

# Targeting of *Salmonella typhimurium* to Vesicles Containing Lysosomal Membrane Glycoproteins Bypasses Compartments with Mannose 6-Phosphate Receptors

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**Abstract.** *Salmonella typhimurium* is an intracellular bacterial pathogen that remains enclosed in vacuoles (SCV) upon entry into the host cell. In this study we have examined the intracellular trafficking route of *S. typhimurium* within epithelial cells. Indirect immunofluorescence analysis showed that bacteria initiated fusion with lysosomal membrane glycoprotein (lgp)-containing compartments ~15 min after bacterial internalization. This process was completed ~75 min later and did not require microtubules. Cation-independent (CI)- or cation-dependent (CD)-mannose 6-phosphate receptors (M6PRs) were not observed at detectable levels in SCV. Lysosomal enzymes showed a different distribution in SCV: lysosomal-acid phosphatase (LAP) was incorporated into these vacuoles with the same kinetics as lgps, while cathepsin D was present in a low proportion (~30%) of SCV. Uptake experiments with fluid endocytic tracers such as fluorescein-dextran sulphate (F-DX) or horseradish-peroxi-

dase (HRP) showed that after 2 h of uptake, F-DX was present in ~75% of lgp-containing vesicles in uninfected cells, while only ~15% of SCV contained small amounts of the tracer during the same uptake period. SCV also showed only partial fusion with HRP-preloaded secondary lysosomes, with ~30% of SCV having detectable amounts of HRP at 6 h after infection. These results indicate that SCV show limited accessibility to fluid endocytic tracers and mature lysosomes, and are therefore functionally separated from the endocytic route. Moreover, the unusual intracellular trafficking route of *S. typhimurium* inside epithelial cells has allowed us to establish the existence of two different lgp-containing vesicles in *Salmonella*-infected cells: one population is separated from the endocytic route, fusogenic with incoming SCV and may arise from a secretory pathway, while the second involves the classical secondary or mature lysosomes.

**L**YSOSOMES are acidic organelles considered to be terminal degradative compartments in the endocytic route (Kornfeld and Mellman, 1989). Lysosomes contain hydrolytic enzymes and characteristic lysosomal membrane glycoproteins (lgps)<sup>1</sup> (von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989; Fukuda, 1991). Lgps

are heavily glycosylated proteins which are major components of the lysosomal membrane (Fukuda, 1991). No specific function has been assigned to these glycosylated proteins, although they may function in protecting lysosomal membranes from hydrolytic enzymes present in the lumen of this organelle. Recent studies have also shown that lgps are overexpressed on the cell surface of activated platelets (Febbraio and Silverstein, 1990) and on certain carcinoma cells (Hefferman et al., 1989; Saioth et al., 1992). These surface-expressed lgps can also mediate adhesion to activated endothelial cells via interactions with E-selectin (Sawada et al., 1993), or be involved in cell migration related to metastasis and altered glycosylation of the lgp (Garrigues et al., 1994).

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1. **Abbreviations used in this paper:** CD-M6PR, cation-dependent mannose 6-phosphate receptor; CI-M6PR, cation independent mannose 6-phosphate receptor; F-DX, fluorescein dextran sulphate, HRP, horseradish-peroxidase; LAP, lysosomal acid phosphatase; lgp, lysosomal membrane glycoprotein; M6P, mannose 6-phosphate; SCV, *Salmonella typhimurium*-containing vacuole.

To date, the most accepted model of lysosome biogenesis includes the passage of lysosomal components through the late endosome/prelysosomal compartment (Griffiths et al., 1988, 1990; Kornfeld and Mellman, 1989). This compartment is rich in mannose-6-phosphate receptors (M6PRs) (Griffiths et al., 1988, 1990; Rabinowitz et al., 1992).

M6PRs are not present in secondary or mature lysosomes (Geuze et al., 1988; Griffiths et al., 1988, 1990). Two different classes of these receptors exist: the cation-independent (CI)-M6PR (215-kD receptor) (reviewed by Kornfeld, 1992), and the cation-dependent (CD)-M6PR (46-kD receptor) (Stein et al., 1987; Dahms et al., 1987). These two receptors transport newly synthesized soluble enzymes containing M6P from the TGN to late endosomes via clathrin-coated vesicles (von Figura and Hasilik, 1986; Kornfeld, 1987, 1992; Kornfeld and Mellman, 1989).

Many intracellular pathogens interact with the prelysosomal and lysosomal compartments of phagocytic cells. Bacteria such as *Coxiella burnetii* persist and multiply in a phagolysosome of low pH (Maurin et al., 1992). *Chlamydia psittaci* and *Legionella pneumophila* block phagosome-lysosome fusion (Zeichner et al., 1983; Horwitz, 1983). This blockage was also reported for *Salmonella typhimurium* (Buchmeier and Heffron, 1991). *Mycobacterium tuberculosis* may reside in fused phagolysosomes, from which they may escape into nonfused vacuoles or the cytoplasm (McDonough et al., 1993). Some intracellular pathogens are able to attenuate the acidic pH in the compartment where they reside. Examples include *L. pneumophila* (Horwitz and Maxfield, 1984), *S. typhimurium* (Alpuche-Aranda et al., 1992), *Toxoplasma gondii* (Sibley et al., 1985), and *M. avium* (Sturgill-Koszycki et al., 1994). This latter study showed that *M. avium* resides in a mild acidic pH (6.0–6.5) phagosome which is rich in Igps yet lacks vacuolar-proton ATPase (Sturgill-Koszycki et al., 1994). *Leishmania mexicana* is also targeted to lysosomes in macrophages (Russell et al., 1992). Phagosomes containing the parasite have Igps and lysosomal enzymes at early times of the infection, and CI-M6PR accumulates progressively in these compartments, with large amounts of this receptor detected at 14 d after infection (Russell et al., 1992).

The phagocytic route has also been studied using a different experimental model: the uptake of inert latex beads by macrophages. Latex beads are targeted to a tubulo-reticular compartment which show markers corresponding to a late endosome/prelysosomal compartment, such as Igps and CI-M6PR, without fusing with secondary or mature lysosomes (Rabinowitz et al., 1992). Desjardins et al. (1994) have more recently reported an extremely dynamic process of acquisition and liberation of endocytic markers in the membrane of phagosomes containing latex beads. Markers such as the Igp lamp-2 and the small GTPase rab 7 are incorporated progressively in the compartment, while the amount of rab 5, a small GTPase involved in vesicular fusion in the early endosome, decreases over time.

Targeting of intracellular pathogens to lysosomes also occurs in non-phagocytic cells. Some examples are bacteria of the genera *Salmonella*, *Yersinia*, and *Chlamydia*, and eucaryotic parasites such as *T. gondii* and *Trypanosoma cruzi* (Joiner et al., 1990; Tardieux et al., 1992). *S. typhimurium* and *Y. enterocolitica*, enclosed in vacuoles during the entire intracellular stage (Falkow et al., 1992; Finlay and Falkow, 1989), are targeted to Igp-containing compartments upon invasion of epithelial cells (Garcia-del Portillo et al., 1993a,b). *S. typhimurium* has the unique capacity of altering the distribution of Igps in the host cell, inducing the formation of filamentous structures containing these glycosylated proteins (Garcia-del Portillo et al., 1993a,b). The eucaryotic parasite

*T. gondii* is targeted to lysosomes within CHO cells only if killed or obligated to enter the cell via a Fc-mediated pathway (Joiner et al., 1990). *Trypanosoma cruzi* is targeted to lysosomes of epithelial cells from the time it makes contact with the host cell surface and initiates the entry in the host cell (Tardieux et al., 1992).

Comparing all the information related to the intracellular trafficking routes used by intracellular pathogens, *S. typhimurium* appears to be the only pathogen capable of triggering within the host cell an extensive redistribution of Igps, which results in the formation of filaments containing these proteins which are connected to the *S. typhimurium*-containing vacuoles (SCV) (Garcia-del Portillo et al., 1993a,b). The appearance of these filaments correlates with the initiation of intracellular bacterial proliferation. For this reason, we have been interested in analyzing the intracellular trafficking route of SCV within epithelial cells, since it may provide information about the nature of host cell organelles that *S. typhimurium* fuses to, and in addition indicate whether intracellular bacteria follow a receptor-mediated uptake route. The data presented here illustrate the unconventional nature of the intracellular route used by *S. typhimurium*, including direct fusion of the pathogen to Igp-containing vesicles yet bypassing compartments containing CD-M6PR or CI-M6PR, and remaining functionally separated from the endocytic route.

## Materials and Methods

### Cell Culture

Human HeLa epithelial cells (ATCC CCL2; American Type Culture Collection, Rockville, MD) were grown in Minimal Dulbecco's Essential Medium (MEM) supplemented with 10% FBS. Cells were used for bacterial infection experiments between passage number 10 and 30.

### Bacterial Infection of Epithelial Cells

The *S. typhimurium* strains used were SL1344, a highly invasive mouse virulent wild type strain (Hoiseth and Stocker, 1981) and 3-11, an intracellular replication defective mutant unable to trigger the formation of filamentous lysosomes (Garcia-del Portillo et al., 1993a). Bacteria were grown overnight in LB medium in static, non-shaking, conditions at 37°C. HeLa cells were seeded on cover slips at a density of  $5-8 \times 10^4$  cells per well. The next day, cells were washed with PBS, pH 7.4, and fresh MEM/5% FBS medium was added. Bacteria were added at a ratio of 10:1 to epithelial cells, and incubated in a 5% CO<sub>2</sub> atmosphere for 15 min. Infected cells were further washed three times with PBS, and incubated in fresh MEM/5% FBS containing 100 µg/ml gentamicin. This antibiotic kills extracellular bacteria without affecting viability of intracellular bacteria (Finlay and Falkow, 1988). At different times after gentamicin addition, infected cells were fixed and processed for immunofluorescence labeling as described below.

### Antibodies

Polyclonal rabbit anti-bovine CI-M6PR, which cross-reacts with the human CI-M6PR (Dr. S. R. Pfeffer, Stanford University, Stanford, CA); affinity purified polyclonal rabbit anti-human CD-M6PR and polyclonal rabbit anti-human LAP (Dr. Kurt von Figura, Universität Göttingen, Göttingen, Germany); H4B4, monoclonal mouse anti-human h-lamp-2, originally produced by Dr. T. August (Johns Hopkins University, Baltimore, MD) and obtained from the Developmental Studies Hybridoma Bank; polyclonal rabbit anti-human cathepsin D (Scripps Research Laboratories, San Diego, CA); monoclonal IgA mouse anti-*S. typhimurium* lipopolysaccharide (LPS) (Dr. J. Slauch, Harvard Medical School, Boston, MA); and polyclonal rabbit anti-*S. typhimurium* LPS (Difco Laboratories, Detroit, MI) were used. Secondary antibodies used were: FITC-goat anti-mouse, FITC-goat anti-rabbit, FITC-goat anti-mouse IgA, Texas red goat anti-mouse,

Texas red goat anti-rabbit, lissamine-rhodamine goat anti-HRP, and Cy5 goat anti-mouse (all supplied by Jackson ImmunoResearch Laboratories Inc., Bio/Can Scientific, Mississauga, ON).

