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Salmonella typhimurium SifA Effector Protein Requires Its Membrane-anchoring C-terminal Hexapeptide for Its Biological Function*

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SifA is a *Salmonella typhimurium* effector protein that is translocated across the membrane of the *Salmonella*-containing vacuole by the *Salmonella* pathogenicity island 2-encoded type III secretion system. SifA is necessary for the formation of *Salmonella*-induced filaments and for the maintenance of the vacuolar membrane enclosing the pathogen. We have investigated the role of the C-terminal hexapeptide of SifA as a potential site for membrane anchoring. An *S. typhimurium* strain carrying a deletion of the sequence encoding this hexapeptide (*sifA*Δ6) was found to be attenuated for systemic virulence in mice. In mouse macrophages, *sifA*Δ6 mutant bacteria displayed a reduced association with vacuolar markers, similar to that of *sifA* null mutant bacteria, and exhibited a dramatic replication defect. Expression of SifA in epithelial cells results in the mobilization of lysosomal glycoproteins in large vesicular structures and Sif-like tubules. This process requires the presence of the C-terminal hexapeptide domain of SifA. Ectopic expression of truncated or mutated versions of SifA affecting the C-terminal hexapeptide revealed a strong correlation between the membrane binding capability and the biological activity of the protein. Finally, the eleven C-terminal residues of SifA are shown to be sufficient to target the *Aequorea* green fluorescent protein to membranes. Altogether, our results indicate that membrane anchoring of SifA requires its C-terminal hexapeptide domain, which is important for the biological function of this bacterial effector.

Salmonellae are the etiological agents of a variety of diseases ranging from gastroenteritis to enteric fever. *Salmonella typhimurium* is the principal agent of food poisoning in humans and causes a typhoid fever-like disease in mice and immuno-compromised humans (1, 2). Ingested *S. typhimurium* crosses the intestinal epithelial barrier through M cells of Peyer's patches (3). Following invasion, the bacterium survives and replicates within macrophages of the spleen and the liver (4, 5). Bacterial multiplication within host cells is essential for virulence, because mutants defective for intracellular replication are attenuated in mice (6, 7). Intracellular replication of *Salmonella* takes place in a membrane-bound compartment, the *Salmonella*-containing vacuole (SCV).[1] Maintenance of the bacteria within this vacuolar enclosure is a key aspect of the virulence process (8, 9).

The nascent vacuole resulting from the bacterium internalization is diverted from the phagocytic pathway and undergoes a specific biogenesis process. The mature SCV is highly enriched in vacuolar ATPase and lysosomal membrane glycoproteins (Lgps) such as Lamp1, Lamp2, and CD 63 (10, 11). However, SCVs are essentially devoid of soluble lysosomal contents (10, 12).

Intravacuolar bacterial replication initiates 3–5 h after infection and correlates with the formation in epithelial cells of unusual tubular membranous structures termed Sifs (for *Salmonella*-induced filaments) (13). Sifs extend from and connect SCVs and are characterized by a high enrichment in Lgps. The formation of Sifs requires a functional type III secretion system (TTSS) encoded by the Salmonella pathogenicity island-2 (SPI-2) (8).

Several Gram-negative pathogens use TTSS to inject proteins, termed bacterial effectors, into the host cell, were they subvert specific host functions. SifA is a SPI-2 TTSS effector (14) which is essential for Sif formation (15). In addition, *sifA*$^-$ mutant bacteria progressively lose their vacuolar membranes in cultured macrophages and epithelial cells, as well as in splenocytes *in vivo* (5, 8, 9). Mutation of *sifA* causes virulence attenuation in mice and a strong replication defect in macrophages (8, 15–17). It is not known whether the absence of Sifs and vacuolar membrane result from the same loss of function.

Other SPI-2 TTSS-translocated proteins, SseF, SseG and SpiC, have also been found to be necessary for the formation of Sifs (17). SpiC was originally described as an inhibitor of interactions between SCV and late endocytic compartments (18). SPI-2-encoded SseF and SseG are present on SCVs and Sifs (19, 20). The role of these proteins in the biogenesis of Sifs remains undetermined. PipB, SseJ, and SifB are other effector

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[1] The abbreviations used are: SCV, *Salmonella*-containing vacuole; Lgp, lysosomal membrane glycoprotein; Sif, *Salmonella*-induced filament; TTSS, type III secretion system; SPI2, *Salmonella* pathogenicity island 2; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; LB, Luria-Bertani (medium); CI, competitive index; FCS, fetal calf serum; PBS, phosphate-buffered saline.

proteins secreted by the SPI-2 TTSS that have been found to localize to Sifs and SCVs (20–22). Transfection of HeLa cells with a plasmid encoding SifA fused to GFP complements the defect of a *sifA*$^-$ strain to induce Sif formation and maintain the vacuolar membrane (8). Furthermore, expression of a GFP·SifA fusion protein in non-infected cells induces the vacuolation of Lamp1-positive compartments and the appearance of tubular structures that resemble Sifs (16).

