A Salmonella typhimurium Effector Protein SifA Is Modified by Host Cell Prenylation and S-Acylation Machinery*

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SifA is a Salmonella effector protein that is required for maintenance of the vacuolar membrane that surrounds replicating bacteria. It associates with the Salmonella-containing vacuole but how it interacts with the membrane is unknown. Here we show by immunofluorescence, S100 fractionation and Triton X-114 partitioning that the membrane association and targeting properties of SifA are influenced by a motif encoded within the C-terminal six amino acids. This sequence shares homology with both CAAX and Rab geranylgeranyl transferase prenylation motifs. We characterized the post-translational processing of SifA and showed that the cysteine residue within the CAAX motif is modified by isoprenoid addition through the action of protein geranylgeranyl transferase I. SifA was additionally modified by S-acylation of an adjacent cysteine residue. Similar modifications to host cell proteins regulate numerous functions including protein targeting, membrane association, protein-protein interaction, and signal transduction. This is the only known example of a bacterial effector protein that is modified both by mammalian cell S-acylation and prenylation machinery.

Salmonella enterica serovar Typhimurium is a leading cause of gastroenteritis in humans and initiates a systemic disease in mice resembling typhoid fever. It is a facultative intracellular pathogen that, after oral ingestion, invades through cells of the intestinal epithelium to survive and replicate within macrophages of the liver and spleen (1, 2). In non-phagocytic cells it enters the cytosol (4). Uptake of bacteria is associated with remodeling of the host cell cytoskeleton (reviewed in Ref. 5).

Upon invasion, Salmonella resides within a membrane-bound compartment known as the Salmonella-containing vacuole (SCV). Maintenance of bacteria within this compartment is necessary for replication and survival (6). There are differing views concerning the biogenesis of the SCV and this may in part reflect differences in the nature of the cell type studied. A unifying theme is that in all cell types investigated the SCV is considered a modified endosomal compartment that diverts from the normal phagocytic pathway by a process that involves modulation of the host intracellular environment (7–9). In epithelial cells, the SCV rapidly loses early endocytic markers such as EEA1 and the transferrin receptor and acquires a subset of late endosomal/lysosomal membrane marker proteins such as LAMP-1, LAMP-2, CD63, and vATPase (10–13). Lysosomal enzymes that require targeting by the mannose-6-phosphate receptor are largely excluded and direct contact with late endosomal compartments appears not to occur (7).

The early stages of infection and SCV maturation are followed by a lag phase, after which bacteria start to replicate. A second TTSS, encoded by Salmonella pathogenicity island 2 (SPI-2), is required for maintenance of the SCV during this period of intracellular replication. Several effector proteins have been identified and are encoded both within and outside the SPI-2 pathogenicity island (14). Their secretion can be induced in vitro by acidic conditions suggesting that reductions in pH, usually associated with phagosome maturation (15), may trigger SPI-2 translocation in vivo (16).

SifA is responsible for inducing the formation of tubular membrane structures (Sifs), which appear to be extensions of SCVs (17, 18) and is necessary for Salmonella virulence (18). It has recently been implicated in the down-regulation of surface MHC in Salmonella-infected cells (19). SifA is proposed to control membrane fusion events and function by providing sufficient membrane to form the growing SCV that surrounds replicating bacteria (20). Transport and fusion between late endosomes and lysosomes is controlled by the small GTPase, Rab7. Ectopic expression of RILP, a Rab7 effector, has recently been shown to recruit the dynin/dynactin motor complex to the SCV (21). This resulted in fusion of the SCV with lysosomes suggesting that Salmonella may modulate SCV maturation by interfering with dynin-mediated vesicular transport (21). SifA has been shown to interact with Rab7 and may function by uncoupling Rab7 from RILP thereby enhancing kinesin-driven Sif formation (22). SifA-mediated uncoupling of Rab7 from RILP would provide a mechanism for influencing SCV fusion with lysosomes.

The mechanism by which SifA localizes to the SCV is unknown. However, a putative prenylation signal similar to that found on members of the Rab family of proteins is present in the C-terminal region of the protein (23). This is intriguing...
given the localization of Rab7 to the SCV and the recent demonstration of a physical interaction between Rab7 and SifA.

In this study we analyze the potential prenylation motif in the C-terminal region of SifA and characterize its requirement for membrane association of SifA. We show by sequence analysis that the motif aligns with consensus motifs utilized by all known prenyltransferases, protein geranylgeranyl transferase I (RGGT), protein farnesyl transferase (PFT), and Rab geranylgeranyl transferase (GGT). Our studies conclude that SifA is modified by prenylation, through the action of geranylgeranyl transferase I. We also show that it is further modified by S-acylation, an addition that is regarded as a reversible second signal in similarly modified proteins.