### Fluid Endocytic Tracers

HRP (2 mg/ml) (type II; Sigma Chem. Co., St. Louis, MO), fixable fluorescein-dextran-sulphate (F-DX, molecular weight 70,000 kD) (1 mg/ml) (Molecular Probes, Eugene, OR), and biotinylated *Datura stramonium* lectin (100 µg/ml) (ICN Biomedicals Canada Ltd., Mississauga, ON) were used as endocytic fluid tracers. *D. stramonium* lectin recognizes lactosaminyl residues present in Igps (Carlsson et al., 1988; Garcia-del Portillo et al., 1993a). For immunofluorescence studies, HRP was detected with a lissamine-rhodamine goat anti-HRP antibody, and biotin-*D. stramonium* lectin with FITC-streptavidin. To pre-load secondary lysosomes, epithelial cells were pulsed with HRP (2 mg/ml) for 4 h and chased for 16 h (Rabinowitz et al., 1992) before bacterial infection.

### Immunofluorescence Microscopy

Cells grown on coverslips were fixed with 3% paraformaldehyde for 20 min at room temperature, and after three washes with PBS, incubated serially with appropriate dilutions of primary, and secondary antibodies in a PBS/10% goat serum/0.2% saponin solution (60 min each incubation at room temperature). Cells were further washed three times with PBS and coverslips mounted on glass slides onto a drop of mounting medium (Sigma Chem. Co.). Indirect immunofluorescence was examined using a Zeiss Axioskop microscope under oil immersion. To detect simultaneously the distribution of three different markers, a fluorescein-labeled endocytic tracer (F-DX) and two additional different secondary antibodies conjugated to lissamine-rhodamine and Cy-5 were used. Cells were analyzed in a scanning confocal fluorescence microscope (MRC600; BioRad Laboratories, Cambridge, MA) for detection of the three fluorochromes as described (Paddock et al., 1993).

### Uptake of Latex Beads

To promote uptake of inert polystyrene latex beads (average diameter 1.1 µm; Sigma Chem. Co.) by non phagocytic cells, HeLa epithelial cells were grown in MEM/1% FBS for 16 h (serum-starving condition) prior to the addition of EGF (50 ng/ml) for 10 min. Latex beads were added (approximately at a ratio of 100 beads/epithelial cell) simultaneously with EGF and cells centrifuged for 10 min at 2,000 rpm in a centrifuge (GPKR; Beckman Instruments, Inc., Palo Alto, CA). This step facilitates contact of beads with cells at the time ruffles are formed in the membrane of the epithelial cells. The EGF-induced membrane ruffles promote uptake of inert particles into non-phagocytic cells (Francis et al., 1993). After the centrifugation step, medium containing beads was removed, and cells were washed extensively with PBS and incubated with fresh MEM/5% FBS without EGF and beads. Cells were fixed at different times after addition of beads and processed for immunofluorescence microscopy.

To determine the percentage of cell-associated latex beads contained in phagosomes with the different markers used, we first quantitated in the phase contrast image a minimum of 100 latex beads which remained associated to the cell after extensive washing. These beads were further observed by fluorescence microscopy to analyze the fraction of beads showing label. Considering that some of the cell-associated latex beads might be in extracellular locations (i.e., attached, but not internalized by the cell), the above percentage represented an underestimation of the real percentage of latex beads phagosomes containing the marker. Nevertheless, these values were optimal for showing relative differences regarding the incorporation of the diverse markers into latex bead-containing phagosomes versus SCV.

### Colocalization of Endocytic and Lysosomal Markers with SCV

To quantitate the percentage of SCV containing different endocytic fluid tracers or lysosomal markers, infected cells were visualized directly in the fluorescence microscope. First, a series of SCV were counted in the phase contrast image, followed by analysis of the fluorescent images which determined the numbers of those vacuoles that contained the corresponding marker. A minimum of 100 SCV were analyzed for each treatment and postinfection time. In the case of fluid endocytic tracers or soluble lysosomal enzymes such as cathepsin D, SCV were considered positive for the pres-

ence of the marker when they contained detectable amounts of it, even if the intensity of the label was weak (see Figs. 4 and 8 for examples).

Since the quantitation of vesicles in uninfected cells by microscopic observation was more difficult than with the SCV due to the difference in size, quantitation of vesicles containing diverse markers in uninfected cells was performed on a Macintosh Quadra 840AV computer using the public domain NIH Image program (written by Wayne Rasband at the U.S. National Institutes of Health and available from the internet by anonymous ftp from zippy.nimh.nih.gov.). This program permits quantitation of "dots" present in the image obtained from the scanning confocal microscope (MRC600; BioRad) (for each of the markers analyzed), as well as the numbers of dots which are present in more than one label ("colocalization").

### Ammonium Chloride and Nocodazole Treatments

Ammonium chloride (25 mM) was used to deplete M6PRs from the sorting site in the TGN (Brown et al., 1986). In control experiments, we determined by immunofluorescence analysis that it was necessary to incubate cells with 25 mM NH<sub>4</sub>Cl for 4 h to ensure a change in the distribution of M6PRs in HeLa epithelial cells. Therefore, in all experiments with bacteria-infected cells or latex beads uptake, a pretreatment of 4 h with the lysosomotropic weak base was done before bacteria or latex beads addition. The weak base was maintained in the tissue culture medium throughout the experiment.

To disrupt microtubules, epithelial cells were preincubated for 1 h at 4°C followed by 1 h at 37°C with 10 µg/ml of nocodazole (Sigma Chem. Co.) before addition of bacteria and/or fluid endocytic tracer. The drug was maintained throughout the period of incubation with bacteria or endocytic tracer. We have previously shown that such treatments destroy the microtubule network (Garcia-del Portillo et al., 1994b).

### Results

#### *S. typhimurium* Is Targeted to Igp-containing Vesicles yet Bypasses Compartments Containing Mannose 6-Phosphate Receptors

Our first analysis focused on characterization of the SCV in

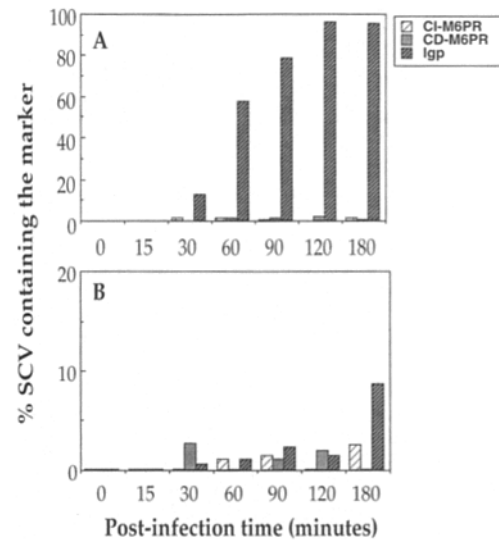
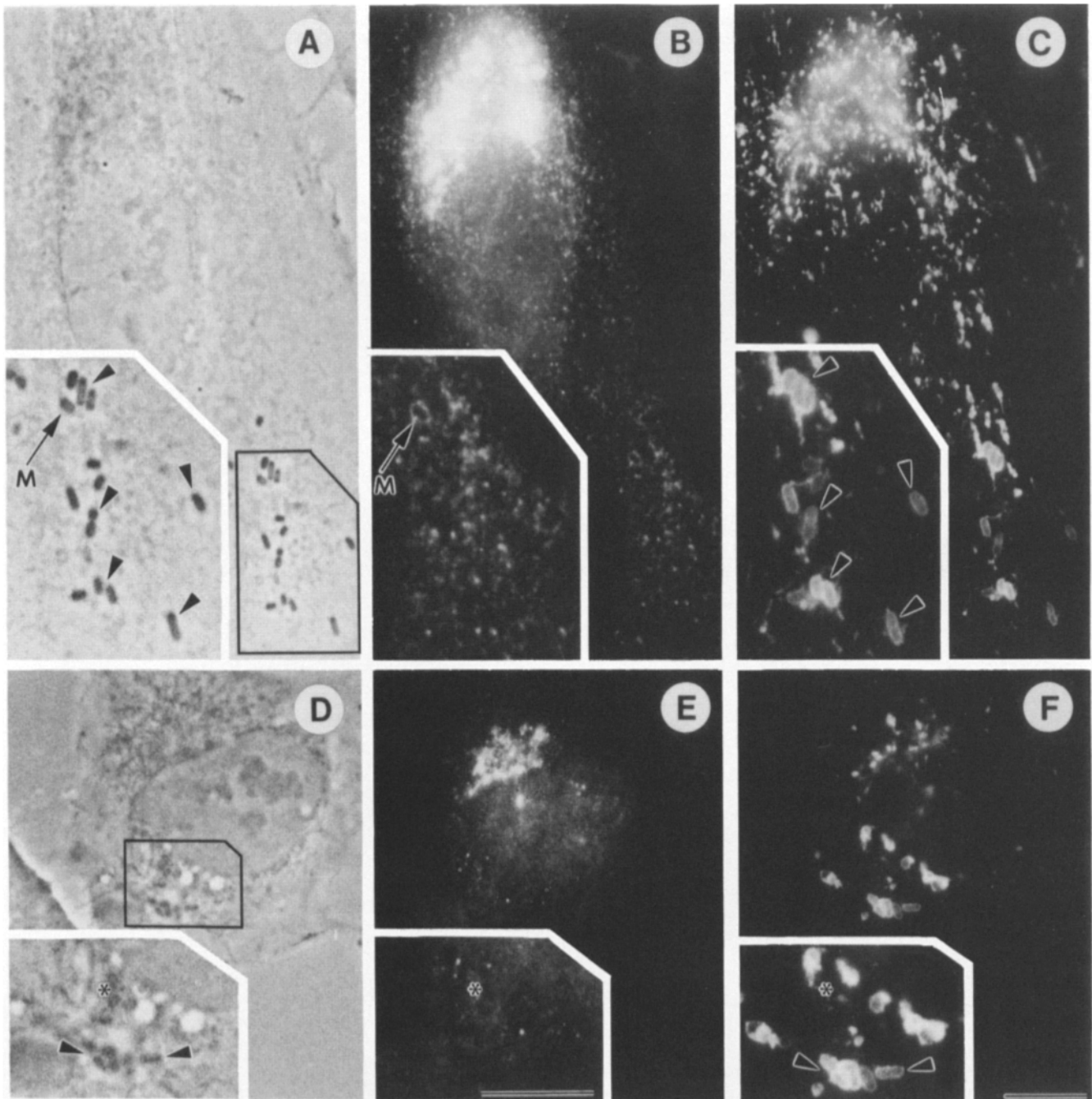


