time of antibiotic addition,* hr	% infected cells with filaments†	% viable intracellular bacteria‡
None	—	65.2	—
Tetracycline (15 μg/ml)	0.5	0	17
	1	0	26
	2	0	11.2
	3	32.7	17.4
	4	59.5	26.5
	5	61.0	78.5
Rifampicin (16 μg/ml)	0.5	0	42
	1	0	75
	2	0	44
	3	20.9	48.4
	4	55.5	28
	5	61.3	76

*Antibiotic concentrations were maintained in the medium after this time until 6 hr.

†Data are representative of three experiments and were calculated as described in *Materials and Methods* by using anti-hlamp-2 and anti-lipopolysaccharide in cells fixed 6 hr after infection.

‡Calculated as viable intracellular bacteria at 6 hr after infection versus viable intracellular bacteria at the time of antibiotic addition.

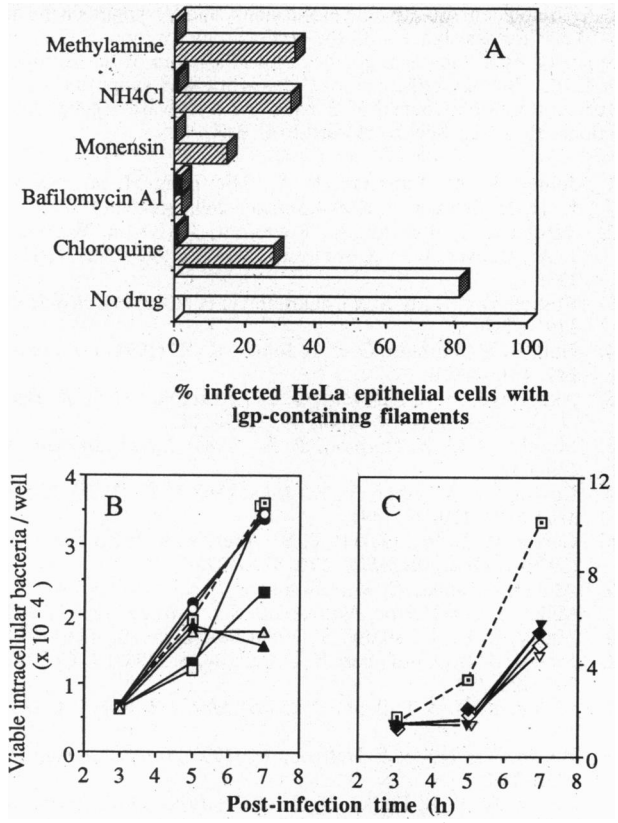

FIG. 4. Effect of vacuolar acidification inhibitors on the formation of lgp filaments and intracellular bacterial replication. (*A*) Percentage of *S. typhimurium* SL1344-infected HeLa cells with hlamp-2-containing filaments 7 hr after infection, after treatment with the vacuolar acidification inhibitors shown for 2 hr (▨, 3–5 hr after infection) or 4 hr (■, 3–7 hr after infection). Infected cells with no inhibitor added served as control (□). (*B* and *C*) Viable intracellular bacteria in *S. typhimurium*-infected cells treated with 5 mM methylamine (●, ○), 20 mM NH_4Cl (■, □), 20 μM monensin (▲, △), 1 μM bafilomycin A_1 (◆, ◇), or 10 μM chloroquine (▼, ▽). Filled symbols, treatment 3–5 hr after infection; open symbols, treatment 3–7 hr after infection. ⊡, Infected cells with no inhibitor added.

the cellular vacuolization induced by weak bases). The effect of these compounds on bacterial viability was minimal since, when tested in minimal M9 medium, none had bactericidal effect when added at concentrations 50–100 times higher than those used with HeLa cells.

lgp-Containing Filaments Induced by *Salmonella* Are Different from Tubular Lysosomes. Tubular lysosomes are usually accessible to fluid endocytic tracers such as Lucifer yellow and colloidal gold particles (22, 27, 29, 30), although with one specific cell line treated with brefeldin A, tubular lysosomes were not accessible to the endocytic tracer rhodamine-ovalbumin (21). In all cases, it has been shown that stability of tubular lysosomes depends on the integrity of the microtubule network (21, 22, 26–30).