To gain some insight in the molecular mechanisms of action of SifA, we analyzed the role of a C-terminal, cysteine-rich, hexapeptide in the subcellular localization and biological function of this virulence factor. In the present paper, we show that this domain is necessary for systemic virulence in mice, intracellular replication, and reorganization of the host endocytic compartment. Collectively, our results indicate that SifA requires its C-terminal hexapeptide to achieve its function.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The rabbit antiserum against the Rab7 GTPase has been described previously (23). The mouse anti-Lamp1 H4A3 and rat anti-Lamp2 ABL-93 monoclonal antibodies, both developed by J. T. August, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health, and maintained by the University of Iowa (Department of Biological Sciences, Ames, IA). Dr. S. Kornfeld (St. Louis, MO) kindly provided rabbit polyclonal antibody against cathepsin D. Mouse monoclonal anti-GFP antibody JL-8 was purchased from Clontech. Polyclonal rabbit anti-*S. typhimurium* lipopolysaccharide was purchased from Difco Laboratories. Goat anti-mouse peroxidase was purchased from Sigma. The secondary antibodies Goat anti-rabbit, anti-rat or anti-mouse IgG conjugated to Alexa 488, Alexa 594, or Alexa 680 were purchased from Molecular Probes.

Cell Lines and Culture Conditions—HeLa human epithelial cells and RAW 264.7 mouse macrophage cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), supplemented with 10% fetal calf serum (FCS, Life Technologies, Inc.), 2 mM glutamine, and non-essential amino acids at 37 °C in 5% CO_2.

Bacterial Strains—The *S. typhimurium* strains used in this work were 12023 wild-type strain (NTCC), HH109 (*ssaV*::*aphT*) (24), P3H6 (*sifA*::*mTn5*) (8), and HH215 (*sifA*Δ*6*). HH215 was constructed following the method described in Ref. 25, using pKD4 as a template for PCR amplification with SIFAD1 (5′-GTT AAC CAC GCT ACA CGT TCG CTC AGA ACA ACA AAG CGG CTA ATA AGT GTA GGC TGG AGC TGC TTC-3′) and SIFAD2 (5′-GAC CGA TCA TTC AAG TTC CAC CTT CTT ATT CAG AGG ATG GGG CAT ATG AAT ATC CTC CTT AG-3′) as primers, to generate a chromosomal deletion of *sifA* that lacks the sequence encoding the C-terminal last six amino acids of the resulting protein. The mutated gene was amplified by PCR, and the deletion confirmed by sequencing.

Plasmid pFVP25.1, carrying *gfpmut3A* under the control of a constitutive promoter, was introduced into bacterial strains for fluorescence visualization (26). Bacteria were grown in Luria-Bertani (LB) medium supplemented with ampicillin (50 μg/ml), kanamycin (50 μg/ml), or chloramphenicol (50 μg/ml) as appropriate.

Mouse Mixed Infections—Mice were inoculated intraperitoneally with 10^5 colony-forming unit per mouse, as described previously (27). The spleens were aseptically removed 48 h after inoculation, and bacteria recovered and enumerated after plating a dilution series onto LB agar and LB agar with the appropriate antibiotics. Each competitive index (CI) value is the mean of three independent mice infections and is defined as the ratio between the mutant and wild-type strains within the output (bacteria recovered from the mouse after infection) divided by their ratios within the input (initial inoculum) (28, 29).

Bacterial Infection of HeLa Cells—HeLa cells were seeded onto glass coverslips (12-mm diameter) in 10-cm dishes at a density of 10^6 cells per dish 24 h before infection. Bacteria were incubated overnight at 37 °C with shaking, diluted 1:33 in fresh LB broth and incubated in the same conditions for 3.5 h. The cultures were diluted in Earle's buffered salt solution, pH 7.4 and added to the HeLa cells at a multiplicity of infection of ~100:1. The infection was allowed to proceed for 10 min at 37 °C in 7% CO_2. Cells were washed three times with DMEM containing fetal calf serum (FCS) and 100 $\mu g \cdot ml^{-1}$ gentamicin and incubated in this medium for 1 h, after which the gentamicin con centration was decreased to 10 $\mu g \cdot ml^{-1}$.

Bacterial Infection of Macrophages and Survival Assays—Macrophages were seeded at a density of 10^5 cells per well in 24-well tissue culture plates 24 h before use. Bacteria were cultured overnight at 37 °C with shaking. The cultures were opsonized in DMEM containing FCS and 10% normal mouse serum for 20 min. Bacteria were added to the cells at a multiplicity of infection of ~100:1 and incubated for 20 min at 37 °C in 7% CO_2. Macrophages were washed three times with DMEM containing FCS and 100 $\mu g \cdot ml^{-1}$ gentamicin and incubated in this medium for 1 h. The medium was replaced with DMEM containing FCS and 10 $\mu g \cdot ml^{-1}$ gentamicin for the remainder of the experiment. For enumeration of intracellular bacteria, macrophages were washed three times with PBS and lysed with 0.1% Triton X-100 for 10 min, and a dilution series was plated onto LB agar. Plates were incubated overnight at 37 °C. Colonies were counted. Each time point was performed in triplicate, and each individual experiment was performed three times or more.

Expression of GFP·SifA in HeLa Cells—The full *sifA* ORF was amplified from *S. typhimurium* genomic DNA by PCR using the primer 1 (5′-AAA AAA GAA TTC CAC CAC CAT GCC GAT TAC TAT AGG GAA TGG-3′) and primer 2 (5′-AAA AAA CCC GGG TTA TAA AAA ACA ACA TAA ACA-3′). The PCR product was subcloned into the unique *Eco*R1 and *Xma*I sites of pEGF-C1 vector (Clontech) into the same reading frame as EGFP, generating p*gfp*::*sifA*. The C-terminal deletion or substitution mutants were generated using the primer 1 and second primers designed so that they correspond to the different C-terminal sequences. For fusing the 11 C-terminal residues of SifA to GFP, the following primers (5′-AATTCTGAACAACAAAGCGGCTGTTT ATGTT-GTTTTTTATAG-3′ and 5′-GATCCTATAAAAAACAACATAAACAGC CGCTTTGTTGTTCAG-3′) were annealed and cloned into the *Eco*R1 and *Bam*H1 sites of pEGFP-C1 generating GFP·SifA-(326–336). HeLa cells were transfected with FuGENE 6 (Roche Molecular Biochemicals) following the manufacturer's instructions. Cells were further incubated for 24 h.