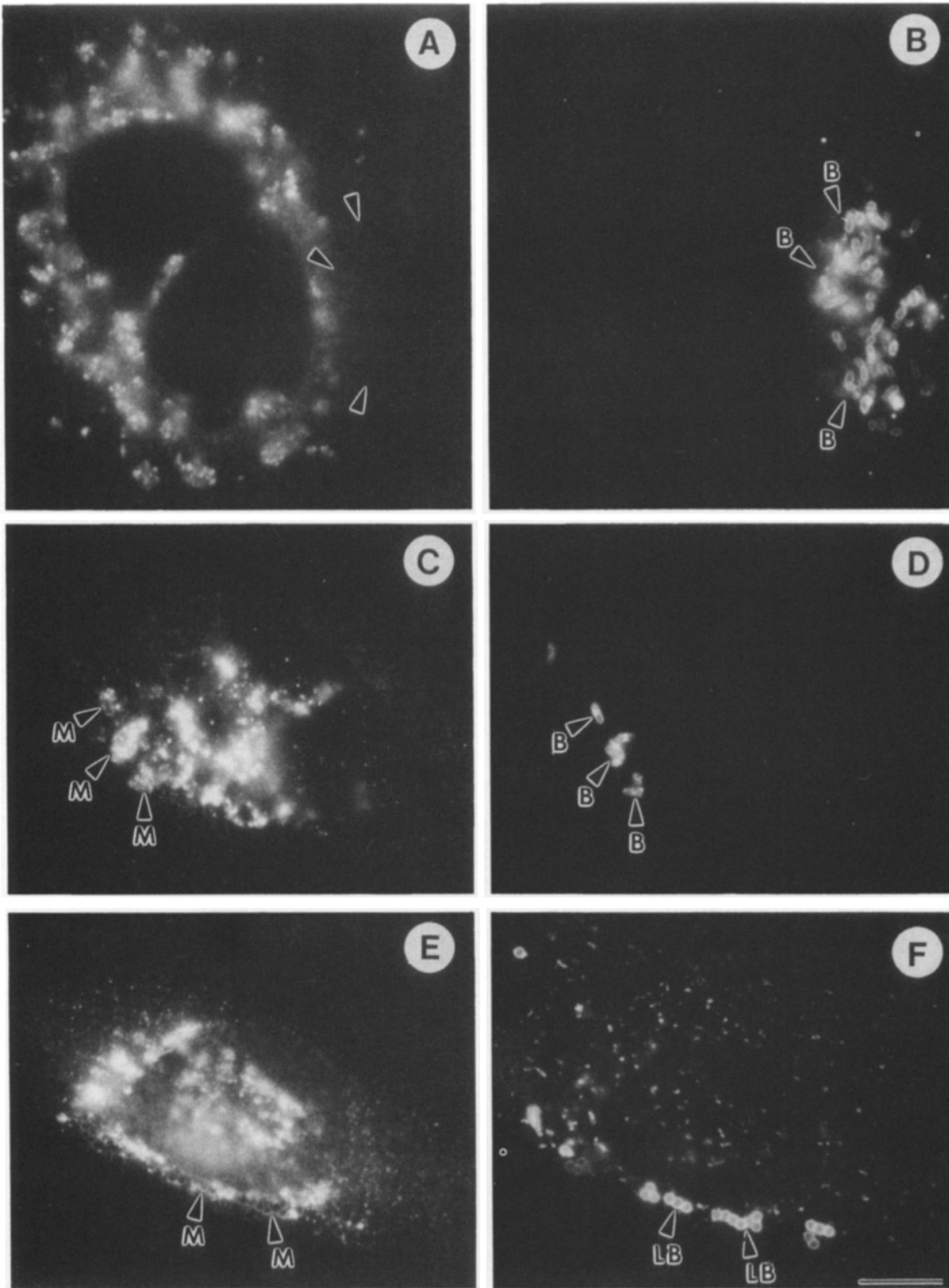
Figure 1. Kinetics of the acquisition of Igps and M6PRs by SCV. HeLa epithelial cells were non-treated (A), or treated with 25 mM NH<sub>4</sub>Cl for 4 h (B), before infecting with *S. typhimurium* (15 min), washed and further incubated in fresh medium with gentamicin (100 µg/ml). At the different postinfection times shown, cells were fixed and processed for indirect immunofluorescence, analyzing distribution of: CI-M6PR, CD-M6PR, and Igp in SCV. Note the different scale in the ordinates of graphics A and B. Percentage of SCV with the markers was calculated as described in Materials and Methods. Data are the average of two different experiments.



**Figure 2.** Mannose-6-phosphate receptors are not abundant in SCV. HeLa epithelial cells were infected with *S. typhimurium* (15 min) and processed at 90 min after infection for double indirect immunofluorescence analysis. Distribution of: (B) CD-M6PR, (E) CI-M6PR, and (C and F) lgp. (A and D) Phase contrast images of the cells shown in B and C and E and F, respectively. Insets are enlargements of areas containing groups of bacteria localized with markers. Note the prominent lgp label in SCV (arrowheads), and the absence of CD-M6PR or CI-M6PR in these vacuoles. In the cell shown in A-C a rare single bacterium is labeled with CD-M6PR (arrow, M). In the cell shown in D-F a group of SCV do not contain either lgp or CI-M6PR (asterisk). Bar, 5  $\mu\text{m}$ .

HeLa epithelial cells. The time that these cells were infected with bacteria was limited to 15 min to attempt to synchronize the formation of new SCV. After this time, non-internalized bacteria were washed away with buffer, and fresh medium containing gentamicin (100  $\mu\text{g}/\text{ml}$ ) was added to prevent further bacterial uptake (Finlay and Falkow, 1988). Infected cells were then fixed at different postinfection times and processed for immunofluorescence labeling with antibodies

against the human lgp, h-lamp2. Fig. 1 A shows that the time course of formation of lgp-containing SCV is progressive during the first 75 min after bacterial entry (90 min after infection). 15 min after gentamicin addition (30 min after infection),  $\sim 15\%$  of SCV already contain lgp, and at 120 min after infection  $>95\%$  of SCV contained lgps. The process of *S. typhimurium* appearance in lgp-containing compartments, which was almost complete 75 min after bacterial internal-



**Figure 3.** Distribution of CD-M6PR in *S. typhimurium*-infected cells pretreated with ammonium chloride. HeLa epithelial cells were treated with 25 mM NH<sub>4</sub>Cl for 4 h prior to bacterial infection (15 min). Cells were fixed at 120 min after bacterial addition, and labeled for double indirect immunofluorescence with rabbit polyclonal anti-CD-M6PR antibody (A and C), and mouse monoclonal IgA anti-*S. typhimurium* LPS (B and D). (A and B) Cell in which bacteria do not colocalize with CD-M6PR. This distribution was observed in most of the infected cells. (C and D) Colocalization of bacteria (marked as B) in vacuoles containing CD-M6PR (marked as M). This distribution was observed in ~2% of the total of infected cells screened. Similar results were obtained with the CI-M6PR. (E and F) Cell not treated with NH<sub>4</sub>Cl, which ingested latex beads via activation with EGF. Cells were fixed at 120 min after ingestion of the beads and labeled for CD-M6PR (E) and IgG (F). Note the colocalization of CD-M6PR (marked as M) and IgG in vacuoles containing the beads (marked as LB). Bar, 5  $\mu$ m.

Table I. Kinetics of Incorporation of M6PRs and lgp to Latex Bead Phagosomes in HeLa Epithelial Cells

Treatment*	Time after bead addition	Percent cell-associated latex beads with the marker†			Percent latex bead phagosomes with both markers	
		CI-M6PR	CD-M6PR	lgp	CI-M6PR/lgp	CD-M6PR/lgp
	<i>min</i>					
None	60	12	5	15	82	34
	120	8	12	28	28	43
	180	8	20	61	13	33
NH <sub>4</sub> Cl	60	0.4	2	7	—	—
	120	1	2	9	—	—
	180	3	3	12	—	—

\* NH<sub>4</sub>Cl 25 mM was added to the cell 4 h before beads and EGF.

† Calculated as described in Materials and Methods.

ization (Fig. 1 A), is considerably shorter than the estimated 2 h required for a fluid endocytic tracer to be delivered to lgp-rich secondary lysosomes (Rabinowitz et al., 1992).

We next asked whether SCV contained M6PRs, a marker of the late endosome/prelysosomal compartment (Griffiths et al., 1988, 1990; Rabinowitz et al., 1992). Bacteria-infected cells were fixed at different postinfection times, and labeled for CD-M6PR or CI-M6PR. Fig. 1 A shows that SCV do not contain appreciable amounts of either M6PR during the period of time analyzed (0–180 min after infection), with only ~2% of these vacuoles containing detectable CD- or CI-M6PR. Fig. 2 shows an example of an immunofluorescence image of *S. typhimurium*-infected cells at 90 min after infection, in which SCV lack abundant CD- or CI-M6PRs (Fig. 2, B and E), yet contain large amounts of lgp (Fig. 2, C and F). In one of the cells shown (Fig. 2 B), a rare single bacterium is labeled with CD-M6PR, but lacks lgp, demonstrating that the indirect immunofluorescence method is sensitive enough to detect the presence of M6PRs in SCV. In control experiments to measure the amount of extracellu-

lar bacteria, we determined that at any post infection time only 0.2% of cell-associated bacteria remained accessible to anti-*S. typhimurium* LPS antibodies when cells were labeled in the absence of detergent (data not shown). This low amount of extracellular bacteria gave validity to the above percentages of SCV containing different markers, since cell-associated bacteria analyzed by our counting process corresponded almost entirely to intracellular bacteria.