We determined whether Lucifer yellow (1 mg/ml) or rhodamine-ovalbumin (500 μg/ml) was delivered to the lgp filamentous structures. To ensure labeling of lysosomes, tracers were added from 4 to 6 hr after infection. Neither Lucifer yellow nor rhodamine-ovalbumin was present in filamentous structures in infected live cells. Although this suggests that the filamentous structures do not receive incoming material from the external medium, label of lgps could not be done in cells treated with both tracers since they leak from the cell upon fixing and permeabilization. Therefore, we examined whether the *Datura stramonium* lectin, which binds to *N*-acetyllactosaminyl residues present in lgps

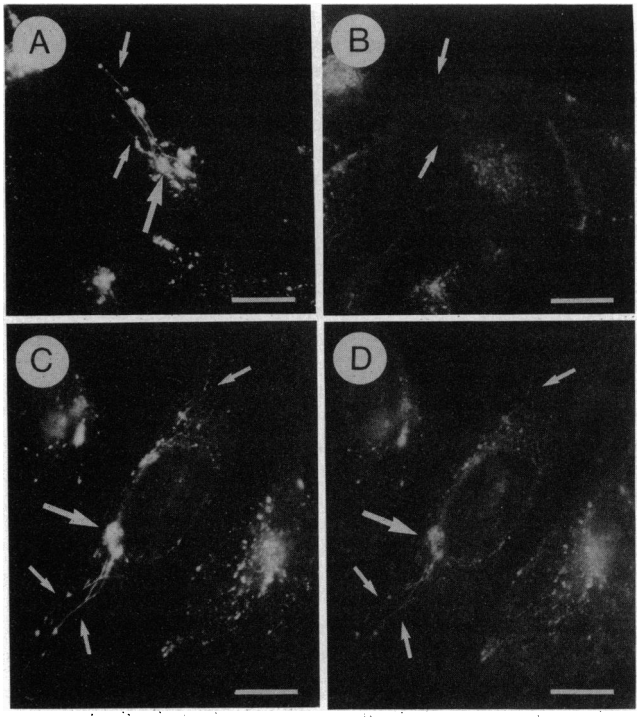

FIG. 5. Inaccessibility of lgp filaments to the extracellular medium. (*A* and *C*) Distribution of hlamp-2. (*B* and *D*) Distribution of the *D. stramonium* lectin conjugated to biotin detected with streptavidin–fluorescein isothiocyanate conjugate. Note absence of lectin in lgp filaments of *S. typhimurium* SL1344-infected HeLa cells in which lectin (100 μg/ml) was added to the culture medium 4 hr after infection (*A* and *B*). Lectin was present in lgp filaments and bacteria-containing vacuoles when added to fixed and permeabilized cells (*C* and *D*). In all samples, cells were fixed and permeabilized 6 hr after infection. Large arrows indicate bacteria-containing vacuoles and small arrows show lgp-containing filaments. (Bar = 10 μm.)

(18), could be delivered to the lgp-containing filaments in live cells. Lectin conjugated to biotin (100 μg/ml) was added to the tissue culture medium of *S. typhimurium* SL1344-infected cells from 4 to 6 hr after infection. Under these conditions, the lectin labeled neither the lgp-containing filaments nor the bacteria-containing vacuoles (Fig. 5 *A* and *B*). When the biotin-conjugated lectin was added to cells that were previously fixed and permeabilized (6 hr after infection), it colocalized with lgps and appeared in both the lgp-containing filaments and the bacteria-containing vacuoles (Fig. 5 *C* and *D*). These data confirm that the lgp-rich filamentous structures induced by *Salmonella* are not accessible to incoming material from the external medium.