Immunofluorescence—Cells grown on coverslips were fixed with 3% paraformaldehyde, pH 7.4, in PBS at room temperature for 10 min. Fixed cells were washed three times in PBS and permeabilized by incubating in PBS containing 0.1% saponin. Saponin 0.1% was included in all subsequent incubation steps. Primary and secondary antibodies were diluted in PBS containing 0.1% saponin and 5% normal horse serum. Coverslips were incubated with primary antibodies for 20 min at room temperature, washed in PBS containing 0.1% saponin, and then incubated with appropriate Alexa goat secondary antibodies. Coverslips were mounted onto glass slides using Mowiol (Aldrich). Cells were observed with a Leica epifluorescence microscope or a confocal laser-scanning microscope LSM510 Zeiss.

Subcellular Fractionation—For each chimeric GFP a 10-cm dish of transfected HeLa cells was chilled on ice and washed three times in ice-cold PBS. Scraped cells were pelleted for 5 min at 100 × *g* in a clinical centrifuge, overlaid with 3 ml of homogenization buffer (250 mM sucrose, 3 mM imidazole, pH 7.4) and centrifuged for 5 min at 1800 × *g*. Cells were resuspended in 0.4 ml of homogenization buffer containing 1 mM phenylmethylsulfonyl fluoride and homogenized by three passages through a 22-gauge needle. After centrifugation for 10 min at 1800 × *g*, the post-nuclear supernatant was collected and centrifuged at 100,000 × *g* for 20 min. The soluble fraction was saved, and the pellet was resuspended in a volume of homogenization buffer. For high pH extraction, the pellet was resuspended in a volume of 100 mM NaOH, pH 11, 50 mM NaCl, incubated for 15 min on ice, and centrifuged at 100,000 × *g* for 20 min. Fractions were loaded on a 12% SDS-PAGE and transferred on Immobilon-P for Western blotting.

RESULTS

A sifAΔ6 Mutant Strain Is Attenuated in Mouse Virulence—The C-terminal hexapeptide of SifA (CLCCFL) contains three cysteine residues that may serve as recognition sites for lipidations. These post-translational modifications are important for membrane attachment and the biological functions of many proteins. To investigate the role of the C-terminal hexapeptide in the function of SifA, a mutant strain (HH215) was constructed carrying a deletion of *sifA* (*sifA*Δ6) in the chromosome. The truncated gene lacks the sequence encoding the C-terminal last six amino acids of SifA. The virulence of this strain was tested by comparing it with the wild-type strain in mixed infections of mice. Bacteria from infected spleen were recovered 48 h after intra-peritoneal inoculation, and the competitive

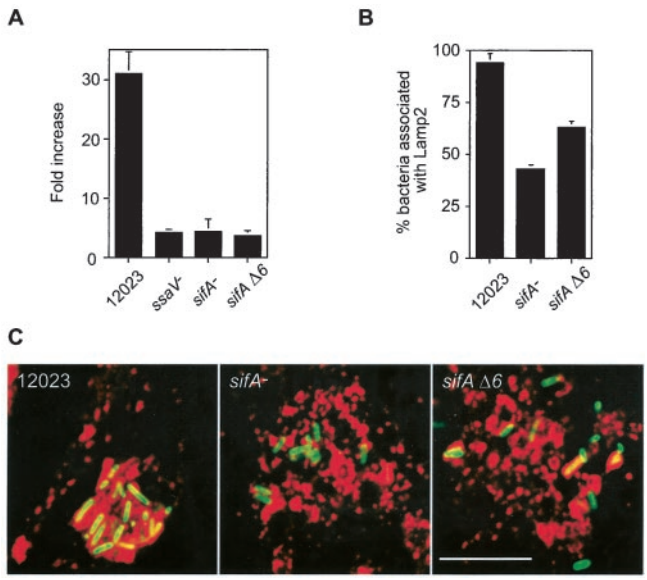

FIG. 1. **A *sifAΔ6* mutant strain is defective for replication and for association with Lamp2 in RAW 264.7 macrophages.** Macrophages were allowed to phagocytose either opsonized wild-type (12023), a SPI-2 secretion defective mutant (*ssaV*$^-$), *sifA*$^-$, or *sifAΔ6* bacteria and were either lysed for enumeration of intracellular bacteria or fixed and examined by epi or confocal fluorescence microscopy. *A*, this graph shows the fold increase of various strains at 16 h post-uptake. The fold increase was calculated as the number of intracellular bacteria at 16 h after uptake divided by their number at 2 h. *B*, the graph shows the percentage of bacteria associating with Lamp2 at 10 h post-uptake. Results in *panels A* and *B* are the means ± S.E. of three independent experiments. *C*, confocal microscopic images of macrophages infected with various strains expressing GFP (*green*) and immuno-labeled for Lamp2 (*red*) show recruitment of cellular Lamp2 to vacuoles containing wild-type but not to vacuoles containing *sifA*$^-$ mutant bacteria. *sifAΔ6* mutant bacteria show an intermediate phenotype. *Bar*, 10 μm.