We next used the method described by Brown et al. (1986) to block transit of M6PRs from the late endosome to TGN by incubating the cells with the lysosomotropic weak base ammonium chloride. Under these conditions, the bulk of M6PRs is localized in expanded vesicular structures and in the late endosome/prelysosomal compartment (Brown et al., 1986). If the intracellular route of SCV includes a transient passage or fusion with M6PR-containing compartments, these receptors should become more easily detectable in SCV in NH<sub>4</sub>Cl-treated cells. Preincubation of HeLa epithelial cells with the weak base did not increase CD-M6PR labeling in SCV (Fig. 1 B). More than 95% of SCV were

Table II. Accessibility of SCV to Fluid Endocytic Tracers

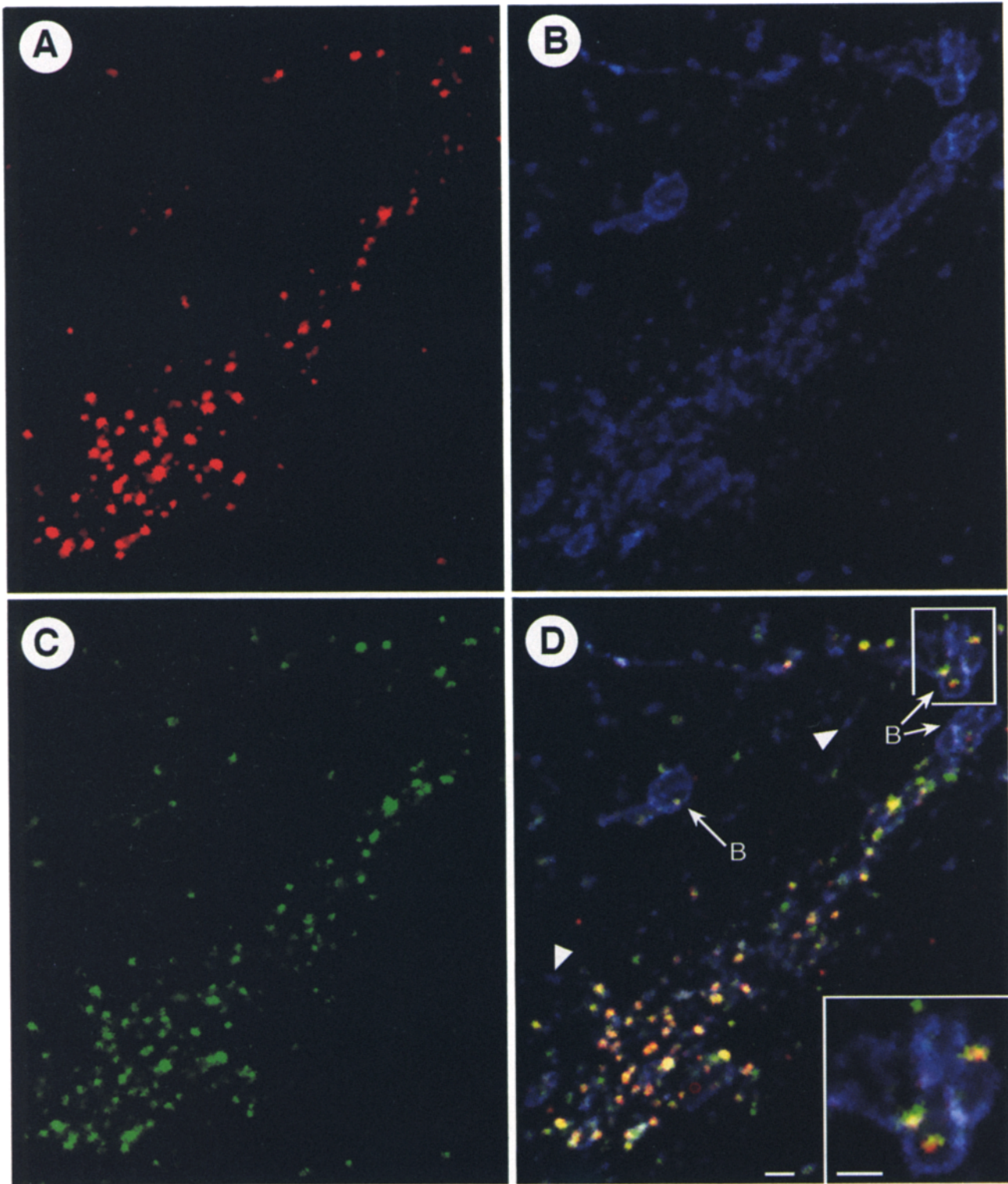
Compartment	F-DX70 uptake	Bacterial infection	Colocalization with markers		
			F-DX70	lgp	HRP
	<i>h</i> *	<i>h</i> †		%§	
lgp-containing vesicles (HRP preloaded in pulse/chase, uninfected cells)	None	—	—	—	78
	2	—	72	—	81
SCV (no HRP added)	0.5–2.5	2.5	19	95	—
	0.5–4.5	4.5	39	91	—
	0.5–6.5	6.5	41	84	—
	0.5–2.5	6.5	70	96	—
	2–4	6	52	88	—
	4–6	6	12	95	—
SCV (HRP-preloaded cells)†	—	2	—	96	22
	—	4	—	98	31
	—	6	—	98	34

\* Used at 1 mg/ml final concentration. In *S. typhimurium*-infected cells, periods of uptake indicated are postinfection times.

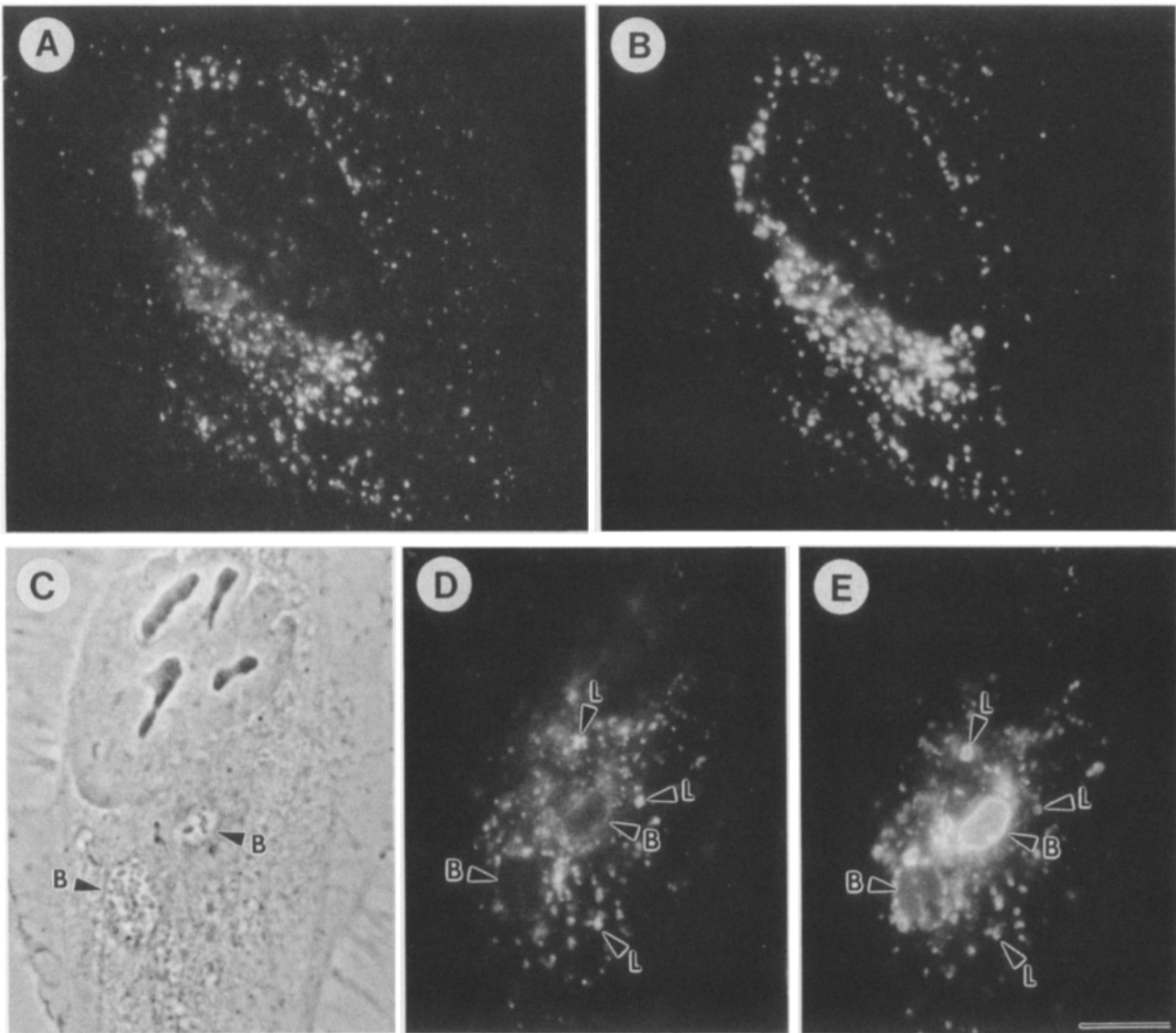
† HeLa epithelial cells were incubated with bacteria for 15 min. After washing, infection was continued in fresh medium containing gentamicin (100 µg/ml). Indicated are times at which cells were fixed and processed for immunofluorescence analysis.

§ Percentage of lgp-containing vesicles with HRP or F-DX70 in uninfected cells was analyzed using the NIH Image program. In infected cells, percentages of SCV with the endocytic tracers were calculated by direct visualization of cells in the fluorescence microscope.

|| HeLa epithelial cells were pulsed with HRP (2 mg/ml) for 4 h and chased overnight.



**Figure 4.** SCV containing Igps are not readily accessible to fluid endocytic tracers. HeLa epithelial cells were pulsed with HRP (2 mg/ml) for 4 h and chased overnight to label secondary lysosomes. Cells were further infected with *S. typhimurium* (15 min) and 15 min later fixable fluorescein-dextran-sulphate (F-DX, 1 mg/ml) was added to the medium. Cells were fixed 4 h after F-DX addition, and processed for scanning confocal immunofluorescence microscopy. (A) Distribution of HRP (red), detected with anti-HRP-lisamine-rhodamine conjugated antibody; (B) distribution of Igp (blue), detected with mouse monoclonal anti-Igp and anti-mouse-Cy5 conjugated antibodies in the same cell; (C) distribution of fluorescein-dextran-sulphate (F-DX, green) in the same cell; (D) distribution pattern of the three markers merged. SCV (arrows, blue, marked as B) show only small amounts of both endocytic tracers (see inset), while most HRP-containing vesicles show prominent label of F-DX (yellow). Also, note the presence of Igp-containing vesicles which do not contain any fluid tracer (arrowheads). Bar, 1  $\mu$ m.