DISCUSSION

Our data provide evidence that an intracellular pathogen which resides within a vacuole (i.e., *Salmonella*) triggers the formation of lgp-containing filamentous structures connected to the bacteria-containing vacuole (Fig. 1). Although the targeting of intracellular pathogens to lgp-containing vacuoles has been described for other parasites, no specialized structure containing lgps was reported (1–3). Our results support the concept that intracellular *Salmonella* have the capacity to induce the formation of these lgp-containing filaments, since the formation of these filamentous structures is not triggered by nonreplicating invasive bacteria such as *Y. enterocolitica* (Fig. 1), dead intracellular *Salmonella* (Table 1), or certain *Salmonella* mutants that are defective in intracellular replication (Fig. 3).

An intact microtubule network and a low intravacuolar pH are two factors needed for the production of lgp-filamentous structures in *Salmonella*-infected cells. Microtubules and related motor proteins support the traffic and distribution of vacuolar lysosomes (33, 34), as well as tubular lysosomes present in macrophages and brefeldin A-treated cells (21, 22, 28). Our data indicate that intracellular *Salmonella* uses the same host machinery to form and maintain its specialized filamentous structure. Recent data have shown that in macrophages *S. typhimurium* retards the acidification process of the bacteria-containing vacuole, which have lysosomal markers (9). A similar process may occur in epithelial cells. Once the vacuole that contains intracellular bacteria reaches a certain pH, the formation of lgp-containing filaments can be triggered by activation of bacterial genes induced at that pH. The time at which the filaments are formed (4–6 hr after invasion) may reflect the time at which the vacuole reaches the pH necessary to trigger their formation, presumably via viable intracellular bacteria.

Diverse drugs that affect formation of lgp filaments produced different results on intracellular replication. Nocodazole produced a decrease of 30–50% in the number of intracellular bacteria 6 hr after infection (Fig. 2). Since this drug disrupts the formation of lgp-containing filamentous structures (Fig. 2), we postulate that although the lgp filaments are used by *Salmonella* to proliferate, their absence could cause the bacteria to proliferate via a filament-independent process. Similar conclusions can be derived from experiments with vacuolar-acidification inhibitors (Fig. 4). Formation of lgp-rich filamentous structures requires a specific acidic vacuolar pH, but there was not a direct linkage between the absence of filamentous structures and the blockage in intracellular replication. Nevertheless, bafilomycin A_1, monensin, and chloroquine resulted in a 50% reduction in intracellular bacteria (Fig. 4), similar to that obtained with nocodazole. It seems that *Salmonella* may use several strategies to replicate within epithelial cells, one of which depends on the formation of the lgp-containing filaments. The irreversibility observed in bafilomycin A_1-treated cells probably reflects the fact that bafilomycin A_1 and weak bases produce different effects on the formation of vacuoles in eukaryotic cells (35, 36). Although weak bases block lgp-filament formation by their direct effect on the intravacuolar pH, they may also have favorable effects by causing osmotic swelling of intracellular vacuoles (36), providing an extra vacuolar space for bacteria to proliferate. No such effect is produced by bafilomycin A_1.

We are uncertain as to the precise function of the lgp-containing filamentous structures. The morphology of *Salmonella*-induced lgp filaments represents a major redistribution of lgps. These filamentous structures are not labeled by tracers added to the external medium, confirming the unique functional and morphological characteristics of these structures. Intracellular bacteria may also induce the formation of lgp-containing filaments to gain independence from the extracellular medium and at that time establish specific interactions with various organelles in the host cell, which provide the bacterium with nutrients. In conclusion, the formation of lgp-containing filaments in epithelial cells infected with *Salmonella* represents another example of exploitation of host cell machinery by pathogenic bacteria. These interactions will provide further information about the strategies used by invasive bacteria to grow within epithelial cells.

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