Salmonella Co-opts Host Cell Chaperone-mediated Autophagy for Intracellular Growth*

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Salmonella enterica are invasive intracellular pathogens that replicate within a membrane-bound compartment inside infected host cells known as the Salmonella-containing vacuole. How Salmonella obtains nutrients for growth within this intracellular niche despite the apparent isolation is currently not known. Recent studies have indicated the importance of glucose and related carbon sources for tissue colonization and intracellular proliferation within host cells during Salmonella infections, although none have been found to be essential. We found that wild-type Salmonella are capable of replicating within infected host cells in the absence of both exogenous sugars and/or amino acids. Furthermore, mutants defective in glucose uptake or dependent upon peptides for growth also showed no significant loss in intracellular replication, suggesting host-derived peptides can supply both carbon units and amino acids. Here, we show that intracellular Salmonella recruit the host proteins LAMP-2A and Hsc73, key components of the host protein turnover pathway known as chaperone-mediated autophagy involved in transport of cytosolic proteins to the lysosome for degradation. Host-derived peptides are shown to provide a significant contribution toward the intracellular growth of Salmonella. The results reveal a means whereby intracellular Salmonella gain access to the host cell cytosol from within its membrane-bound compartment to acquire nutrients. Furthermore, this study provides an explanation as to how Salmonella evades activation of autophagy mechanisms as part of the innate immune response.

Infections caused by serovars of Salmonella enterica remain one of the major causes of bacterial zoonotic and food-related gastrointestinal diseases worldwide (1–3). Although the human-restricted serovar, Salmonella typhi, is generally associated with severe systemic forms of infections, an increase in the severity and systemic forms of infections has been observed for nontyphoidal serovars (4, 5). Likewise, increasing levels of antibiotic resistance and the emergence of Salmonella serovars for which current vaccines are ineffective (5–8) indicate a better understanding of host-pathogen interactions for this important bacterial pathogen is sorely needed.

Salmonella are facultative intracellular pathogens that infect and proliferate within intestinal epithelial cells, macrophages, and other cell types where they reside within a membrane-bound intracellular compartment known as the Salmonella-containing vacuole (SCV) (9, 10). Although acquisition of nutrients within the infected host and bacterial metabolism play fundamental roles in pathogenesis, little is known about how Salmonella obtains nutrients for growth within this intracellular compartment (11–15). Prior studies in vitro and in vivo have indicated that access to glucose and related sugars is important for full virulence in the host; however, these same studies have also indicated that none appear to be essential (11, 12, 15–17). Indeed, the majority of clinical isolates of S. typhi, the causative agent of human typhoid, are auxotrophic for amino acids and show highly variable capacities for carbon source utilization (18, 19). Furthermore, highly attenuated aroA strains of S. typhimurium recover virulence in mice when metabolites not involved in aromatic amino acid biosynthesis but whose synthesis also require chorismate are included in feed or water (20). These prior studies therefore indicate that in vivo, Salmonella have access to host-derived sources of nutrients supplying both carbon sources and amino acids. However, the mechanisms whereby intracellular Salmonella can access host stores of nutrients remain unknown.

Prototrophic Salmonella acquire amino acids through multiple pathways, including de novo biosynthesis, uptake of free amino acids, and uptake and catabolism of small peptides, which serve as both a source of amino acids and carbon units. Here we show that one means of acquisition of both carbon units and amino acids involves acquisition of one of the host cell’s own cytosolic protein turnover pathways. We show that the LAMP-2A isoform of lysosomal protein LAMP-2 and the

4 The abbreviations used are: SCV, Salmonella-containing vacuole; CQ, chloroquine-diphosphate; CMA, chaperone-mediated autophagy; m.o.i., multiplicity of infection; CDH, cyclohexadienyl dehydrogenase; Man-6-PR, mannose 6-phosphate receptor; TMR, tetramethylrhodamine.
host heat-shock protein Hsc73 are both recruited to the SCV where they are involved in delivery of host cell cytosolic proteins/peptides through their function in host cell chaperone-mediated autophagy (CMA). Furthermore, we demonstrate that CMA is actively involved in supporting growth of the peptide-dependent mutant of Salmonella, and we identify a known substrate of CMA in highly purified SCVs. Our results provide an explanation for the relative independence of Salmonella for specific carbon sources observed in prior studies. The ubiquitous nature of CMA in essentially all host cell types may explain the ability for Salmonella to survive and persist within many different cell types in the host, including cells of the immune system. Furthermore, the results from our study provide an explanation as to how Salmonella avoids the host autophagy innate immune defense during infection of host cells.

Results

To determine to what extent exogenous glucose and amino acid pools contribute to the intracellular growth of S. enterica serovar typhimurium (S. typhimurium), we infected host cells that had been deprived of either amino acids, glucose, or both for 24 h prior to and during infection. Both cell viability and cytotoxicity assays verified that no adverse effects on the cell monolayers other than reduced proliferation rates occurred during the 48 h of deprivation (data not shown). The only significant effects on the intracellular growth of Salmonella were observed in the macrophage cell line in the absence of glucose (Fig. 1B), but were consistent with previous reports (11). Assays for intracellular glucose levels of the host cells verified the absence of measurable glucose in deprived cells (Fig. 1, E and F). However, although statistically significant, these relatively minor effects and the absence of significant reductions in intracellular growth of Salmonella in intestinal epithelial cells indicated that intracellular growth of wild-type S. typhimurium can be independent of exogenous sources of both glucose and amino acids (Fig. 1, A and B). These results were somewhat surprising as prior in vitro and in vivo studies have indicated the importance of glucose and glycolysis for intracellular growth and systemic infections (11, 12, 15–17). To verify that host cell cytosolic stores of glucose were not providing carbon units, we also tested a Salmonella Δglik ΔmanXYZ ΔptsG mutant, which is unable to take up glucose (11, 12). As seen in Fig. 1C, however, no significant reductions in intracellular growth of the mutant compared with the wild-type strain were observed in either intestinal epithelial or macrophage cells. Furthermore, although the mutant showed the same significant reduction in growth in macrophages in the absence of glucose and amino acids as seen for the wild-type strain in Fig. 1B, intracellular growth of the glucose uptake mutant remained comparable with that of the wild-type strain under all conditions (Fig. 1C). The same results were also found for a Salmonella Δglik ΔmanXYZ ΔptsG ΔhphT mutant that additionally is unable to take up glucose 6-phosphate (data not shown). Again, however, the results indicated that neither exogenous sources of glucose nor intracellular host cell stores were essential requirements for intracellular growth of Salmonella, and they indicated that intracellular Salmonella have access to alternative sources of nutrients from within the SCV.