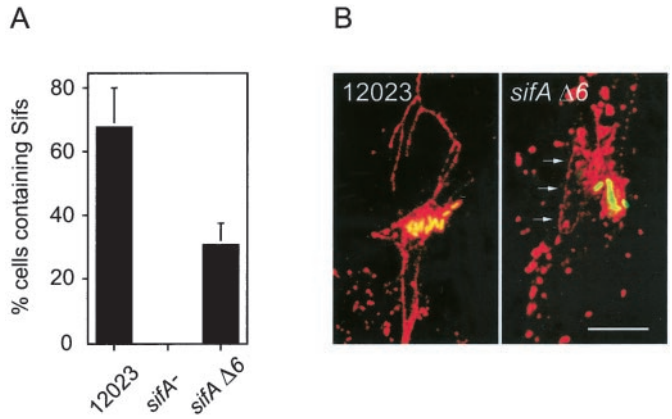

FIG. 2. **A *sifAΔ6* mutant strain is partially defective for Sif formation in HeLa cells.** Cells were infected with either wild-type (12023), *sifA*$^-$, or *sifAΔ6* strains expressing GFP, fixed, and examined by epi or confocal fluorescence microscopy. *A*, each *S. typhimurium* strain was scored for its ability to induce Sif formation 10 h after bacterial entry. Values are given as percentage of infected cells containing Sifs. Results are the means ± S.E. of three independent experiments. *B*, confocal microscopic images of representative examples of cells infected with either wild-type or *sifAΔ6* strains. Sifs in cells infected with the *sifAΔ6* mutant bacteria are shorter and thinner (*arrows*) than in cells infected with wild-type strain. *Bar*, 10 μm.

index was determined as described previously (30). The CI is a sensitive measure of the relative degree of virulence attenuation of a given mutant (27). The CI of the *sifAΔ6* mutant *versus* the wild-type strains was significantly lower than 1.0 (CI = 0.226 ± 0.08) showing that, in the mouse, the net growth of the *sifAΔ6* mutant strain was considerably less than that of the wild-type strain. This result indicates that the C-terminal hexapeptide motif of SifA is required for full virulence in mice and, therefore, SifA function.

A SifAΔ6 Mutant Strain Has a Replication Defect in Macrophages—Because the *sifAΔ6* mutant strain is attenuated in the systemic phase of the infection, and *S. typhimurium* systemic virulence is correlated with its ability to replicate inside host macrophages, this mutant strain was tested for replication over 16 h in the macrophage-like RAW 264.7 cell line. As a control we used an *ssaV*$^-$ mutant strain that is completely defective for SPI-2-mediated secretion and, thus, defective for intracellular replication (31, 32). We observed that the *sifAΔ6* mutant has a replication defect similar to that of *sifA*$^-$ and *ssaV*$^-$ mutant strains (Fig. 1*A*). This indicates that SifA-mediated proliferation in macrophages requires its C-terminal hexapeptide.

Loss of Vacuolar Membrane—SifA is essential to maintain the integrity of the vacuolar membrane, as *sifA*$^-$ mutant bacteria are gradually released into the host cell cytosol (8). The cytosol of macrophages does not support *S. typhimurium* replication (9). Therefore it is possible that the failure of the *sifAΔ6* mutant strain to replicate inside these cells is due to a failure to maintain the vacuolar membrane. To address this question, we compared the level of association with an SCV membrane marker, Lamp2, of *sifAΔ6* mutant bacteria to that of the wild-type and *sifA*$^-$ strain by immunofluorescence analysis. In macrophages, 95% of wild-type and 47% of *sifA*$^-$ bacteria were decorated by the anti-Lamp2 antibody 10 h after bacterial uptake. The *sifAΔ6* mutant displayed an intermediate phenotype with 70% of the bacteria found in association with the membrane marker (Fig. 1*B*). Similar results were obtained in bone marrow-derived macrophages (data not shown).

A drastic reorganization of Lgps to the vicinity of vacuoles occurs in epithelial cells infected by *S. typhimurium*. This process is dependent on the function of SifA (15, 22). In Raw 264.7 macrophages, a similar SifA-dependent phenomenon was observed. Alteration of the distribution of Lamp2 was detected in 65 ± 6 or 5.5 ± 0.7% of macrophages infected with wild-type or *sifA*$^-$ mutant bacteria, respectively (Fig. 1*C*). 27 ± 3% of macrophages infected with the *sifAΔ6* mutant strain exhibited modified Lamp2 distribution (Fig. 1*C*). We concluded that the maintenance of bacteria inside a vacuolar membrane and the recruitment of Lgp-containing compartments require a full-length SifA.

The C-terminal Domain of SifA Plays a Role in the Formation of Sifs—The presence of Sifs is the hallmark of *S. typhimurium* infection of epithelial cells (13), and the *sifA* gene is essential for their formation (15). At 10 h after bacterial entry, Sifs were detected in more than 65% of epithelial cells infected with the wild-type strain, whereas the *sifA*$^-$ mutant strain was unable to induce the formation of such structures (Fig. 2*A*). The *sifAΔ6* mutant strain exhibited an intermediate phenotype, as Sifs were found in ~30% of infected cells (Fig. 2*A*). Where Sifs were observed their morphology was altered, appearing shorter and thinner (Fig. 2*B*). Therefore, we conclude that the C-terminal hexapeptide motif of SifA contributes to the function of this SPI-2 effector in remodeling intracellular compartments.