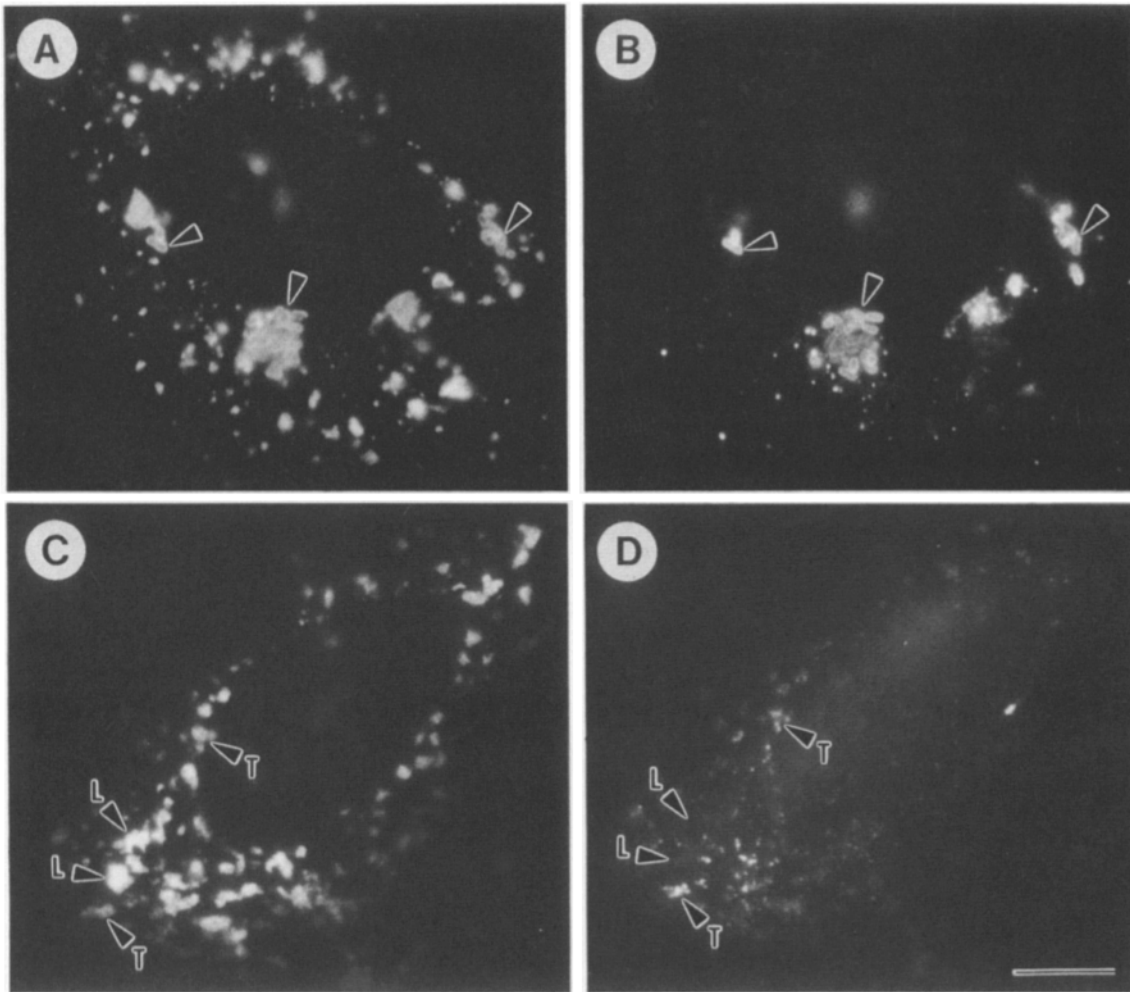
**Figure 5.** Impaired delivery of the lectin *Datura stramonium* to SCV containing Igps. (A and B) Control uninfected HeLa epithelial cells in which biotinylated *D. stramonium* lectin (100  $\mu\text{g/ml}$ ) was added and incubated for 4 h. (C-E) *S. typhimurium*-infected epithelial cell. Bacteria were added for 15 min to the medium, and 15 min later biotinylated *D. stramonium* lectin (100  $\mu\text{g/ml}$ ) was added to the medium and incubated for 6 h. (A and D) Distribution of the lectin detected with FITC-streptavidin; (B and E) distribution of IgG, detected with mouse monoclonal anti-IgG and anti-mouse-Texas red antibodies. (C) Phase contrast image of the cell shown in D and E. Note that in the control uninfected cell nearly all the IgG-containing vesicles are accessible to the lectin, while the SCV (marked as B) remain unlabeled or only faintly labeled with the lectin. Vacuolar lysosomes (marked as L) show prominent label with the lectin in the infected cell. Bar, 5  $\mu\text{m}$ .

devoid of detectable label for CD-M6PR under these experimental conditions (Figs. 1 B and 3, A and B), although in  $\sim 1$ –3% of the SCV the receptor was observed in this compartment (Figs. 1 B and 3, C and D). Analogous results were obtained with CI-M6PR (Fig. 1 B). Fusion of SCV with IgG-containing compartments was also blocked by the ammonium chloride treatment (Fig. 1 B), confirming previous findings that there is an inhibitory effect of this compound on the phagosome-lysosome fusion process (Gordon et al., 1980).

The above results suggested that the majority of SCV bypass M6PR-containing compartments during their fusion with IgG-containing vesicles. This is in contrast with previous reports that showed that in macrophages and non-phago-

cytic cells phagosomes containing other particulate material, such as latex beads, also contained CI-M6PR (Rabinowitz et al., 1992; Jahraus et al., 1994). When HeLa epithelial cells were treated with EGF to facilitate bead uptake (Francis et al., 1993), latex bead-containing vacuoles were labeled with both M6PRs and Igps (Fig. 3, E and F). The percentage of cell-associated latex beads containing M6PRs were  $\sim 10\%$  for CI-M6PR and  $\sim 10$ –20% for CD-M6PR (Table I). In contrast to the situation during bacterial infection, it is possible that a fraction of cell-associated latex beads were not internalized. This would result in an underestimation of the percentage of phagosomes containing these markers. Indeed, we observed that a large fraction of cell-associated latex beads ( $\sim 40\%$ ) remained without IgG staining at 180 min





**Figure 6.** *S. typhimurium* is targeted to IgG-containing compartments in the presence of nocodazole. HeLa epithelial cells pretreated with nocodazole (2 h, 10  $\mu\text{g/ml}$ ) were infected with *S. typhimurium* SL1344 for 15 min, and fixed at 4 h after infection. Nocodazole was maintained in the medium throughout. (A) Distribution of IgG detected with mouse monoclonal anti-IgG and anti-mouse Texas red antibodies; (B) label of intracellular bacteria in the same cell using rabbit polyclonal anti-*S. typhimurium* LPS and anti-rabbit-FITC-conjugated antibodies. Arrowheads show location of SCV containing IgG. (C and D) Uninfected cell pretreated with nocodazole for 2 h, then fluorescein-dextran sulphate (F-DX) was added to the medium and incubated for another 4 h. Nocodazole was maintained in the medium throughout. (C) Distribution of IgG; (D) distribution of F-DX in the same cell. Some IgG-containing vesicles show label with the tracer (marked as T) while large condensed IgG-containing vesicles are not accessible to the endocytic tracer (marked as L). Bar, 5  $\mu\text{m}$ .

after bead addition (Table I), implying a probable extracellular location for this fraction of beads. We estimated that the fraction of latex beads containing both IgG and CI-M6PR ranged from 82% at 60 min after bead addition to 13% at 180 min (Table I). Notably, the fraction of beads containing both IgG and CD-M6PR remained constant at all times measured ( $\sim 30\text{--}40\%$ ). Analogous calculations in bacteria-infected cells gave a value of 2% SCV IgG positive showing label for M6PRs at 180 min after infection (Fig. 1 A). Therefore, we conclude that differences may exist in the intracellular targeting of SCV when compared to latex bead-containing phagosomes. Pretreatment of cells with ammonium chloride blocked acquisition of M6PRs and IgGs by phagosomes containing latex beads (Table I), as previously described for SCV (Fig. 1 B).

#### ***SCV Have Limited Accessibility to Fluid Endocytic Tracers and Mature Lysosomes***

Since SCV did not contain detectable amount of M6PRs, it is possible that vacuoles containing these bacteria are separated from the endocytic route. To test this hypothesis, we added endocytic fluid tracers such as fluorescein-dextran (F-DX) to infected cells. We quantified the proportion of SCV containing detectable amounts of this tracer. In this set of experiments, F-DX was added 15 min after SCV formation to prevent simultaneous uptake of bacteria and tracer by the epithelial cell. The results obtained with bacteria-infected cells were compared with analogous uptake times in uninfected cells. As shown in Table II, in uninfected cells  $\sim 75\%$  of IgG-containing vesicles were accessible to F-DX after 2 h

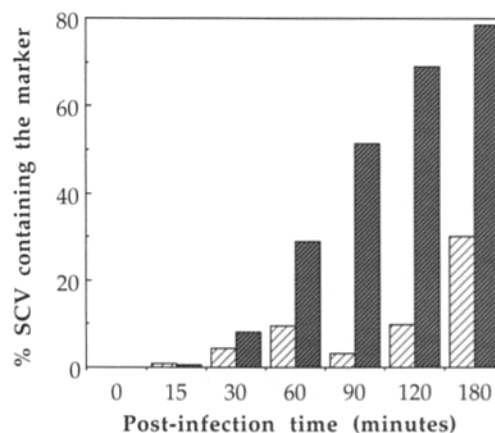
of uptake with the tracer. This is in contrast to that obtained in *S. typhimurium*-infected cells: only 19% of SCV contained the tracer after 2 h of uptake. This proportion was similarly low when F-DX uptake (2 h) was done at a different postinfection time (11%, when F-DX added at 4 to 6 h after infection). In the case where the tracer was detectable in SCV, only small amounts were seen, although SCV contained large amounts of Igps at these postinfection times (Fig. 4). A relative increase in the proportion of SCV containing the endocytic tracer occurred with longer uptake periods (4 or 6 h) or with 2 h uptake followed by 2–4 h of additional chase (Table II). On average, 50–70% of SCV contained detectable amounts of the tracer after these long uptake periods. Nevertheless, the low amounts of F-DX observed in the SCV (Fig. 4 D) was indicative that massive fusion of SCV with compartments of the endocytic route did not occur. All these data suggest that most SCV are not directly linked to the major endocytic route.

The percentage of fusion of SCV with HRP-preloaded secondary lysosomes was also determined. As shown in Table II, SCV showed limited fusion with HRP-preloaded secondary lysosomes, with only ~30% of SCV containing HRP at 2 h after infection. This proportion did not change with longer postinfection times up to 6 h. These results contrast those obtained with Igp acquisition by SCV, since at 2 h after infection ~95% of SCV contained Igps (Fig. 1, Table II). Therefore, SCV are apparently targeted to Igp-containing vesicles that, although lacking M6PRs, cannot be classified as secondary or mature lysosomes. When an analogous HRP-preloading of secondary lysosomes was performed in uninfected cells, 80% of Igp-containing vesicles contained HRP (Table II). This result suggests the existence of Igp-containing compartments not connected directly to the endocytic route (representing the other 20%), and this population of Igp-containing vesicles may be involved in fusing with SCV (Fig. 4, C and D). In *S. typhimurium* infected cells the HRP-preloaded secondary lysosomes were fully accessible to incoming F-DX added during the bacterial infection (Fig. 4, A and B).