Based on these observations, we considered possible alternative host-derived nutrient sources other than glucose and free amino acids. In standard bacteriological growth media, pep-
tides provide not only amino acids but also carbon units and nitrogen (21). However, *Salmonella* is only capable of uptake of peptides of 5 to 6 amino acids in length into the bacterial cytosol and does not express extracellular proteases capable of the degradation of large proteins (22). Within host cells, peptides would therefore have to be available in at least a partially degraded form to serve as a nutrient source. To determine a possible role for peptide catabolism in intracellular growth, we constructed mutants of *Salmonella* defective in both the uptake and de novo biosynthesis of the aromatic amino acids. Deletion of the genes encoding transport systems involved in uptake of the aromatic amino acids (ΔaroP, ΔpheP, ΔtyrP, and Δmtr) combined with mutations affecting the biosynthesis of phenylalanine (ΔpheA), tryptophan (ΔtrpBA), or tyrosine (ΔtyrA ΔtyrB) confers a strict dependence upon peptides as a source of the missing amino acids. These strains do not grow on minimal medium supplemented with the free amino acids but will grow in medium containing oligopeptides (Fig. 2). Consistent with the growth dependence on small peptides, the ΔaroP ΔpheP ΔtyrP Δmtr ΔpheA mutant showed no growth in defined minimal media containing casamino acids dialyzed to remove peptides smaller than 3.5 kDa nor in cell culture medium containing serum (Fig. 2B). In contrast, although clearly dependent upon small peptides for growth in culture, the peptide-dependent mutant showed intracellular growth similar to the wild-type strain in both epithelial and macrophage cell lines, suggesting that oligopeptides were available to *Salmonella* within the SCV. The same results were obtained for strains harboring mutations in the tryptophan and tyrosine biosynthetic pathways, as well as combinations (data not shown). As the SCV has been reported to acidify within a short time post-infection (23, 24), the studies shown were performed with strains harboring the CDH plasmid.

Maturation of the SCV into a lysosomal compartment could provide peptides taken up by endocytosis and degraded by lysosomal proteases. However, although the SCV shares certain host cell markers common to lysosomes, prior studies have excluded conventional lysosomal maturation of the SCV (26–29). Alternatively, other studies have indicated that in cell culture models of infection, a fraction of intracellular *Salmonella* may escape from the SCV and proliferate within the host cell cytosol (30–33). It was therefore possible that the continued growth observed in the absence of exogenous nutrients was the result of SCV escape and replication within the host cytosol. To determine the vacuolar status of *Salmonella*, we performed infections in the presence of chloroquine, which has been used to discriminate between vacuolar and cytosolic populations of intracellular pathogens (30). As shown in Fig. 3, however, a >90% inhibition of intracellular growth was observed for up to 24 h post-infection, indicating a constant access of chloroquine to vacuolar *Salmonella*. Treatment of host cells with wortmannin has also been reported to increase the cytosolic fractions of intracellular *Salmonella* post-infection (33). However, wortmannin treatment of the host cells prior to and during infection did not increase the levels of chloroquine-resistant bacteria in the cell lines used here (Fig. 3). As also seen in Fig. 3, the chloroquine-mediated inhibition of intracellular growth remained the same at both 4 and 24 h post-infection, indicating no “hyper-replication” of intracellular *Salmonella*, which has been reported for cytosolic growth (32). These results are consistent with other studies showing recruitment of other host proteins to the SCV membrane, despite the absence of LAMP-1, the latter of which is an indicator of SCV membrane integrity (34, 35). More recent studies have also shown that intracellular *Salmonella*-induced filaments can be associated with membrane that does not contain LAMP-1 (36, 37). As LAMP-1 co-localization is often used to determine the vacuolar status of intra-
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A, LoVo intestinal epithelial cells were deprived of glucose (−glc) and free amino acids (−aas) for 24 h prior to and throughout infection with S. typhimurium wild-type (wt) or the peptide-dependent mutant strain (mut) in the presence of normal or dialyzed serum (dial. serum), as indicated below the graph. 4 h prior to infection, cells were treated with either DMSO (white bars) or a protease inhibitor mixture in DMSO (black bars) at the same dilutions and were present for 2 h post-infection. At the time points indicated, cells were lysed, and the intracellular CFU were determined. Shown are the relative fold increases (rel.-fold incr.) in intracellular CFU between 4 and 24 h post-infection. Data shown are the means and standard deviations of at least three independent experiments. B, effect of the protease inhibitor (PI) mixture used in A on host cell endogenous protease activity was determined from whole cell lysates or trypsin as a control. Data shown are the means and S.D. of at least three independent experiments. *p > 0.05, non-significant (n.s.); **, p < 0.05; ***, p < 0.01; ****, p < 0.001.

FIGURE 4. Minimal contribution of low molecular weight serum proteins and host-derived peptidases as a nutrient source for intracellular Salmonella. A. The continued intracellular growth of wild-type Salmonella might provide an explanation for the minimal effects observed with protease inhibitors in whole cell lysates seen in Fig. 4B, where the substrate protein used, casein, may be too large to result in generation of TCA-soluble fragments. We therefore performed the same protease inhibitor studies using serum dialyzed to remove peptides and/or oligopeptides smaller than 3.5 kDa, representing possible substrates for both host cell brush-border peptidases as well as direct uptake by Salmonella peptide uptake systems (22). As shown in Fig. 4A, the substitution of dialyzed serum in the cell culture medium reduced the intracellular growth of wild-type Salmonella by ~70% and that of the peptide-dependent mutant by 80%. Although the addition of protease inhibitors did not result in any additional reductions in growth, which would have been suggestive of the loss of a small peptide substrate fraction, the intracellular growth of the peptide-dependent mutant was not significantly different compared with that of the wild-type strain (Fig. 4B). It therefore appeared that host cell processing of extracellular serum proteins did not provide a major contribution to the intracellular growth of Salmonella under these conditions.

The continued intracellular growth of the peptide-dependent mutant in the absence of essentially all extracellular sources of nutrients also indicated that there must be additional intracellular sources of small peptides capable of supporting cellular Salmonella, this might suggest some studies may overestimate the fraction of cytosolic Salmonella. The results shown in Fig. 3 indicated that in the epithelial cell lines used, loss of SCV membrane integrity and escape of Salmonella into the host cell cytosol did not contribute significantly to the observed intracellular growth.