### MATERIALS AND METHODS

#### Cell Culture, Transfection, and Immunofluorescence—

293T cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin at 37 °C in 5% CO₂. Transfection of plasmid constructs was performed using Effectene reagent following the manufacturer's instructions (Qiagen). Immunofluorescence analysis was performed as previously described (24). Samples were analyzed using a Zeiss Axiohot fluorescence microscope (100× objective).

**Plasmid Construction—** SifA-GFP was generated by PCR amplification of SifA from *S. enterica serovar Typhimurium* genomic DNA using SifA-GFPfor and SifA-GFPrev primers. This introduced an HA tag into SifA. PCR products were cloned into the PCR2.1-TOPO vector, and clones verified by DNA sequence analysis.

**Generation of Salmonella Strains—** Strains *sifA*:331S and *sifA*:333S were generated by site-directed mutagenesis of *Salmonella* 12023 using the suicide vector pDS132 (25). A 1830 bp SifA insert, containing all 1011 bp of coding sequence of SifA from the start ATG to stop TAA codons and an additional 819 bp of sequence 3' of the stop codon, was cloned into a SacI/XbaI-digested pDS132 as a three insert ligation. This was cloned into a BamHI fragment excised from the GFP-SifA vector, encoding SifA from the initiating ATG codon to the BamHI site at position 834; 2) a BamHI-EcoRI fragment of SifA generated by PCR of HA-SifA-CLCCFL, HA-SifA-CLSCFL, or HA-SifA-SLCCFL and coding for wild type, −CLSCFL, and −SLCCFL sequences, respectively; 3) a S22 bp EcoRI to XbaI fragment encoding genomic sequence 3 of the SifA gene and generated by PCR of genomic DNA using primers 5'-AGAATTCACATCAGACGACGCTTTCTTGACCAGTCA-3' and 5'-TGAATTCACATCAGACGACGCTTTCTTGACCAGTCA-3' (restriction sites in bold). This placed the −CLCCFL region centrally within the insert. All restriction site junctions and PCR fragments were confirmed by DNA sequence analysis. Plasmid integration into the wild-type 12023 recipient chromosome was selected for by plating on chloramphenicol. Selection for the second recombination event was through growth on 5% sucrose without NaCl. Identification of homologous recombinants was by PCR using primers SifA22 5'-CCGGAACCCGCTTCAATACGCG-3' and substSifA22 5'-CCGGAACCCGCTTCAATACGCG-3'. Mutated genes were amplified by PCR using primers SifA25 5'-AGCAGATCTACCATGTACCCATACGACGTCCCAGACTACGCTCC-3' and SifA26 5'-CCAGGTTTATCTTCTTTTTATAAGAATTC-3' and substitution of the various cysteine residues confirmed by DNA sequence analysis.

**Mouse Survival and Macrophage Replication Assays—** Macrophage infections were performed essentially as described by Beuzon et al. (20). Bacterial viable counts were performed on spleen and liver homogenates on day 5 and the significance of results assessed by Student’s t-test analysis.

### TABLE I

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence</th>
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<tr>
<td>SifA-GFPfor</td>
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<td>SifA-GFPrev</td>
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* Restriction sites used in cloning of PCR products are underlined.

**Triton X-114 Partitioning—** Triton X-114 extractions were performed essentially as described by Bordier (26). Briefly, cells were harvested and resuspended in 1% Triton X-114 containing protease inhibitors (Complete Mini EDTA-free, Roche Applied Science). Proteins were ex-
tracted by rotating for 1 h at 4 °C. Insoluble debris was removed by microcentrifugation at 13,000 rpm for 10 min at 4 °C, the pellet reextracted and the two detergent supernatants combined. Phase separation was achieved by incubating the detergent supernatants at 37 °C for 5 min, followed by microcentrifugation at 13,000 rpm for 10 min at 25 °C. The aqueous phase was removed, adjusted to 1% Triton X-114 and repartitioned as above. The detergent phases were combined and adjusted to the same volume as the aqueous phase. Proteins were precipitated by addition of trichloroacetic acid to 10% and pellets washed in acetone. Aqueous and detergent fractions were resuspended to the same volume prior to analysis.