GFP·SifA but Not GFP·SifAΔ6 Induces the Formation of Enlarged, Late Endosomal Vesicles and Sif-like Structures—Ectopic expression of SifA in epithelial cells complements the failure of a *sifA*$^-$ mutant strain to induce Sif formation (8) and results, in non-infected cells, in the reorganization of late endocytic compartments (16). To investigate the specific contribution of each of the six C-terminal residues to SifA function, we generated a series of *sifA* alleles carrying deletions or substitutions in the sequence encoding the C-terminal region of the protein and ligated them into a vector for transfection from

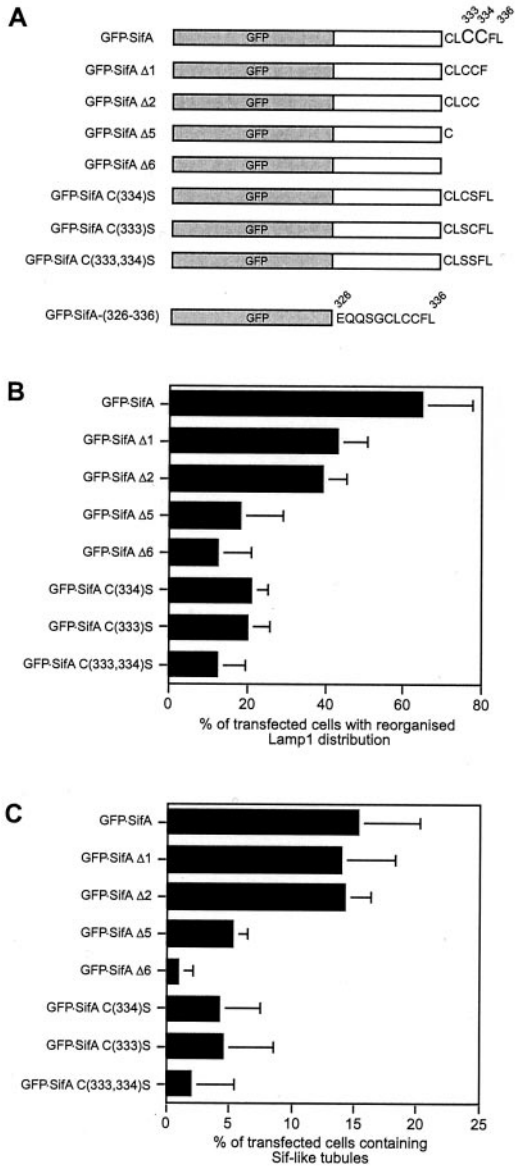

FIG. 3. **C-terminal deletion and substitution mutants and their effects on SifA activities.** *A*, schematic representation of deletion and substitution mutants of GFP·SifA. The positions of cysteine-333 and cysteine-334 and terminal leucine-336 residues are indicated. The number following the Δ symbol indicates the number of C-terminal residues deleted. For GFP·SifA(326–336), residues 326 to 336 corresponding to the eleven C-terminal residues of SifA were fused to GFP. *B* and *C*, mean values ± S.E. of three independent experiments where HeLa cells (n = 100 for each experiment) transfected with the various constructs were scored for the presence of large Lamp1-positive vesicles (*B*) or the formation of Sif-like tubules (*C*). Values are given as the percentage of transfected cells displaying the phenotype.

which GFP-tagged versions of each proteins were expressed (Fig. 3A).

Transfection of epithelial cells with a vector encoding GFP·SifA induces the formation of large Lgp-enriched vesicles and filamentous structures resembling Sifs (16). Compared with non-transfected cells (*star symbol* in Fig. 4*A*), we observed similar large Lamp1-positive vesicles and tubules in cells expressing GFP·SifA (Fig. 4*A*). Tubules were more numerous but thinner than Sifs in infected cells (compare *inset* in Fig. 4*A* with Fig. 2*B*) and will thereafter be referred to as Sif-like tubules. Large Lgp-enriched vesicles and Sif-like tubules were observed in 65 ± 13% and 15 ± 5%, respectively, of GFP·SifA transfected cells. GFP·SifA co-localized with Lamp1 on vesicles and tubules (Fig. 4*A*), and appeared essentially membrane bound.