We therefore considered alternative processes whereby vacuolar, intracellular Salmonella might acquire host-derived peptides. From a topological point of view, the inside of the SCV represents the exterior face of the host cell, and host cell uptake systems present in the plasma membrane would be oriented to transport nutrients out of the SCV. However, membrane-bound intestinal epithelial brush-border enzymes, including aminopeptidases, are found at the outer surface of the intestinal epithelia (38) and would be oriented within the lumen of the post-infection SCV once Salmonella is internalized. It was therefore possible that host brush-border peptidases present within the SCV might process serum proteins present in the cell culture medium into oligopeptides capable of uptake by Salmonella. Continued interaction with early endocytic pathways (28, 39, 40) could maintain the supply of proteins to support intracellular growth. To test this possibility, we pre-treated host cells that had been deprived of both glucose and free amino acids with broad specificity protease inhibitors prior to and during infection with either wild-type or peptide-dependent strains of Salmonella. As shown in Fig. 4A, although protease inhibitor treatment reduced the intracellular growth under these conditions ~25%, the effect was essentially the same in both wild-type and mutant strains and was only significant for the wild-type strain. Likewise, whereas the protease inhibitor mixture was clearly active against purified trypsin, only minimal effects were observed on protease activity in whole cell lysates (Fig. 4B).
growth. We therefore considered other potential host cell processes that might be involved in providing peptides to intracellular Salmonella. One possible mechanism we chose to investigate was chaperone-mediated autophagy (CMA), a selective host cell protein turnover pathway involved in the transport of cytosolic proteins into lysosomes for degradation (43, 44). An estimated 30% of all cytosolic proteins are turned over through this mechanism (45). Two key protein components of CMA are the LAMP-2 isoform LAMP-2A at the lysosomal membrane and the heat shock protein Hsc73, which acts both as a chaperone for cytosolic proteins as well as a luminal lysosomal protein involved in transport of target proteins across the lysosomal membrane (46–48).

Standard markers for the SCV or phagolysosome include the lysosomal proteins LAMP-1 and LAMP-2, which are acquired early during the invasion process (49). As only one of the three human isoforms of LAMP-2 is involved in CMA (46), we were interested to know which isoform(s) of LAMP-2 co-localized with the SCV membrane. As shown in Fig. 5A, antibodies against LAMP-1 were found to co-localize with the SCV, as expected. In contrast, although antibodies specific for the LAMP-2A isoform were found to co-localize with the SCV, antibodies against the LAMP-2B isoform showed no co-localization. Notably, both isoforms of LAMP-2 localize to lysosomes, but in different membrane microdomains (50), suggesting the LAMP-2A present at the SCV membrane did not originate through lysosomal fusion. The same co-localization pattern for LAMP-2A was also observed in macrophages as well as intestinal epithelial cells (data not shown), indicating acquisition of the LAMP-2A isoform is a general feature of the SCV. We next determined whether the other key component of the CMA complex, Hsc73, was also present. As shown in Fig. 5A,
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FIGURE 6. Inhibition of chaperone-mediated autophagy reduces the intracellular growth of a peptide-dependent Salmonella mutant but not the wild-type strain in intestinal epithelial cells. LoVo intestinal epithelial cells were pre-incubated for 4 h prior to infection with ammonium chloride (NH4Cl), 10 mM 3-methyladenine (3MA), or 10 μg/ml cycloheximide (CHX), or 10 μg/ml anti-Hsc73 antibodies (α-Hsc73) and then infected with either the wild-type (white bars) or peptide-dependent mutant strain (black bars) at an m.o.i. of 5 in standard invasion (infection) assays. The relative fold increase in intracellular bacterial CFU between 4 and 24 h is shown. The results shown are the means ± S.D. of three independent assays, with significance determined using the Student’s unpaired t test where p > 0.05 is considered non-significant (n.s.), *p < 0.05; **p < 0.01; ***p < 0.001.

antibodies specific for Hsc73 were also found to co-localize with the SCV of intracellular Salmonella. Although the absence of significant co-localization with LAMP-2B suggested no fusion with lysosomal compartments, both LAMP-2A and Hsc73 are normal constituents of lysosomal compartments. We therefore performed additional experiments using antibodies against the mannose 6-phosphate receptor (Man-6-PR) and lysosomal integral membrane protein 2 (LIMP-2), the lysosomal receptor for mannose 6-phosphate-independent transport of lysosomal hydrolases (51). As shown in Fig. 5A, no significant co-localization with the SCV was observed for either Man-6-PR, as reported previously (26, 29), for LIMP-2. Quantification of co-localization studies from three independent experiments indicated that 80–90% of intracellular Salmonella were associated with LAMP-1, LAMP-2A, and Hsc73, whereas little or no co-localization was observed for LAMP-2B, Man-6-PR, or LIMP-2 (Fig. 5B). Co-localization of Man-6-PR with the SCV was included as a control, as it is known not to co-localize with the SCV (26, 29). These results supported the suggestion that fusion with lysosomal compartments was unlikely to serve as a source of peptides for the mutant.

We next examined the intracellular growth of the peptide-dependent mutant in the presence of inhibitors of CMA and other forms of autophagy. Here, we reasoned that if host cell CMA was providing peptides to intracellular Salmonella, the intracellular growth of the peptide-dependent mutant should be affected by loss or inhibition of CMA, whereas the wild-type strain would remain capable of using alternative sources of carbon units and amino acids as well as de novo biosynthesis (15). As shown in Fig. 6, 3-methyladenine, an inhibitor of macroautophagy, showed no significant effects on growth of the mutant in intestinal epithelial cells, whereas pre-treatment of host cells with cycloheximide at concentrations used to inhibit CMA (52) resulted in a significant growth inhibition of the mutant compared with the wild-type strain. Likewise, pre-loading of cells prior to infection with antibodies against Hsc73 to inhibit CMA (47) significantly inhibited intracellular growth of the mutant while leaving the wild-type unaffected (Fig. 6).

As the preceding results were suggestive of the idea that host cell CMA was involved in providing intracellular Salmonella with peptides within the SCV, we sought to verify these results in another cell line, HEK293T, including knockdown of expression of LAMP-2A. As seen in Fig. 7, A and B, consistent with the results in the LoVo intestinal epithelial cell line (Fig. 6), only cycloheximide treatment or pre-loading cells with neutralizing antibodies against Hsc73 showed significant reductions in growth of the peptide-dependent mutant. Control experiments pre-loading cells with anti-LAMP-1 antibodies showed no effect on intracellular growth (Fig. 7B). Finally, we also performed knockdown experiments of LAMP-2A expression and compared the intracellular growth of the mutant and wild-type strains. As LAMP-2A is an essential component of host cell CMA activity, knockdown of LAMP-2A expression is considered the “gold standard” for determining the role of CMA in cytosolic protein turnover. As shown in Fig. 7C, reduced expression of LAMP-2A significantly inhibited the growth of the peptide-dependent mutant strain compared with the wild type, despite the presence of serum proteins that could also contribute to intracellular growth (Fig. 4). Western blotting verified a clear reduction in LAMP-2A expression in cells transfected with the LAMP-2A shRNA construct (Fig. 7D). Note that in Fig. 7D, LAMP-1 was used as a loading control because the levels of LAMP-1 have been found not to be affected by loss of LAMP-2, whereas other housekeeping protein levels such as actin can show large reductions (53).

Although the LAMP-2A knockdown experiments had been repeated a total of six times, independently, and the reduction in intracellular growth of the peptide-dependent mutant was found to be statistically significant, the residual growth of the mutant suggested either an additional source of peptides or incomplete inhibition of CMA activity. We therefore determined the levels of CMA activity in control, LAMP-1, or LAMP-2A knockdown cell lines. Co-localization of a Halo-Tag variant of the CMA substrate protein, GAPDH, with lysosomes has been used to monitor the effects of activators, inhibitors, as well as LAMP-2A knockdown expression on CMA activity (54–56). Cells previously transfected with either a vector (scrambled RNA) control, or LAMP-1, or LAMP-2A shRNA constructs for knockdown expression of LAMP-1 or LAMP-2A, respectively, were co-transfected with a GAPDH-HaloTag construct, incubated in the presence of the red fluorescent HaloTag ligand, tetramethylrhodamine (TMR), and subsequently stained with a green fluorescent marker for lysosomes.