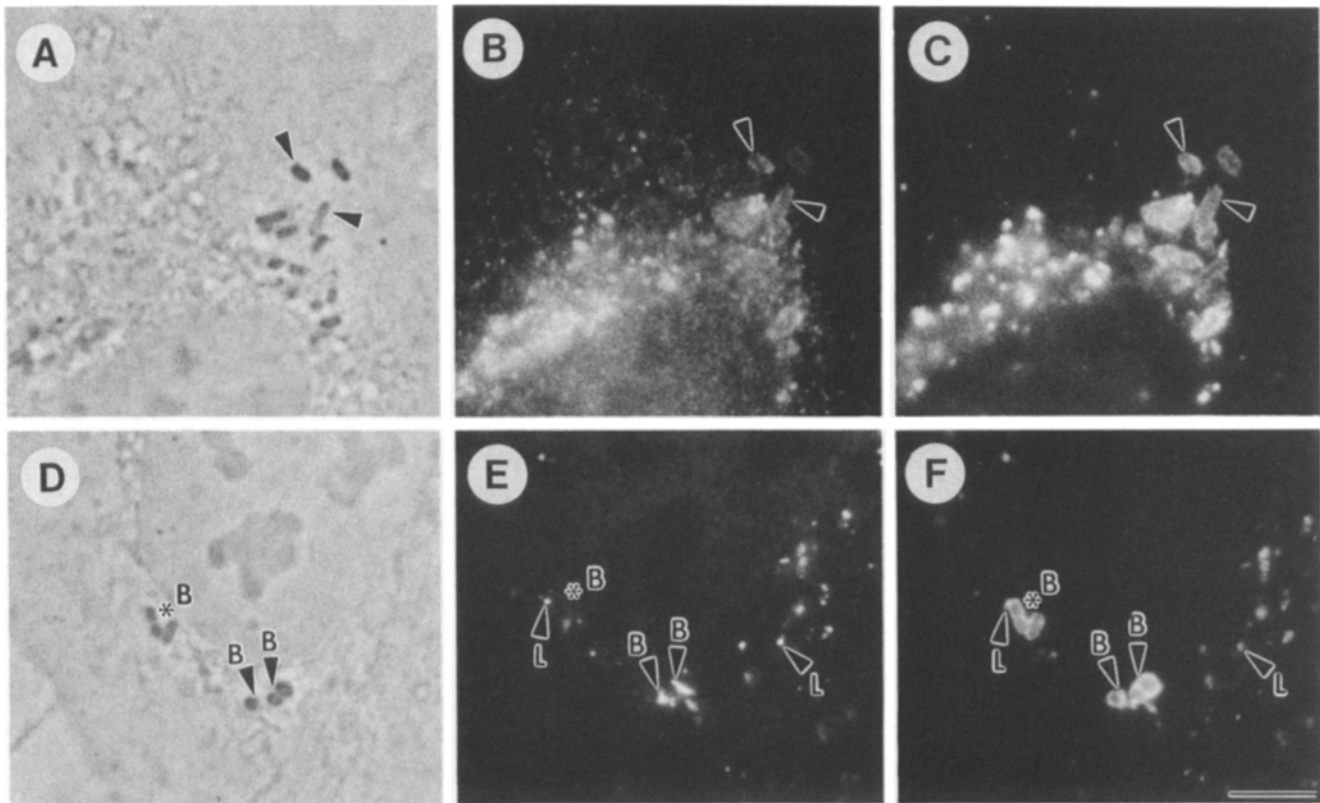
To substantiate the results obtained with the fluid endocytic tracers, we determined whether the uptake of a third tracer, which binds to Igps, would provide similar results. Biotinylated *Datura stramonium* lectin was used for this purpose. This lectin binds specifically to lactosiminy residues, which are present in Igps (Carlsson et al., 1988). As with the fluid endocytic tracer F-DX, *D. stramonium* lectin was added to the medium 15 min after bacteria internalization. Fig. 5 shows that in uninfected cells there is a complete overlap between the distribution of the internalized lectin and Igps after 4 h of uptake, i.e., the incoming lectin is delivered to most of the Igp-containing vesicles (Fig. 5, A and B), while in *S. typhimurium*-infected cells the lectin labeled vacuolar lysosomes but was absent or present only in low amounts of the SCV, even when long periods of lectin uptake (6 h) were used (Fig. 5, C–E). As we previously reported, this lectin intensely labeled Igps in the SCV if added to fixed and permeabilized infected cells (Garcia-del Portillo et al., 1993a). The lack of delivery of *D. stramonium* lectin to SCV during bacterial infection provides additional evidence for functional separation between SCV and the endocytic route.

### Targeting of SCV to Igp-containing Vesicles Does Not Require Microtubules

Several reports have shown that transfer of fluid and membrane material to deeper stages of the endocytic route is dependent on the integrity of microtubules. The transport step from the early endosome to the late endosome/prelysosome compartment is mediated by carrier vesicles that move along microtubules (Kornfeld and Mellman, 1989). It is also known that movement of lysosomes is supported by the microtubule network (Matteoni and Kreis, 1987; Swanson et al., 1987b; Hollenbeck and Swanson, 1990), and that transfer of Igp to compartments containing latex beads is microtubule dependent (Desjardins et al., 1994). We then tested whether the incorporation of Igp into SCV would have the same requirement for microtubules. HeLa epithelial cells were preincubated with nocodazole (2 h) and further infected with *S. typhimurium*. At early postinfection times (60 min), a small number of SCV were labeled with Igp, while at later times (from 2 h after infection), a large number of SCV contained Igp. Fig. 6 (A and B) shows the distribution of Igps at 4 h after infection in a *S. typhimurium*-infected cell, and the colocalization of this marker in all the SCV. We further performed a control experiment in which the endocytic fluid tracer F-DX was added to the medium of cells treated with nocodazole for the same period of time (4 h). Under these conditions, most of the Igp-containing vesicles of the cell, which were clustered due to the effect of microtubule depolymerization, contained no tracer (Fig. 6, C and D), indicating that normal endocytosis was disrupted. These results again suggest that targeting of SCV to Igp-containing vesicles occurs via a different pathway than incoming material that is delivered to secondary lysosomes connected with the endocytic route (Desjardins et al., 1994).



**Figure 7.** Delivery of lysosomal enzymes to SCV. HeLa epithelial cells were infected with *S. typhimurium* (15 min) and further incubated in fresh medium with gentamicin (100  $\mu$ g/ml). At the different postinfection times shown, cells were fixed and processed for indirect immunofluorescence, and the distribution of cathepsin D (▨), or LAP (■) was analyzed in SCV. The percentage of SCV containing the markers was calculated as described in Materials and Methods. Data are average of two different experiments. Note that LAP is incorporated into SCV with the same kinetics as Igp (see Fig. 1) while cathepsin D is present in only a small percentage of these vacuoles.

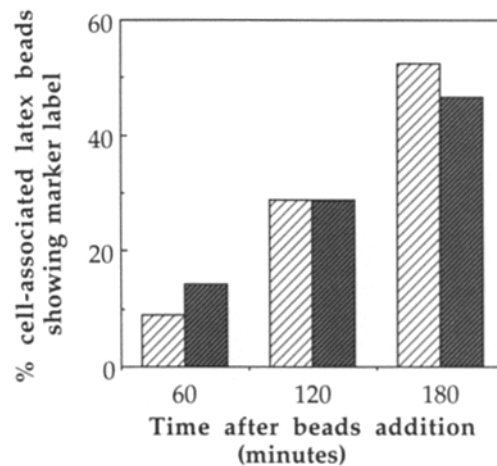


**Figure 8.** Localization of lysosomal enzymes in SCV. HeLa epithelial cells were infected with *S. typhimurium* (15 min) and fixed at 90 min (LAP) or 120 min (cathepsin D) and processed for indirect immunofluorescence. (B) Distribution of LAP; (C and F) distribution of lgp; (E) distribution of cathepsin D. A and D are the corresponding phase contrast images of the cells shown in B and C and E and F, respectively. lgp and LAP colocalize in SCV, while cathepsin D is present in a few SCV (marked as B), but absent from others (asterisk). In contrast, cathepsin D colocalizes with lgp in all vacuolar lysosomes (some examples marked as L). Bar, 5  $\mu$ m.

### Differential Presence of Lysosomal Enzymes in SCV and Latex Beads Phagosomes

Some lysosomal enzymes are transported to secondary lysosomes via M6PRs while others are targeted to this compartment via a M6PR-independent pathway (von Figura and Hasilik, 1986). We analyzed the distribution of cathepsin D, a lysosomal enzyme which contains M6P residues (Gieselmann et al., 1983), and LAP, which traffics to secondary lysosomes without recognition of M6PRs, in *S. typhimurium* infected epithelial cells. LAP was incorporated into SCV with similar kinetics as lgps (Fig. 7). However, cathepsin D was only present in  $\sim$ 20–30% of SCV (Fig. 7). Fig. 8 (A–C) illustrates a cell in which SCV contain both lgp and LAP. In contrast, cathepsin D is absent from some SCV and present in others (Fig. 8, D–F). The low proportion of SCV containing cathepsin D was similar at all postinfection times analyzed.

We also determined the acquisition of these lysosomal markers by latex bead-containing phagosomes. This type of compartment acquired both lap and cathepsin D, with  $\sim$ 50% of cell-associated latex beads showing label for both enzymes (Fig. 9). In all our microscopic observations these two enzymes colocalized in latex bead phagosomes containing lgps. These data contrast the lack of cathepsin D in SCV (Figs. 7 and 8). Therefore, we conclude that latex bead-con-



**Figure 9.** LAP and cathepsin D are equally present in latex bead-containing phagosomes. Serum-starved HeLa epithelial cells were activated with EGF (50 ng/ml) to induce latex bead internalization as described in Materials and Methods. At the times shown after beads addition, cells were fixed and labeled for cathepsin D (▨), and LAP (■). Note the similar percentage of cell-associated latex beads showing label for both lysosomal enzymes.

**Table III. SCV with the Intracellular Replication Mutant 3-11 Has a Similar Targeting Route as the Wild Type Strain**

Relevant phenotype	<i>S. typhimurium</i> strain	
	SL1344 (wild type)	3-11 (mutant)
Targeting to lgp-vesicles*	++ (90-120 min)	++ (90-120 min)
Fusion with CI- or CD-M6PR-containing compartments†	—	—
Accessibility to endocytic tracers (DX-70)‡	—	—
Percent SCV containing cathepsin-D (2 h/3 h after infection)	28.0/43.5	42.0/32.1

\* Time in brackets represents the postinfection time at which >95% of SCV have fused with lgp-containing vesicles.