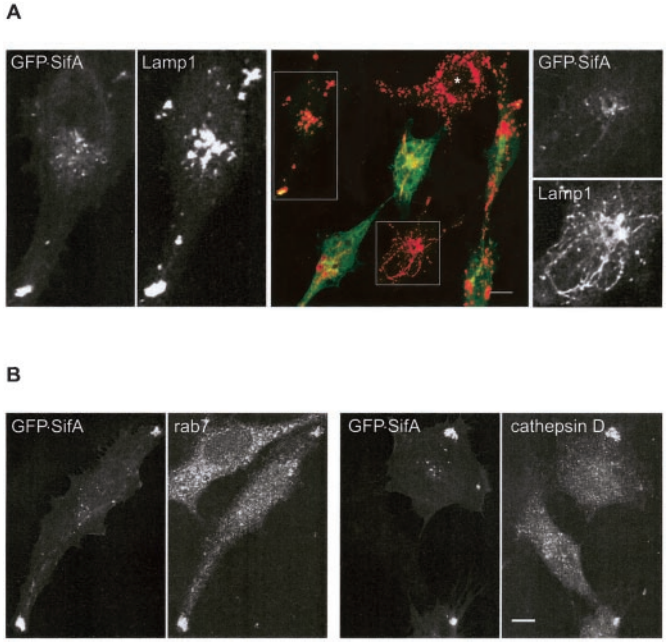

FIG. 4. **Ectopic expression of GFP·SifA in HeLa cell induces the reorganization of late endocytic compartment.** HeLa cells transiently expressing GFP·SifA were fixed, immuno-labeled for various markers, and observed by confocal microscopy. *A*, the color image shows GFP·SifA (*green*) transfected cells immuno-labeled for Lamp1 (*red*). *Gray* scale images present GFP·SifA and Lamp1 labeling of *insets* magnified twice. GFP·SifA induces the formation of large Lamp1-positive vesicles (*top left inset*) and the formation of Sif-like tubules (*bottom middle inset*). For comparison, a non-transfected cell with a typical lysosomal pattern for Lamp1 is marked (*). *B*, HeLa cells expressing or not expressing GFP·SifA and labeled for Rab7 or cathepsin D. Both Rab7 and cathepsin D are present in GFP·SifA-induced vesicles. *Bar*, 10 or 20 μm for magnified *insets*.

To investigate the composition of the SifA-induced structures in more detail, we examined the effect of ectopic expression of these chimeras on the distribution of several other markers of the endocytic pathway. EEA1, a marker for early endosomes, was unaffected by the cellular expression of SifA (data not shown). We also analyzed the effects on the distribution of late endosomal (Rab7 and the cation-independent mannose 6-phophate receptor) and lysosomal (lysobisphosphatidic acid, Lamp2, CD63, vATPase and cathepsin D) markers. In non-transfected cells these two groups of markers were distributed to distinct compartments, and no co-localization of late endosomal and lysosomal markers could be detected by confocal microscopy (data not shown). In contrast, cells expressing GFP·SifA were characterized by the presence of both Rab7 and cathepsin D in the large vesicular compartments in which they co-localized with the over-expressed protein (Fig. 4*B*). This was also the case for other late endosomal and lysosomal protein and lipid markers (data not shown). Therefore, ectopic expression of SifA induces a profound reorganization of late endocytic structures and results in the formation of an unusual vesicular and tubular compartment.

We next examined the intracellular localization and the consequences of the expression of GFP·SifAΔ6 in transfected cells. Compared with cells expressing the full-length version of SifA, a drastic reduction in the frequency of redistribution of Lamp1-containing structures was observed, and Sif-like tubules were very rarely seen (Fig. 3, *B* and *C*). Confocal imaging revealed that the GFP·SifAΔ6 fusion protein was localized both in the nucleus and the cytoplasm (Fig. 5), contrasting with the membrane distribution of GFP·SifA (Fig. 4). Therefore, we conclude that both membrane association and SifA activity in transfected cells are dependent on its C-terminal hexapeptide motif.

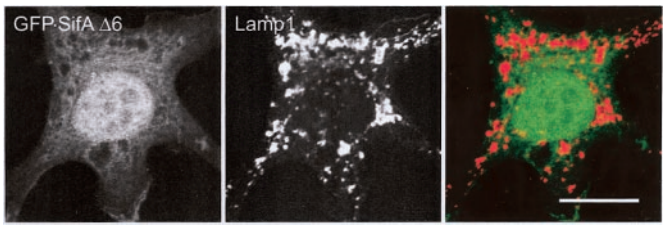

FIG. 5. **GFP·SifAΔ6 does not alter the distribution of Lamp1 in transfected cells.** HeLa cells transiently expressing GFP·SifAΔ6 were fixed, immuno-labeled for Lamp1, and observed by confocal microscopy. *Bar*, 10 μm.

The C-terminal Domain of SifA Contains a Signal for Its Association to Membranes—To gain insight in the function of the C-terminal motif, the distribution of wild-type and mutant versions of the GFP-tagged version of SifA between soluble and membrane fractions of transfected cells was analyzed (Fig. 6). GFP·SifA was found to associate with the membrane fraction, whereas the majority of GFP·SifAΔ6 was found in the cytosol (Fig. 6*A*), consistent with fluorescence microscopic observations (Fig. 5). Removal of the last or the last two amino acid residues did not result in a significant change in the distribution of the protein. By contrast, the deletion of the five C-terminal amino acid residues rendered the protein mostly cytosolic (Fig. 6*A*).

The C-terminal hexapeptide of SifA contains three cysteine residues, two of them being removed in the GFP·SifAΔ5 mutant (Cys-333 and Cys-334, see Fig. 3*A*). To determine the relative importance of each of these residues, we substituted each cysteine with serine, either individually or two at a time, and tested their ability to mediate association with membranes in transfected cells. We found that the membrane association of the double C333S/C334S and both of the individual C333S and C334S mutants was strongly reduced and comparable with that of the GFP·SifAΔ5 mutant protein (Fig. 6*C*). We conclude from these results that both Cys-333 and Cys-334 are required for the membrane association of SifA.

Cysteine residues in position 333 and 334 could act as isoprenylation or palmitoylation sites. Such protein lipidations stably anchor proteins to membranes, rendering them resistant to extraction by alkali. To investigate the possibility that SifA is tightly associated with membranes, membrane fractions from cells expressing GFP·SifA were treated with 0.1 M sodium hydroxide. As shown in Fig. 6*C*, the membrane association of GFP·SifA resisted such high pH treatment. As expected, similar results were obtained for Rab7, which associates with membranes by virtue of isoprenylation, whereas calreticulin, which associates with membranes by binding to the KDEL receptor, was extracted by this treatment (data not shown). These results suggest that SifA undergoes lipidation involving its cysteine residues.