To further verify that co-localization was due to CMA activity, we also determined the response to serum deprivation, a treatment known to result in elevated CMA activity (43, 57) As shown in Fig. 8, whereas LAMP-1 knockdown expression showed no major effects on co-localization of GAPDH with lysosomes compared with the vector control, loss of LAMP-2A expression showed significant reductions in co-localization...
The observations that chemical inhibition, neutralizing antibodies against Hsc73, and knockdown expression of LAMP-2A and CMA activity were the only treatments found to affect the growth of the peptide-dependent Salmonella strain, as well as the observation that only the LAMP-2A isoform co-localized with the SCV, all supported the idea that CMA contributed to the intracellular growth of Salmonella. In an additional effort to verify this possibility, we sought to determine whether known substrates of the CMA system could also be identified co-localizing with the SCV. We therefore performed co-localization experiments in cell lines harboring the GAPDH-HT construct that had been infected with wild-type Salmonella expressing GFP and subsequently incubated in the presence of the TMR ligand. As shown in Fig. 9, D and E, in the presence of the TMR ligand, the characteristic punctate patterns of red fluorescence were observed co-localizing with the SCV, similar to the patterns observed when transfected cells were simultaneously stained with both the TMR ligand and dyes labeling lysosomes (Figs. 8 and 9B). In contrast, the TMR ligand itself showed only a diffuse distribution within cells (Fig. 9C), as reported previously (54). Quantification of the co-localization of the GAPDH-HT fusion with intracellular Salmonella also indicated that CMA-activating conditions (serum deprivation) resulted in significantly higher co-localization of the CMA substrate with the SCV (Fig. 9F).

In a second approach, we constructed an additional vector harboring an N-terminal fusion of the CMA-targeting motif of a second known CMA substrate, RNase A (56) with DsRed2, and we performed additional co-localization studies. As shown in Fig. 10, the DsRed2 fusion with the RNase A N-terminal CMA-targeting motif showed the same characteristic punctate pattern of localization within host cells (Fig. 10C), which also increased after serum deprivation (Fig. 10, A and B). As seen in Fig. 10C, the N-terminal CMA targeting fusion also showed the same co-localization with Salmonella-containing vacuoles as seen with the GAPDH-HT CMA reporter fusion (Fig. 9), whereas the control DsRed2 vector showed only a diffuse cytosolic distribution with no co-localization with SCVs (Fig. 10C). Together with the CMA inhibitor studies, these results further supported the suggestion that not only were the host cell components necessary for CMA transport of cytosolic proteins present at the SCV membrane, but genuine substrates of CMA were also actively transported to the SCV membrane.

Finally, to further verify the presence of components of the CMA transport system at the SCV membrane, we purified Salmonella-containing vacuoles and performed Western blotting for SCV-associated host proteins. Wild-type Salmonella expressing green fluorescent protein (GFP) were bound with carbon-coated paramagnetic cobalt nanoparticles and used to infect macrophages, followed by recovery of the bacteria after lysis of host cells using a magnetic field. As a control, nanoparticle-bound Salmonella were heat-killed and incubated with macrophage for uptake by phagocytosis. After removal of cell lysate supernatants under magnetic field conditions, the SCV
preparations were incubated in the presence of antibodies against LAMP-1 or LAMP-2A and examined by fluorescence microscopy to verify the integrity of the SCV membrane. As shown in Fig. 11A, GFP-expressing *Salmonella* recovered in this manner from cell lysates showed exterior labeling with both LAMP-1 and LAMP-2A, indicating intact SCV membranes. Control experiments using the secondary antibodies alone showed no labeling (data not shown). The SCV preparations were then subjected to SDS-PAGE followed by Western blotting against various lysosomal markers. As expected, and consistent with the results shown in Fig. 5, LAMP-1, LAMP-2A, and Hsc-73 were shown to be present in purified SCV preparations, whereas LAMP-2B and LIMP-2 were significantly reduced or absent compared with control phagosomes contain-

**FIGURE 8.** Inhibition of CMA substrate protein GAPDH targeting to lysosomes in LAMP-2A knockdown expression cell lines. A, HEK293T cells transfected with constructs harboring either control (scrambled RNA), LAMP-1, or LAMP-2A shRNA vectors were cultivated in the presence or absence of serum for 24 h prior to co-transfection with a HaloTag derivative of GAPDH (GAPDH-HT) and incubated in the presence of TMR ligand (red) and counterstained for lysosomes with LysoTracker Green (green). Co-localization of GAPDH-HT with lysosomes is seen as overlapping red and green fluorescent puncta (yellow). B, targeting of GAPDH-HT to lysosomes was quantified by determining the total number of GAPDH-HT-positive lysosomes per cell in cells co-transfected with control (con.), LAMP-1 shRNA (L1), or LAMP-2A shRNA (L2A) constructs and the GAPDH-HaloTag construct. The insets to the panels for the LAMP-2A knockdown cell line show sections of the same cells in the merged images that have been amplified for the red fluorescent channel for visualization purposes due to the diffuse (non-punctate) distribution of GAPDH-HT in this cell line. The data shown are the means ± S.D. of three independent experiments counting puncta in 25 co-transfected cells for each cell line (n = 75 cells/cell line), p > 0.05, non-significant (n.s.); *, p < 0.05; **, p < 0.01; ***, p < 0.001.
ing dead Salmonella (Fig. 11B). Control preparations using heat-killed Salmonella showed the presence of all markers, as expected for mature lysosomes. In addition, we also observed significantly higher amounts of both LAMP-2A and Hsc-73 in SCV preparations with live Salmonella compared with phago-cytosed dead Salmonella, suggesting an enrichment of these proteins at the SCV membrane (Fig. 11C). Furthermore, consistent with the co-localization studies (Fig. 9), the known CMA substrate, GAPDH, was also present in low but appreciable levels in the SCV preparations (Fig. 11, B and C).