† Tested from 0 to 180 min after infection.

‡ <20% SCV contained DX-70 up to 4 h uptake after bacterial internalization.

taining phagosomes simultaneously acquire M6PR-dependent and -independent enzymes such as cathepsin D and LAP (Fig. 9). Both the incorporation of these lysosomal enzymes to latex bead phagosomes and the acquisition of LAP by SCV were blocked by pretreating the cells with ammonium chloride (data not shown).

### ***Intracellular Replication Defective S. typhimurium* Mutants Do Not Have Apparent Differences in the Intracellular Targeting Route to lgp-containing Vesicles**

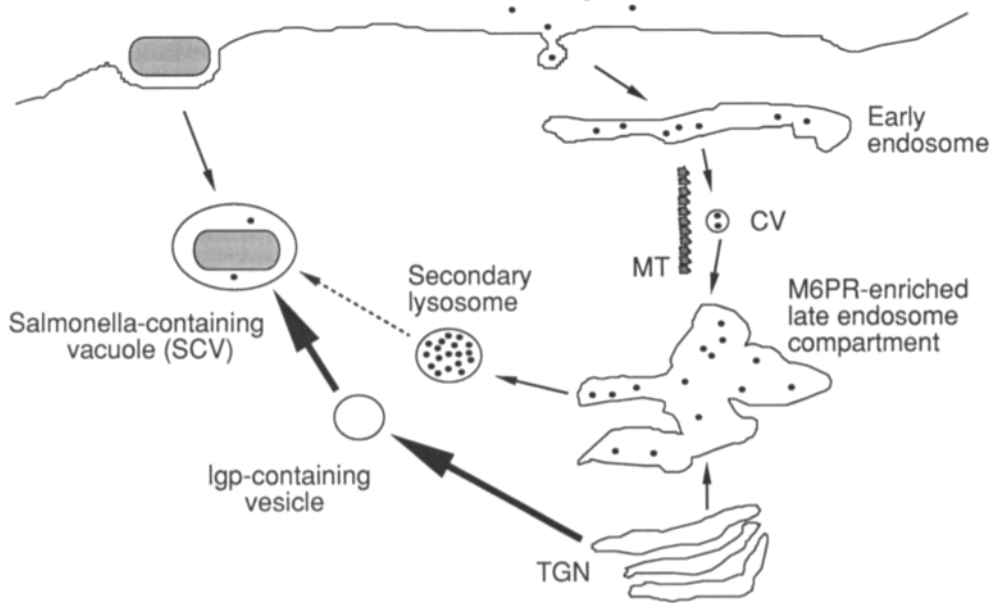
Certain *S. typhimurium* intracellular replication defective mutants are unable to trigger the formation of filamentous lysosomes (Garcia-del Portillo et al., 1993a). This phenotype might be caused by differences in the biogenesis of the SCV. To assess this possibility, we used a representative mutant, the prototrophic strain 3-11 (Garcia-del Portillo et al., 1993a), to analyze biogenesis of SCV in HeLa epithelial cells infected with this mutant. Table III summarizes several phenotypic properties tested with this mutant. The kinetics of incorporation of lgps to SCV containing mutant 3-11 was similar to that described with the wild type strain, with >90% of SCV exhibiting lgp label at 120 min after infection. No detectable amounts of CD-M6PR or CI-M6PR were observed in SCV containing this mutant. Other properties shared by SCV containing 3-11 and the parental wild type strain include the lack of accessibility of fluid endocytic tracers such as F-DX when added after bacterial internalization (up to 4 h uptake of the tracer), and the relatively low percentage of SCV showing detectable levels of cathepsin D, with values close to 30–40% of total number of SCV at 2 and 3 h after infection. In conclusion, the mutation associated with 3-11, which impairs this strain for the capacity to induce filamentous structures containing lgps, does not seem to alter the initial stages in the biogenesis of SCV containing this mutant, which apparently bypasses M6PRs-containing compartments and maintains a functional separation from the endocytic route.

### **Discussion**

We have presented evidence here that *S. typhimurium* exists in an unusual intracellular location inside epithelial cells, defined by a rapid appearance of lgp in SCV without detectable interactions with compartments containing M6PRs. This suggests that intracellular bacteria rapidly fuse with a lgp-containing compartment yet bypass the normal endo-

cytic route. These two properties run counter to current models about the formation of phagosomes, which dictate that any incoming material, including large particles such as latex beads or intracellular pathogens, interact with compartments of the endocytic route, resulting in vacuoles containing membrane marker profiles representative of the late endosome compartment or secondary lysosomes (Rabinowitz et al., 1992; Russell et al., 1992; Desjardins et al., 1994). Nevertheless, our results cannot rule out the possibility of transient interactions between M6PR-containing compartments and SCV. Sorting of contents to maintain distinct compositions may occur, resulting in the maintenance of distinct marker profiles.

The fusion of SCV with lgp-containing compartments of epithelial cells is a relatively rapid process. Macrophages, which harbor much more endocytic activity than non-phagocytic cells (Swanson et al., 1987a), require a half time of 2 h for delivery of externally added fluid endocytic tracers to secondary lysosomes (Rabinowitz et al., 1992). SCV, which do not contain appreciable amounts of M6PRs, initiate fusion with lgp-containing vesicles as early as 15 min after bacterial entry into the epithelial cell. This fusion process is essentially complete 75 min later. Three studies have previously reported the kinetics of fusion with lgp-containing compartments. These studies include the maturation of macropinosomes in macrophages (Racoosin and Swanson, 1993), the mode of entry of the parasite *T. cruzi* in epithelial cells (Tardieux et al., 1992), and the interaction of compartments containing latex beads with elements of the endocytic route (Desjardins et al., 1994). Macropinosomes are large vacuoles formed as a result of massive uptake of the extracellular fluid. Macropinosomes can be induced by cell activation with macrophage colony-stimulating factor (MCS-F) or phorbol myristate (Racoosin and Swanson, 1989), or during *S. typhimurium* invasion of phagocytic and non-phagocytic cells (Alpuche-Aranda et al., 1994; Garcia-del Portillo and Finlay, 1994a). The recent study of Racoosin and Swanson (1993) has shown that fusion of macropinosomes with lgp-containing compartments in macrophages is initiated as early as 4 min after macropinosome formation. In contrast to our observations with SCV in epithelial cells, macropinosomes acquire CI-M6PR 9 min after macropinosomes are formed, and fuse with tubular lysosomes, which represent the functional secondary lysosomes in macrophages (Racoosin and Swanson, 1993). *T. cruzi* also interacts with lgp-containing vesicles upon contact of the parasite with the epithelial cell surface. The parasite is able to recruit lgp-



**Figure 10.** Proposed model of the intracellular location of *S. typhimurium*, and route of fusion of lgp-containing vesicles with SCV in epithelial cells. SCV fuse with lgp-containing vesicles that bypass the late endosome M6PR-enriched compartment. These secretory lgp-containing vesicles may arrive directly from the TGN, and are therefore not connected to the classical endocytic route represented by the early endosome, carrier vesicles (CV), late endosome, and secondary lysosomes. The fusion event of SCV with lgp-containing vesicles does not require microtubules (MT). Limited fusion of secondary lysosomes with SCV may also occur to a lesser extent (fluid endocytic tracer is represented by dots).

containing vesicles at the site of entry, being internalized into lgp-containing vacuoles (Tardieux et al., 1992). The recent analysis of Desjardins et al. (1994) on the incorporation of a lgp, lamp 2, to compartments of macrophages containing latex beads, showed that it was necessary to pulse for 20 min with the beads and chase for 120 min to detect large amounts of lamp-2 in the latex bead-containing compartments. Nevertheless, lgps are detected in these compartments 20 min after ingestion. Therefore, the fusion of SCV with lgp-containing compartments is more closely related to the fusion of latex beads with lgp-containing vesicles rather than macrophosome maturation or formation of *T. cruzi*-containing vacuoles. However, SCV do not fuse extensively with M6PR-containing compartments (only 2% of SCV are lgp<sup>+</sup>, M6PR<sup>+</sup>, Fig. 1 A), contrary to what had been previously reported for latex beads containing phagosomes (Rabinowitz et al., 1992; Jarhaus et al., 1994).

In our system with HeLa epithelial cells, we have been able to detect a higher percentage of latex bead phagosomes containing lgp than M6PRs (Table I). This higher percentage raises the possibility that at least a fraction of internalized beads fuse first with lgp-containing vesicles devoid of both M6PRs, as occurred with SCV. We also determined that at early times (60 min after bead addition) 82% of latex bead phagosomes contain both lgp and CI-M6PR (Table I), while at 180 min this percentage decreases to 13% (Table I). These values agree with previous reports that show fusion of latex bead phagosomes with the late endosome/prelysosomal compartment (Rabinowitz et al., 1992; Jahraus et al., 1994), but also suggest that these phagosomes may mature further into compartments containing lgp but devoid of CI-M6PR. The fact that the percentage of latex beads phagosomes containing both lgp and CD-M6PR remained constant at all times analyzed indicates that CD-M6PR is not equally excluded during the biogenesis of the latex bead phagosome.

We also found that fusion of latex bead phagosomes with

lgp- and M6PR-containing compartments is sensitive to weak bases such as ammonium chloride (Table I). Acquisition of lgps by SCV is also blocked by this compound (Fig. 1 B). These results suggest that early fusion processes of incoming particulate material with lgp-containing vesicles devoid of M6PRs (in the case of SCV and also probably a fraction of latex bead phagosomes), or the late endosome/prelysosomal compartment (latex bead phagosomes), are blocked by treatment with the weak base. It is interesting to note that Brown et al. (1986) reported delivery of fluid endocytic tracers such as Lucifer yellow to CI-M6PR-containing endosomes in NH<sub>4</sub>Cl-treated rat clone 9 hepatocytes. These endosomes were devoid of lgps (Brown et al., 1986). In our system, we never observed latex bead phagosomes labeled with M6PRs but devoid of lgps. We conclude, in contrast to endocytic fluid tracers, that particulate material such as bacteria and probably latex beads do not enter the normal endocytic route, fusing at a certain stage with lgp-containing compartments devoid of M6PRs. Latex bead phagosomes, in contrast to SCV, would undergo an additional fusion event (either before or after fusion with lgp-containing compartments) with the late endosome/prelysosomal compartment.