The C-terminal Domain of SifA Is Sufficient to Target GFP to Membranes—SifB is another *S. typhimurium* SPI-2 TTSS effector (20). In contrast to GFP·SifA, GFP·SifB was found to be cytosolic upon expression in HeLa cells (data not shown). SifB exhibits 26.4% identity to SifA over the length of the proteins. However the last 11 amino acid residues of the C-terminal domain of SifA are absent in SifB. To examine the effectiveness of this domain as a membrane targeting signal, we fused the 11 C-terminal residues of SifA to GFP or GFP·SifB, generating GFP·SifA-(326–336) (see Fig. 3*A*) or GFP·SifB·SifA-(326–336), respectively. As shown in Fig. 7*A*, a fluorescence microscopic observation of transfected HeLa cells revealed that GFP·SifA-(326–336) appeared to be essentially membrane associated contrasting with the cytosolic distribution of GFP. Biochemical analysis of fractionated transfected cells confirmed that GFP·SifA-(326–336) (Fig. 7*B*) and GFP·SifB·SifA-(326–336)

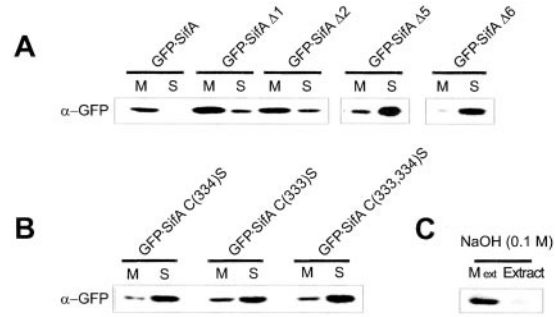

FIG. 6. **The C-terminal domain of SifA contains a signal for membrane attachment.** *A* and *B*, HeLa cells expressing either GFP·SifA or various deletion or substitution mutant in the C-terminal domain of SifA were homogenized. Post-nuclear supernatants were fractionated into membrane (*M*) and soluble (*S*) fractions, run on a SDS-PAGE, and Western blotted with the use of a mouse monoclonal anti-GFP antibody. *C*, total membranes of HeLa cells expressing GFP·SifA were extracted at high pH and fractionated again into extracted membrane (*M ext*) and extract fractions.

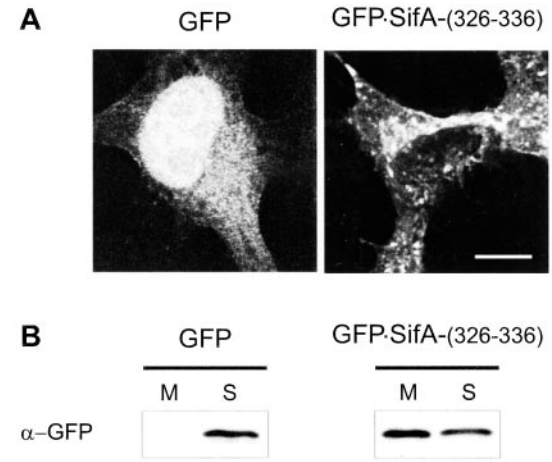

FIG. 7. **The eleven C-terminal residues of SiFA are sufficient to target GFP to membranes.** Intracellular distribution of ectopically expressed GFP or GFP·SifA-(326–336) was observed by microscopy or cellular fractionation. *A*, typical fluorescence microscopy images of HeLa cells expressing each construct. *Bar*, 10 μm. *B*, transfected cells were homogenized. Post-nuclear supernatants were fractionated into membrane (*M*) and soluble (*S*) fractions, run on a SDS-PAGE, and Western blotted using a mouse monoclonal anti-GFP antibody. GFP·SifA-(326–336) is essentially membranous, whereas GFP is cytosolic.

(data not shown) partitioned to the membrane fraction and resisted to high pH extraction (data not shown). These experiments demonstrate that the eleven C-terminal residues of SifA are sufficient to target cytosolic proteins to membranes.

Membrane Association of SifA Is Required for Its Biological Activity—Removal of the last or two last residues only slightly reduced the biological activity of SifA, as measured by its ability to induce formation of Lgp-enriched vesicles and Sif-like tubules in transfected cells (Fig. 3, *B* and *C*). This ability was greatly decreased when the C-terminal 5 residues were removed. Interestingly, some residual activity was still detected in cells transfected with GFP·SifAΔ5 that was consistently higher than that detected in cells transfected with GFP·SifAΔ6. Ectopic expression of SifA alleles carrying mutations in either Cys-333, Cys-334, or both, induced residual reorganization of Lgp-compartments in a manner similar to that induced in cells expressing the GFP·SifAΔ5 mutant protein. These results establish a direct correlation between the membrane association of the various deletion or substitution mutants and their ability to reorganize late endocytic compartments. We conclude from

these results that the biological activity of SifA depends on its membrane binding capacity.

DISCUSSION

In the present paper we have investigated the structure-function relationship of SifA. This *Salmonella* protein is synthesized after bacterial entry into host cells and is translocated across the vacuolar membrane into host cell membranes by the SPI-2-encoded TTSS (8, 33). SifA was originally described as essential for the formation of Sifs in epithelial cells (15). We have previously shown that, in absence of this effector protein, *S. typhimurium* is unable to preserve the integrity of the vacuolar membrane in which it is enclosed and is progressively released into the host cytoplasm (8).