Discussion

The results of this study indicate that one means whereby intracellular Salmonella acquire nutrients for growth within infected host cells is by co-opting host cell CMA-dependent cytosolic protein turnover to obtain access to peptides that can be used as a source of both carbon units and amino acids. Using a combination of chemical, neutralizing antibody, LAMP-2A knockdown expression, and intracellular co-localization studies for key components of the host cell CMA complex and CMA substrates, we have shown that membrane-bound (SCV) intracellular Salmonella not only acquire LAMP-2A and Hsc73 but the CMA complex is also actively involved in supporting the growth of intracellular Salmonella. As noted by other authors, wild-type Salmonella uses multiple metabolic pathways simultaneously, and loss of one source of nutrients can be compensated through other pathways (15). The identification of host cell CMA activity providing nutrients to membrane-bound
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Intracellular Salmonella was made possible through use of a peptide-dependent mutant strain defective in both uptake and de novo biosynthesis of essential amino acids. Because of the ubiquitous nature of CMA in nearly all cell types (44), intracellular Salmonella would therefore have access to an intracellular source of pre-formed amino acids in essentially all cells of the infected host, including cells of the immune system. Infection of host cells deficient in LAMP-2A or treatments with inhibitors of CMA indicate that this host cell pathway provides a significant contribution to the intracellular growth of Salmonella.
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and it provides an explanation for the observations that the intracellular growth of Salmonella can be independent of both exogenous carbon sources and de novo amino biosynthesis. Although our study does not exclude additional pathways or processes whereby intracellular Salmonella acquire nutrients, to our knowledge this is the first host cell process identified shown to be used by Salmonella to access intracellular sources of nutrients from within the SCV.

Autophagy has previously been implicated in host cell restriction of intracellular bacterial growth or killing of intracellular pathogens by targeting them to the autophagolysosome (58–63). Among the different autophagy pathways involved in defense against bacterial pathogens, the majority involve recognition of cytosolic bacterial pathogens or vacuolar intracellular pathogens such as Salmonella with damaged vacuolar (SCV) membranes, followed by formation of an autophagosomal compartment surrounding the bacterium which eventually fuses with lysosomes. Cytosolic Salmonella can be recognized by both ubiquitin-dependent and -independent pathways (58–61). Although a number of intracellular pathogens are capable of either evading or even subverting the autophagosomal pathways for intracellular growth (62, 63), with regard to Salmonella, autophagy has been generally seen as a host defense mechanism. It should be noted that a recent publication has suggested that the classical autophagy pathways involving p62, LC3, Atg5, and Atg16L1 were involved in supporting the intracellular growth of Salmonella, which are released from the SCV into the host cytosol in the HeLa cell line (64). However, in our study we have specifically examined the role of chaperone-mediated autophagy on membrane-bound (SCV) intracellular Salmonella, as verified by the continued sensitivity to chloroquine up to 24 h post-infection (Fig. 3). Furthermore, wortmannin, a standard inhibitor of autophagy, has also been reported to promote release of Salmonella from the SCV in HeLa cells (33, 65). Here, wortmannin treatment did not show a reduction in the sensitivity to chloroquine, as would be expected if Salmonella

FIGURE 11. Purified SCVs of live but not heat-killed Salmonella show differential acquisition of key components of CMA. A, representative immunofluorescence pictures of purified SCVs recovered from THP-1 macrophage infected with paramagnetic nanoparticle-labeled GFP-expressing Salmonella and stained for LAMP-1 (left) or LAMP-2A (right). The white scale bars are 3.2 and 6 μm in the left and right panels, respectively. B, purified SCVs recovered from THP-1 macrophage infected with either live (SCV) or heat-killed (killed) GFP-expressing Salmonella were subjected to SDS-PAGE and Western blotting using antibodies against the indicated host proteins. Loading controls consisted of uninfected host cell lysates used as an internal standard or GFP as a loading control for samples containing Salmonella. C, quantification of the relative amounts of the indicated host proteins present in SCVs purified from macrophage infected with either live (open bars) or heat-killed Salmonella. Signal intensities were determined relative to a constant amount of non-infected host cell lysates loaded on the same gels and shown for representative purposes on the right. The data shown are the mean and S.E. of at least two independent experiments. Statistical significance was determined by a two-way analysis of variance, where p > 0.5 is considered non-significant (n.s.); *, p ≤ 0.05; **, p < 0.01; ***, p < 0.001.
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had been released into the host cell cytosol (Fig. 3). It remains possible that the role of the classical autophagy pathways on the intracellular growth of Salmonella may be cell line- or type-specific, as suggested previously (64). In contrast to the classical autophagy pathways, CMA is an exceptional form of autophagy in that it does not involve ubiquitylation or autophagy receptors such as p62, Galectin 8, or NDP52, nor does it involve membrane mobilization and autophagosome formation (43, 44).

As noted in the Introduction, prior studies based on mutants of Salmonella have indicated that although glucose and glycolysis as well as other sugars are important for growth within the host, they are not essential (11–17). Unfortunately, very little is known about the actual intracellular concentrations of nutrients, which might be available to Salmonella within infected host cells in vivo. As shown here, the absence of exogenous (and intracellular) glucose did not have major effects on the intracellular growth of Salmonella within either epithelial or macrophage cells. The observation that a peptide-dependent mutant of Salmonella is still capable of intracellular proliferation indicates that peptides clearly contribute to growth within host cells. Although serum proteins appeared to be a plausible source of nutrients for intracellular Salmonella, at least in vitro, we found no significant differences in the intracellular growth of a peptide-dependent mutant compared with wild-type Salmonella in cells cultured in normal or dialyzed serum (Fig. 4A).

Furthermore, it would seem unlikely that this would play an important role in tissues other than intestinal epithelium where brush-border peptidases might provide peptides capable of uptake by Salmonella.

In addition to the brush-border peptidases, the intestinal epithelium also possess brush-border enzymes, such as lactase, maltase, and sucrose, involved in hydrolysis of oligosaccharides to mono- and disaccharides for uptake (38). As with proteolytic enzymes, Salmonella does not secrete glycolytic enzymes capable of degradation of higher oligosaccharides to forms capable of uptake. However, it has been shown that extracellular Salmonella benefits from the glycosidic activities of the gut microbiota in the intestinal lumen, which can generate sugars from the intestinal mucin or glycosylated proteins of the intestinal epithelia (66, 67), although the role of oligosaccharides on the intracellular growth is not known. Although oligosaccharides and other sugars do not play a role in our in vitro studies, it would seem plausible that in vivo the brush-border enzymes involved in hydrolysis of oligosaccharides, which would also be present within the SCV, could also generate mono- and disaccharides for uptake from glycolytic intermediates originating either from the host amylase activity or the gut microbiota.

Although fusion with lysosomes could provide a source of peptides and amino acids, our results are consistent with a more restricted interaction with lysosomal compartments, as indicated by the presence of LAMP-2A but the exclusion of LAMP-2B, both isoforms of which are present at lysosomal membranes but within particular microdomains (50). Likewise, the lysosomal marker LIMP-2 was not found to co-localize with the SCV, nor was it present in significant amounts in purified SCV membranes. It would therefore appear likely that acquisition of the LAMP proteins may occur through interactions with the recently described post-Golgi LAMP protein-containing vesicles that are independent of vesicles involved in delivery of lysosomal enzymes through Man-6-PR-dependent pathways (68). Interaction of the SCV with vesicles originating from the trans-Golgi network has previously been described (69). Interestingly, our study also indicates a high degree of selectivity in the SCV acquisition of the LAMP-2A isoform of LAMP-2, while excluding the LAMP-2B isoform, although both are found at lysosomal membranes (50). Our discovery that intracellular Salmonella co-opts the host’s own cytosolic protein turnover system, CMA, provides an explanation for both the source and mechanism of acquisition of peptides that can support the growth of this intracellular pathogen in the absence of exogenous nutrient sources.