The proposed bypassing of the M6PR-enriched late endosome/prelysosomal compartment by SCV has notable consequences on the fate of this specialized compartment: it establishes a functional separation from the major endocytic route. To our knowledge, this is the first report that demonstrates a compartment which contains large particles, such as latex beads or intracellular pathogens, yet does not contain detectable amounts of M6P receptors at any postentry time. In one of the few cases in which the presence of this marker has been analyzed in phagosomal compartments, it was shown that *L. mexicana*-containing phagosomes have increasing amounts of CI-M6PR during the course of the infection (Russell et al., 1992). M6PRs are also present in the plasma membrane in a proportion of ~10–20% of the total

amount of the receptors in the cell (Geuze et al., 1988; Braulke et al., 1987). Since we did not detect M6PRs in SCV even at very early times after bacterial internalization (0-min internalization, 15 min after infection), it is possible that invading bacteria prevent fusion with M6PR-containing compartments, or alternatively selectively remove M6PRs from the SCV. In a recent study, we have shown that *S. typhimurium* induces selective aggregation and internalization of surface host proteins such as class I MHC heavy chain (Garcia-del Portillo et al., 1994b). Other surface proteins such as  $\beta_2$ -microglobulin,  $\alpha_5\beta_1$ -integrin, or CD-44 are aggregated by the bacteria but are present in a low proportion of SCV (Garcia-del Portillo et al., 1994b). A similar case may occur with M6PRs, and the bacteria might prevent the incorporation of plasma membrane M6PRs from entering the forming SCV. The large amounts of class I MHC heavy chain present in SCV during the first 90 min after infection (Garcia-del Portillo et al., 1994b) may also provide SCV with an unusual membrane composition, not recognized by the endocytic machinery and therefore not competent for fusion with M6PR-containing compartments, yet able to recognize lgp-containing compartments devoid of these receptors.

Another recent study has reported that giant organelles present in cells of patients with the lysosomal disease Chediak-Higashi syndrome are enriched in lgps and cathepsin D, but negative for CI-M6PR and rab 7 (Burkhardt et al., 1993). This giant organelle is accessible to fluid endocytic tracers, although it required 4–6 h uptake to observe a substantial amount of the tracer. These compartments are also labeled when pulsed and chased with an endocytic tracer. Therefore, it was concluded that the mutation related to this syndrome specifically affects interactions between late endosomes and lysosomes, and that the giant organelle is located downstream in the endocytic route (Burkhardt et al., 1993).

A different result was obtained in our study with SCV. SCV do not contain detectable amounts of M6PRs and they remain relatively separated from the endocytic route, showing limited accessibility to endocytic tracers. After 2 h of F-DX uptake during *S. typhimurium* infection of epithelial cells, only 12–19% of SCV contain detectable amounts of the tracer. Similarly, there is only a small amount of fusion between SCV and HRP-preloaded lysosomes. Although the relative number of SCV containing F-DX increases after longer incubation times, we were unable to detect an increase in the amount of this tracer present in SCV (Fig. 4). As expected, at this time all the HRP-preloaded vacuolar lysosomes contained large amounts of the tracer. Thus, SCV exhibit limited or transient interactions with secondary lysosomes located at the terminal end of the endocytic route, which would explain the presence of low amounts of the tracer. Indeed, it has been shown that lysosomes are highly fusogenic organelles, at least with regards to interchanging membrane components (Deng et al., 1991). Our assumption of a functional separation between SCV and the endocytic route was supported by uptake experiments with the *D. stramonium* lectin, which binds to lgps (Carlsson et al., 1988), but failed to label SCV when added externally (Garcia-del Portillo et al., 1993a). We have been also unable to detect large amounts of endocytic fluid tracers in the lgp-filaments induced by *S. typhimurium*, even when the tracers were maintained in the medium from 30 min to 6 h after infection, a

time at which these structures are induced by intracellular bacteria (Garcia-del Portillo et al., 1993a; data not shown). Two previous findings also support the disconnection of SCV with the endocytic route. First, we have shown that the *iroA* gene of *S. typhimurium*, induced at low concentrations of  $Fe^{2+}$ , is expressed at high levels by intracellular bacteria located in SCV even when large amounts of iron is added to the extracellular medium (Garcia-del Portillo et al., 1992). Secondly, intracellular *S. typhimurium* is able to proliferate inside the SCV (Finlay and Falkow, 1989) under experimental conditions in which a bactericidal concentration of gentamicin (100  $\mu g/ml$ ) is maintained in the extracellular medium. In contrast, a recent report by Drevets et al. (1994) has shown that when *Listeria monocytogenes* does not escape to the cytoplasm in macrophages, it is killed by the delivery of extracellular gentamicin to the bacteria-containing phagosome. This finding was also supported by the access of a fluid endocytic tracer, Lucifer yellow, to the *L. monocytogenes*-containing phagosome (Drevets et al., 1994). *S. typhimurium* is also capable of surviving inside macrophages in medium containing gentamicin (Fields et al., 1986).

An additional two features of the intracellular route followed by SCV are different than classical endocytic routing: SCV fusion with lgp-containing vacuoles still occurs when microtubules are disrupted; and a lysosomal enzyme normally recognized by M6PRs, cathepsin D, is only present in a small fraction of SCV (~20–30%).

To our knowledge, this is the first demonstrated fusion of vacuoles containing large particles with lgp-containing vesicles that occurs independent of microtubules. Latex beads require microtubules to be targeted to lgp-containing vacuoles (Desjardins et al., 1994), and macropinosome formation with the concomitant rapid fusion with lgp-containing vacuoles does not take place unless microtubules remain intact (Racoosin and Swanson, 1992). It is therefore tempting to speculate that the nature of the lgp-containing compartments that are fusogenic with SCV, are secretory vesicles whose final destination is the plasma membrane. It is known that secretory vesicles do not have a strict requirement for microtubules for trafficking to the surface of the cell. Moreover, considering the segregation of SCV from the endocytic route, it is possible that the source of lgp-containing vesicles that fuse with SCV are formed via a direct transport route from the TGN, which bypasses the late endosome/prelysosomal compartment, and may be involved in an exocytic pathway to the plasma membrane. This route, which is diagrammed in a model in Fig. 10, derives support from several recent observations. For example, it is known that certain lgps and lysosomal enzymes such as LAP traffic to the plasma membrane before targeting to lysosomes (Lippincott-Schwartz and Fambrough, 1986, 1987; Braun et al., 1989; Furuno et al., 1989; Mane et al., 1989; Mathews et al., 1992; Akasaki et al., 1993), and that a mutation in the rab 9 protein, which blocks return of M6PRs to the TGN from the late endosome compartment, stimulate a "default" pathway of vesicles from the TGN containing large amount of lgps and lysosomal enzymes (Riederer et al., 1994). Among these lysosomal enzymes, which are secreted to the extracellular medium by the mutant cells, are soluble enzymes which contain M6P such cathepsin D,  $\beta$ -glucuronidase, and hexosaminidase. The altered secretion of these en-

zymes suggest that the "default" pathway probably connects with the plasma membrane. Other studies have also demonstrated that traffic of lysosomal enzymes to the plasma membrane includes a route from the site of synthesis to the early endosome compartment and plasma membrane (Ludwig et al., 1991). It is also known that overexpression of Igps results in increased amounts of Igps in the plasma membrane (Williams and Fukuda, 1990; Harter and Mellman, 1992), which may be transported via our proposed pathway. The transport of Igps to the plasma membrane seems not to occur in other cell lines. For example, different reports have demonstrated that the rat and mouse Igps are targeted to lysosomes without appearance on the plasma membrane (D'Souza and August, 1986; Green et al., 1987; Harter and Mellman, 1992). It is obvious that further analysis of the intracellular route of SCV in these cell lines will provide information about our proposed fusion of SCV with secretory vesicles containing Igps.

The low proportion of SCV containing cathepsin D cannot be explained by the presence of dead intracellular bacteria, since >95% of the starting inoculum was viable as evidenced intact bacterial motility observed under the microscope, and intracellular bacterial death is not expected since bacterial numbers do not decrease over time (Finlay and Falkow, 1988; Garcia-del Portillo, F. and B. B. Finlay, unpublished observations). The majority of SCV lacking cathepsin D may be derived from the existence of some Igp-containing vesicles that lack M6P-containing enzymes, or, alternatively, the transfer of membrane lysosomal components but not the luminal contents to the SCV. The former explanation is supported by a recent study that showed large granules of lymphoblastic cells of patients with Chediak-Higashi syndrome contain elevated amounts of Igps but do not have cathepsin G, a soluble lysosomal enzyme (Jones et al., 1992). In contrast, apparent transfer of lysosomal membrane components preceding transfer of luminal contents has been described for the maturation of macropinosomes, which acquire Igps at 4 min after formation but do not exhibit detectable levels of cathepsin D until 9 min after formation (Racoosin and Swanson, 1993). Interestingly, in our system with HeLa epithelial cells we did not observe such a difference in the incorporation of these lysosomal markers to latex beads phagosomes. Both Igp and cathepsin D were simultaneously incorporated to latex bead phagosomes (Fig. 9), in contrast to what was observed with SCV. Another example of Igp-containing compartments devoid of some lysosomal markers is the *M. avium*-containing phagosome, which does not have the vacuolar-proton ATPase, which is involved in acidification of the vacuolar compartments (Sturgill-Koszycki et al., 1994). These authors postulated an active process of elimination of this membrane component that is induced by the bacteria, or the existence of "non-classical lysosomes" without this membrane component, which may form directly from the TGN.

The results presented in this study indicate that the existence of a trafficking route of Igp-containing vesicles moving from the TGN, which bypasses the late endosome and does not intersect with incoming material from the extracellular medium. It is probable that this route also functions in uninfected cells. We detected Igp-containing vesicles (~20% of total) which were not loaded with HRP after a pulse and overnight chase with the tracer (Table II). When a second fluid tracer was added to HRP-preloaded cells, it was deliv-

ered to ~80% of Igp-containing vesicles, confirming that the same fraction of Igp-containing vesicles may not be connected to the endocytic route. A precedent for this population of Igp-containing vesicles is found in the study of Geuze et al. (1988), which observed that 10% of the Igp content of the cell was located in compartments devoid of M6PRs and not accessible to fluid endocytic tracers.

Finally, analysis of the biogenesis of SCV containing the intracellular replication mutant 3-11 did not reveal basic phenotypic differences when compared to the parental wild type strain. These results may indicate that the proposed direct targeting route of intracellular *Salmonella* to Igp-containing vesicles does not strictly correlate with the initiation of bacterial proliferation. Alternatively, it is also possible that this type of mutant fails to respond to specific signal(s) derived from this targeting route.

In conclusion, *S. typhimurium* is a useful biological tool to analyze lysosome biogenesis. The intracellular route of this pathogen corresponds to a unique pathway that is different than the phagocytic and endocytic routes described to date. Similar studies performed with other intracellular pathogens will provide additional information about the mechanisms used by intracellular pathogens to survive or proliferate within eucaryotic cells, in addition to furthering our knowledge about eucaryotic cell processes.

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