We are interested in understanding how SifA interferes with the host cellular machinery. We hypothesize that SifA directly competes with a host component. However, neither a BLASTp against protein databases nor a search for proteins signatures revealed significant similarities with eukaryotic proteins. PSORT analysis of SifA revealed the presence, at the C terminus, of a putative isoprenylation motif similar to those found on Rab proteins (CAA*X* boxes are CC, C*X*C, CC*XX*, CC*XXX*; C is cysteine, A is aliphatic and *X* can be any amino acid residue). Rab GTPases are important regulators of different steps of vesicular transport in eukaryotic cells (for review see Refs. 34–36). Isoprenylation is necessary for membrane binding and biological activities of Rab proteins (37). Therefore, we were interested in exploring the significance of this motif on a prokaryotic protein, SifA, which affects intracellular trafficking in eukaryotic cells.

The functional importance of the C-terminal hexapeptide is illustrated by the virulence attenuation of the *sifAΔ6* mutant strain in the mouse model of systemic infection. In macrophages, the *sifAΔ6* mutant strain displayed a replication defect comparable with that of the *ssaV*$^-$ and *sifA*$^-$ strains. In the case of the *sifA*$^-$ strain, this defect has been shown to be mainly due to its inability to replicate into the macrophage cytosol (9). Despite its strong replication defect, only 30% of *sifAΔ6* mutant bacteria lost their vacuolar membranes. *sifA*$^-$ mutant bacteria that remain enclosed within a vacuole are still not capable of replicating in either macrophages or epithelial cells (9), suggesting that SifA function is also necessary for the maturation of the SCV into a compartment that allows bacterial replication. Our results show that, beyond the phenotype of vacuolar membrane loss, the *sifAΔ6* mutant strain has an additional defect for replication inside the vacuole, suggesting that the C-terminal hexapeptide is also necessary for SCV maturation into a compartment permissive for replication.

In both macrophages and epithelial cells the late phase of infection is marked by a dramatic redistribution of Lgps toward the SCVs, as well as a disappearance of the vesicular pattern of lysosomes. This SifA-dependent event is rarely observed in macrophages infected with the *sifAΔ6* mutant strain. It suggests that, in the absence of its C-terminal hexapeptide, SifA is functionally defective. The low number of cells displaying Sifs and their altered morphology in cells infected with the *sifAΔ6* mutant is likely to result from the same loss of function.

The functional defects observed with the *sifAΔ6* mutant strain, are correlated with the failure of ectopically expressed SifAΔ6 to induce the reorganization of late endocytic compartments that is observed in cells expressing the full protein. GFP·SifA induces the formation of large vesicles or large clusters of vesicles that exhibit both late endosomal and lysosomal markers. In contrast to what has been observed previously with GFP·SifA (16) we found that cathepsin D is present in GFP·SifA-induced vesicles. GFP·SifA also induces the formation of Sif-like tubules. These are more numerous but thinner than the Sifs found in infected epithelial cells. In cells expressing Lamp1·GFP we have observed that lysosomes are very dynamic organelles from which tubules are constantly forming, growing, shrinking, and contacting other tubular or vesicular structures. In contrast, in cells expressing Lamp1·GFP and infected with wild-type *S. typhimurium*, Sifs are essentially non dynamic.[2] The function of SifA could be to stabilize tubular structures, therefore favoring membrane exchanges between the SCV and host compartments. Large vesicles in SifA-expressing cells may result from unbalanced membrane exchanges between late endocytic structures.

GFP·SifA is essentially membrane bound, whereas the functionally inactive GFP·SifAΔ6 is cytosolic. Deletion mapping of the C-terminal domain showed that a SifA mutant protein with a deletion of the last two amino acid residues (GFP·SifAΔ2) retains its membrane binding ability as well as full activity. In contrast, deletion of the pentapeptide motif LCCFL as well as the cysteine to serine substitution of residues 333 and 334 abolished both membrane association and biological functions of SifA. Considering the homology of SifA C-terminal domain with eukaryotic CAA*X* motifs, the crucial role of cysteine residues, and the ability of SifA to resist extraction by alkali, it seems very likely that the cysteine residues 333 and 334 of SifA are sites of lipidation.

To investigate further the contribution of the C-terminal domain of SifA as a membrane targeting signal, its 11 C-terminal residues were fused to the cytosolic proteins GFP and GFP·SifB. The rationale for this experiment is based on the observation that the similarity between SifA and SifB excludes the last 11 amino acids residues of SifA, which are absent in SifB. The membranous localization of both GFP·SifA (326–336) and GFP·SifB·SifA (326–336) demonstrates that the 11 C-terminal amino acid residues of SifA contain a signal that is sufficient to target this protein to membranes. One can hypothesize that this peptide sequence is either recognized by a membrane receptor or contains a consensus sequences for lipidation. Very short peptides containing consensus lipidation sequences (4–16 amino acid residues) have been used to target various GFP variants to membranes (38). The resistance of GFP·SifA and GFP·SifA-(326–336) to alkali extraction and the specific role of cysteine residues 333 and 334 in the partition of SifA are consistent with the lipidation hypothesis. Indeed, the myristoylation of type III effector proteins from *Pseudomonas syringae* (a plant pathogen) by the host cell has been already reported (39). Interestingly, myristoylation targets Avr proteins to membranes, enhances their functions, and is required for virulence. However, lipidation of SifA by the host cells remains to be demonstrated.

Overall, our analysis of mutants shows that SifA must be membrane-bound to be fully functional and has revealed a crucial role of its C-terminal domain as a membrane-targeting signal. Further work is needed to establish the molecular function of SifA and to understand how this function confers a selective advantage to *S. typhimurium* within the host cell.

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Salmonella typhimurium SifA Effector Protein Requires Its Membrane-anchoring C-terminal Hexapeptide for Its Biological Function

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