The results of this study provide explanations for a number of observations regarding the resilience of S. enterica as a facultative intracellular pathogen. Acquisition of host-derived cytosolic peptides as a source of carbon units and amino acids would explain the observation that clinical isolates of Salmonella often harbor amino acid auxotrophies or defects in carbon source utilization, yet they remain virulent and pathogenic for both animals and humans (18, 19, 70). Recent studies have also shown that intracellular Salmonella are able to evade the host autophagy response as an innate immune mechanism of pathogen clearance. The autophagy pathway is activated by amino acid starvation of the host cell during the infection process (71). However, whereas both Salmonella and Shigella infections of host cells result in amino acid starvation of the host, in contrast to Shigella, there is a rapid re-establishment of the host cytosolic amino acid pools in Salmonella-infected cells, which limits the metabolic stress induction of autophagy (71). Our results suggest that Salmonella’s utilization of the host’s own protein turnover system rather than cytosolic amino acid pools as a source of amino acids could be an effective strategy for avoiding this innate immune response mechanism. Whether other intracellular pathogens, which remain within a vacuolar compartment, also subvert the host cell CMA pathway to acquire peptides is not known, but this could represent a common strategy among vacuolar intracellular bacterial pathogens.

It has been observed that the long term asymptomatic carriers of Salmonella, both animal and human, often show re-infections and shedding of the micro-organism under stress conditions (72, 73). Interestingly, CMA is also activated under stress conditions (43, 44), suggesting a means whereby intracellular Salmonella may sense the health status of the infected host and adjust its growth rate accordingly. The basal levels of host cell cytosolic protein turnover would provide a steady but possibly low level source of carbon units and amino acids, permitting intracellular Salmonella to persist within infected host cells, particularly within deeper lymphoid tissues. Stress conditions encountered by the host would be expected to activate CMA as a general organism-wide response and to result in a “nutritional shift-up” for Salmonella within infected host cells, leading to reactivation of otherwise slow-growing but persistent Salmonella for increased proliferation and relapse infections of carriers. Finally, we would point out that cytosolic host proteins degraded by the CMA pathway require a targeting motif (43–45). Our results indicate that fusion of this targeting
Intracellular Growth of Salmonella enterica

TABLE 1

Bacterial strains and plasmids used in this study

<table>
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<th>Strains</th>
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</tr>
<tr>
<td>AT1202</td>
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</tr>
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<td>S. typhimurium hisG64, virulent strain</td>
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<tr>
<td>6414</td>
<td>S. typhimurium 4/74 wild type, virulent strain</td>
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<tr>
<td>6220</td>
<td>SL1344 ΔaroDΔphoPΔphoRΔmitrΔrhaBADia</td>
</tr>
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<td>7752</td>
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<tr>
<td>8220</td>
<td>4/74 Δgln::kan ΔmxyXYZ ΔptsG ΔshpT</td>
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<table>
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<th>Relevant features</th>
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<td>kan P_paci::gfpma3 B6K origin</td>
</tr>
<tr>
<td>pACYC184</td>
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<td>pMHEtetCDH</td>
<td>cat tetOP-tyrC p15a origin</td>
</tr>
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</tr>
<tr>
<td>pCMV-GAPDH-HT</td>
<td>bla neo P_CMV-GAPDH-HT SV40 origin</td>
</tr>
<tr>
<td>pKFERQ-DsRed2</td>
<td>bla neo P_CMV::KFERQ-DsRed2-N1 SV40 origin</td>
</tr>
</tbody>
</table>

The abbreviations used are as follows: bla, β-lactamase; cat, chloramphenicol acetyltransferase; kan, kanamycin resistance; neo, neomycin resistance; tet, tetracycline resistance.

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motif to heterologous proteins can direct such proteins to the SCV, suggesting a means for targeting intracellular Salmonella, and possibly other intracellular pathogens, with antibacterial peptides.

Experimental Procedures

Bacterial Strains and Growth Conditions—Bacterial strains and plasmids used in this study are listed in Table 1. Deletion mutants were constructed by directed non-polar gene deletion/replacement with a kanamycin-resistance cassette using the λ Red recombinase methodology as described previously (74). After PCR verification of gene deletions/replacement, bacteriophage P22 lysates were prepared on the mutant strain(s) using the high transducing P22 phage HT105/1 (75), were subsequently used for transduction of kanamycin resistance back into the isogenic wild-type strain, and were again verified by PCR. Elimination of the kanamycin-resistance cassette was performed by introduction of the FLP-recombinase-expressing plasmid, pCP20, followed by growth at 37 °C to eliminate the plasmid (74, 76). Subsequent deletions/gene replacements were introduced by bacteriophage P22 transductions as above. Sequences of the oligonucleotides used for the gene deletions and PCR screening are available from the authors upon request.

Bacteria were routinely grown in L-broth or M9-defined minimal medium and contain 50 g/ml histidine where required (SL1344 and derivatives). Where indicated, M9 minimal medium was supplemented with phenylalanine at 50 μg/ml, casamino acids at 1% (w/v), or heat-inactivated fetal calf serum at 10% (v/v). Dialedyzed casamino acids were prepared by dialysis of a 20% (w/v) stock solution of casamino acids in dialysis tubing (Serva Membra-Cell MWCO 3500, Heidelberg, Germany) against sterile distilled water. Where used for selection of strains or plasmids, carbenicillin was present in the media at 100 μg/ml, chloramphenicol at 15 μg/ml, kanamycin at 50 μg/ml, and tetracycline at 15 μg/ml. Tetracycline at 0.1 μg/ml was used for intracellular induction of CDH expression from plasmid pMHEtetCDH (25). For generation of growth curves, strains were inoculated at 10⁵ CFU in 0.1 ml of medium into replicate wells of a 96-well plate and growth was monitored by determination of the optical density at 600 nm at 15-min intervals over 24 h with shaking at 37 °C on a heated Synergy HT microtiter plate reader (BioTek).

Construction of the CMA Substrate Targeting Plasmid—The plasmid pKFERQ-DsRed2 encoding a fusion of the N-terminal 15 amino acids of the mature Bos taurus RNase A protein containing the consensus CMA-targeting motif KFERQ (accession number NM_181810) with the Discosoma spp. red fluorescent protein DsRed encoded in pDsRed2-N1 (Clontech) was constructed in two steps. The DNA sequence encoding the full-length fusion protein with an optimized Kozak sequence was synthesized and obtained as a clone in the vector pUC57 (GenScript). An Xhol-XbaI fragment harboring the fusion protein coding and upstream sequences was then subcloned into the Xhol and XbaI sites of the eukaryotic expression vector pcDNA3.1 (+) (Invitrogen), placing expression of the fusion protein under transcriptional control of the CMV promoter for eukaryotic expression. Clones used for transfection studies were verified by sequencing and fluorescence studies after transfection of HEK293T cells.

Chemicals and Reagents—Chloroquine diphosphate, DMSO, Triton X-100, amino acids, paraformaldehyde, 3-methyladenine, wortmannin, protease inhibitor mixture (aprotinin, bestatin, E64, leupeptin, and pepstatin A), and diprotin A (Ile-Pro-Ile) were all obtained from Sigma. Proteinase K was obtained from Qiagen. Amino acid mixtures used in cell culture media were purchased from Biochrom.

Cell Culture and Growth Conditions—The human epithelial cell lines HEK293T (ATCC CRL-11268) and LoVo (intestinal epithelia, ATCC CCL-229) were grown in DMEM/Ham’s F-12 salts (1:1) medium (Biochrom). The human macrophage-like cell line THP-1 (DSMZ ACC-16) was cultivated in Iscove’s modified Dulbecco’s medium (Biochrom). Cell culture media were supplemented with 10% fetal calf serum, and cells were grown under standard tissue culture conditions of 37 °C and 5% CO₂. All experiments in the various cell lines were performed within five passages after seeding of the original frozen stocks.

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Cell culture media without glucose, inositol, and amino acids were purchased as a special formulation (Biochrom) and supplemented with separate stocks of serum, glucose, and/or amino acids as indicated. In experiments using dialyzed serum, heat-inactivated serum was dialyzed twice against 10 volumes of sterile deionized distilled water using 3.5-kDa molecular mass cutoff dialysis tubing (Serva Membra-Cell MWCO 3500, Heidelberg, Germany) and was filter-sterilized using 0.22-μm filters.

Intracellular glucose concentrations of LoVo and THP-1 cells were determined by growth of cells in 24-well cell culture plates (Corning CellBind) by ~90% confluence in full medium containing glucose and amino acids. The cell culture medium was then removed; the cells were washed twice with phosphate-buffered basal salts (PBS), and the culture medium in triplicate wells of cell monolayers was then exchanged for medium either with or without the standard concentrations of glucose (Biochrom). 48 h later, triplicate wells of cells grown in either full medium or lacking glucose were then washed twice with ice-cold PBS and lysed by addition of 0.5 ml of glucose assay buffer (BioVision). The cell lysates representing ~10^6 cells/ml were combined and frozen at −80 °C. The frozen cell lysates were rapidly thawed and filtered to remove endogenous enzymes and proteins by centrifugation using 10-kDa molecular mass cutoff spin columns (BioVision). Determination of the glucose concentrations present in the cell lysates were performed using fluorometric assays as recommended by the manufacturer (BioVision), including supplemented samples, controls without added enzyme, and standard curves performed in duplicate in black 96-well flat-bottomed plates (Corning). Fluorescence was determined in a BioTek Synergy HT fluorometer. The assays were performed three times, independently, for each cell line.

Transfections of cell lines were performed using HiPerFect transfection reagent according to the manufacturer's instructions (Qiagen). Stably transfected cell lines were isolated by puromycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmids and scrambled RNA control vector (Sure Silencing LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmid set, Qiagen). Transfections of cell lines were performed using HiPerFect transfection reagent according to the manufacturer's instructions (Qiagen). Stably transfected cell lines were isolated by puromycin selection for the LAMP-2A (Sure Silencing LAMP-2 shRNA plasmid clone 2, Qiagen) and LAMP-1 shRNA plasmids and scrambled RNA control vector (Sure Silencing LAMP-1 shRNA plasmid set, Qiagen), or neomycin selection for the cpm-GAPDH-HT (54) and pcDNA3.1 (Invitrogen) control plasmids.

**Cell Culture Infection Assays**—Cells grown to a cell density of ~2 × 10^5 cells/well in 24-well cell culture plates (Corning Cell-Bind) were infected at multiplicities of infection (m.o.i.) of 1–5 with *Salmonella* strains grown with aeration at 37 °C to late log/early stationary phase (absorbance at 600 nm of ~2–3). Bacteria were collected from 1 ml of culture by centrifugation, and the resulting cell pellets were resuspended in the same volume of cell culture medium. The absorbance of the resuspended cells was determined, and dilutions were made to provide the final m.o.i. based on the average cell density of the confluent monolayers. Infected cell culture plates were centrifuged at 150 × g for 10 min and incubated at 37 °C, 5% CO₂, for 30 min, followed by a change of cell culture medium containing 50 μg/ml gentamycin and further incubation for 60 min. The cell culture medium was then replaced with medium containing 10 μg/ml gentamycin for the remainder of the experiment. At the time points indicated in the figures, cell culture medium was removed from duplicate wells of infected cells, and the cells were washed twice with phosphate-buffered saline and lysed by addition of freshly prepared sterile 0.1% (v/v) Triton X-100 in deionized distilled water. Dilutions of the cell lysates were then plated to agar plates for determination of the intracellular colony-forming units. Infection of cell cultures was performed with cells that had been passaged a maximum of five times from frozen stocks. The intracellular growth was determined as the ratio of intracellular colony forming units (CFU)/ml of cell lysate at 24 h post-infection relative to the intracellular CFU at 4 h post-infection and reported in the figures as relative fold increase.

Where indicated, cells were pre-incubated for 4 h prior to infection with 3-methyladenine (10 mM), cycloheximide (200 μg/ml), ammonium chloride (10 mM), or anti-LAMP-1 or anti-Hsc73 antibodies (10 μg/ml). The intracellular growth assays with inhibitors were performed at least three times, independently, with statistical significance of the mean relative growth determined using the Mann-Whitney U test. In all other intracellular growth assays, statistical significance was determined by performing a Student’s unpaired, two-tailed t test from at least three independent experiments. Statistical analyses were performed using the GraphPad Prism version 5 statistics software.

In infection assays in host cells deprived of glucose and/or amino acids, 24 h prior to the infection assays, the cell culture medium was exchanged after washing with 1× PBS for either complete medium or medium lacking glucose (and inositol), or amino acids, or both. The following day, bacteria were grown as above, resuspended in cell culture medium lacking both sugars and amino acids, and used to infect cell monolayers grown in the combinations of media as described. One hour post-infection, extracellular *Salmonella* were killed by exchange of the respective media for media containing 50 μg/ml gentamycin. Following a 1-h incubation, the cell culture media were replaced again with media containing 10 μg/ml gentamycin for the remainder of the experiment. In assays involving protease inhibitors, cells were treated with a 1:250 dilution of protease inhibitor mixture in DMSO (Sigma) containing an additional dipeptidyl peptidase IV inhibitor, diprotin A (Ile-Pro-Ile, 1.5 mM), for 4 h prior to and during the 1-h infection and gentamycin treatment periods (total of 6 h). Protease inhibition by the protease inhibitor mixture was verified in reactions containing 10 μg of whole cell lysates or 5 ng of purified trypsin using the protease fluorescent detection kit (Sigma) according to the manufacturer’s instructions.

**Determination of CMA Activity**—CMA activity in cells transfected with control, LAMP-1, or LAMP-2A shRNA vectors was performed as described by Seki et al. (54). In brief, as indicated in the figure legends, stably transfected vector control, LAMP-1, or LAMP-2A knockdown cell lines were additionally transfected with a GAPDH-HaloTag (GAPDH-HT) construct in cells cultured in either complete or serum-deprived media. Lysosomes were then labeled with LysoTracker Green (Invitrogen) and GAPDH-HT labeled with TMR ligand. The level of co-localization of green (lysosomes) and red fluorescence (uptake of GAPDH-HT into lysosomes) was determined by fluo-
rescence microscopy and counting of GAPDH-HT-positive lysosomes using a minimum of 50 individual cells.

Chloroquine Resistance Assays—To determine the cytosolic fraction of intracellular Salmonella, chloroquine resistance assays were performed essentially as described previously (30), but using m.o.i. of \( \leq 5 \). Briefly, cell lines were infected with Salmonella as above, and 1 h post-infection, the cell culture medium was exchanged for medium containing 50 \( \mu \)g/ml gentamycin to kill remaining extracellular Salmonella. After 1 h of treatment, the medium was again exchanged for medium containing 10 \( \mu \)g/ml gentamycin, with or without 50 \( \mu \)M chloroquine-diphosphate (CQ), and duplicate wells were immediately sampled for intracellular CFU after two washes with PBS and cell lysis using 0.1% Triton X-100 in deionized distilled water. Where indicated, host cells were pre-treated with 100 nM wortmannin for 30 min prior to and during the infection, as well as during the 1 h of 50 \( \mu \)g/ml gentamycin treatment. The wortmannin was removed, and cells were washed prior to addition of medium containing 10 \( \mu \)g/ml gentamycin with or without CQ for the remainder of the experiment. The concentration of CQ used (50 \( \mu \)M) was determined in preliminary titration experiments with both the LoVo and HEK293T cell lines as the lowest concentration showing the same degree of inhibition of intracellular growth of Salmonella as higher concentrations, with no effects on host cell viability.

Host Cell Viability and Cytotoxicity Assays—The effects of glucose and/or amino acid deprivation on host cell viability and cytotoxicity were performed with replicate wells of cell cultures using Cell Titer-Fluor Cell Viability and CytoTox-ONE Homogeneous Membrane Integrity assays according to the manufacturer’s instructions (Promega).

Microscopy and Co-localization Studies—For microscopy studies, cells were grown on 12-mm diameter glass coverslips and infected at an m.o.i. of 5 with wild-type S. typhimurium strain SL1344 harboring plasmid pGP704gfp (77) as above, then removed from the wells after washing for either fixation or live cell microscopy. At the indicated times post-infection, cells were washed with pre-warmed 1× PBS and fixed in 4% paraformaldehyde in PBS for 15 min at 4 °C. Fixed cells were washed once with 1× PBS and permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed once with 1× PBS, and blocked with 1.5% BSA in PBS for 1 h. Permeabilized cells were incubated with primary antibodies in blocking solution for either 1 h or overnight at 4 °C. Cells were then washed twice with 1× PBS and incubated with the appropriate fluorescently conjugated secondary antibodies for 1 h at room temperature. Coverslips were washed twice and mounted inverted into Mowiol (Sigma) on glass slides and visualized using a Leica TCS SP-2 confocal laser scanning microscope using a ×63 oil immersion objective at ambient temperature. All primary and secondary antibodies used are given in Tables 2 and 3, respectively. Quantification of the co-localization of host proteins with intracellular Salmonella was based on visual inspection of at least 25 infected cells in different fields harboring at least one bacterium in two (LAMP-2B and LIMP-2) or three (LAMP-1, LAMP-2A, and Hsc73) independent experiments. For each infected cell, the total number of bacteria per cell was first determined followed by the number of bacteria found to co-localize with the respective antibody. In some experiments, images were acquired using an Olympus IX81-ZDC2 inverted stage fluorescence microscope and Volocity acquisition software for automated determination of puncta per cell. Graphic displays of the quantification data were generated using SigmaPlot version 11 (Systat Software, San Jose, CA).

Preparation of SCVs—From a stock solution of 3 mg/ml carboxy-coated paramagnetic cobalt nanoparticles (10–20 nm diameter; TurboBeads, Zurich), a 0.5-ml aliquot was sonicated to disperse aggregates, followed by centrifugation at 1000 \( \times g \) for 30 s. Following centrifugation, 0.1 ml of the resulting supernatant was diluted 1:10 in sterile deionized water prior to use. Salmonella strains grown in L-broth were collected from 1 ml of culture by centrifugation in a microcentrifuge at 16,000 \( \times g \) for 5 min. The resulting bacterial pellets were resuspended in PBS and incubated in the presence of a 5:1 ratio of nanoparticles/bacteria at 37 °C for 20 min with shaking. The bacterial suspension was then filtered through a 0.22-\( \mu \)m filter fitted to a 5-ml syringe. The filters were washed twice with 2 volumes of PBS, then the filter was inverted, and the bacteria were recovered in 1 ml of PBS collected into a sterile microcentrifuge tube.

THP-1 human macrophages grown in 6-well cell culture plates were infected at a cell density of 10\(^6\) cells/well with nanoparticle-labeled Salmonella (m.o.i. = 5) and incubated at 37 °C, 5% CO\(_2\). 24 h post-infection, the cell culture media were removed; infected cells were washed twice with PBS and then lysed by addition of 1 ml/well of 0.1% Triton X-100 in water. The resulting cell lysates were then placed in a magnetic field (Single Place Magnetic Stand, Ambion), and the supernatant was carefully removed, discarded, and replaced by 1 ml of a 1× PBS, 2% sucrose solution. The buffer was again removed and replaced by 0.1 ml of the same buffer to concentrate the SCVs, which were then used either for staining with antibodies or subjected to SDS-PAGE and Western blotting. Western blot-
tting was performed using 12% SDS-polyacrylamide gels in which lanes had been loaded with equivalent total protein concentrations determined separately. Western blotting was carried out according to standard procedures using unlabeled primary antibodies and HRP-conjugated secondary antibodies indicated in the figures and listed in Table 2. Proteins were revealed using the ECL detection kit according to the manufacturer’s instructions (Pierce/Thermo Scientific). Quantification of the band intensities for estimation of the relative levels of host proteins present in purified SCVs was determined by normalizing against the signals obtained for GFP using the ImageJ software package version 1.48 (imagej.nih.gov).

Author Contributions—J. F.-I. and P. S. performed the strain constructions and invasion assays of mutants; V. S. performed the microscopy work, CMA inhibitor assays, transfections, and invasion assays; A. T. and A.-C. H.-C. contributed strains and analyzed data; K. T. conceived the study, analyzed the data, and wrote the manuscript. All authors were involved in discussion of results and approved the final version of the manuscript.

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