Subcellular Fractionation by S100 Centrifugation—Subcellular fractionation was performed as described by Gomes et al. (27). After three freeze-thaw cycles cells were repeatedly passed through a 25-gauge needle. Cell debris was removed by low speed centrifugation (5000 × g for 10 min) and the postnuclear supernatant centrifuged at 100,000 × g for 60 min. The low speed fraction, P100 pellet and S100 cytosolic fractions were adjusted to the same volume, precipitated by addition of trichloroacetic acid to 10%, acetone-washed and resuspended to the same volume.

Antibody Reagents—Antibodies used were anti-HA (clone 3F10 Roche Applied Science), anti-GFP (JL-8 BD Biosciences), anti-HSP70 (Stressgen Biotechnologies, SPA-822) and anti-HLA class I (HC10 courtesy of Dr. P. Lehner). Rabbit anti-mouse HRP conjugate second layer (Stressgen Biotechnologies, SPA-822) and anti-HLA class I (HC10 courtesy of Dr. P. Lehner) were used in Western blotting (DakoCytomation). Immunoprecipitation of HA constructs was performed using anti-HA affinity matrix reagent (Roche Diagnostics).

SDS-PAGE Analysis and Immunoblotting—Proteins were resolved by standard 12.5% SDS-PAGE electrophoresis, as described by Sambrook et al. (28), using a Bio-Rad Mini Protein II (Bio-Rad). Following SDS-PAGE, gels were rinsed in transfer buffer (20 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) and proteins transferred onto Hybond ECL nitrocellulose membrane (Amersham Biosciences). Transfer was confirmed by staining membranes with 0.1% Ponceau S (Sigma). Membranes were washed, incubated in blocking buffer (5% milk powder, 0.2% Tween-20 in phosphate-buffered saline) for 1 h to overnight prior to incubation with antibody in blocking buffer. Detection was by enhanced chemiluminescence (Amersham Biosciences) and exposure to Hyperfilm ECL chemiluminescent film. Quantitation was performed using the AlphaEaseFC imaging system and software.

In Vitro Translation and Prenylation—All constructs were transcribed into mRNA using the RibomAX™ T7 Large Scale RNA Production System (Promega Corporation). Proteins were synthesized using the Nuclease Treated Rabbit Reticulocyte Lysate System (Promega Corporation). Translation efficiency was monitored by labeling with [35S]cysteine and methionine (PRO-MIX, Amersham Biosciences). For [3H]mevalonic acid labeling, 1 µg of RNA was translated in a 50-µl reaction containing 200 µCi of [3H]mevalonic acid (American Radiolabeled Chemicals Inc, code ART 334) as described (29). For immunoprecipitation, the in vitro translation reaction was stopped by addition of 1 ml of 1% Nonidet P-40 Lysis buffer (1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5, 5 mM iodoacetamide, 2 mM phenylmethylsulfonyl fluoride). Samples were incubated with 14°C for 30 min and insoluble material pelleted in a microcentrifuge at 4°C for 10 min at 13,000 rpm. The supernatant was transferred to a fresh tube, and 100 µl of anti-HA Affinity Matrix (Roche Diagnostics) added. Tubes were incubated with rotation at 4°C for 60 min. The matrix was pelleted for 10 s at full speed in a microcentrifuge, washed three times with fresh lysis buffer, and finally resuspended in 50 µl of SDS loading buffer. Samples were resolved by 12.5% SDS-PAGE, gels were fixed (5% methanol, 7.5% acetic acid) for 10 min and impregnated with Amplify fluorographic reagent (Amersham Biosciences). Gels were dried and exposed to preflashed Hyperfilm ECL chemiluminescent film at −80°C.

Prenylation inhibitors, GGTI-298 and PTI-277 (CalBiochem), specific inhibitors of geranylgeranyl transferase I and farnesyl transferase, respectively, were reconstituted immediately prior to use and added directly to the in vitro translation mix immediately prior to incubation at 37°C.

S-Acylation Analysis of HA-SifA Constructs—Protein acylation analysis was undertaken as described by Coligan et al. (29). Briefly, 293T cells were plated to 70% confluency prior to transfection. After 24 h, cell culture medium was replaced with labeling medium containing 5 mM sodium pyruvate. After 60 min [9,10-3H]palmitic acid (PerkinElmer Life Sciences) was added to 250 µCi/ml, and cells were incubated for 7 h. Cells were harvested, lysed by addition of 1 ml of 1% Nonidet P-40 lysis buffer, and immunoprecipitated with 100 µl of anti-HA affinity matrix (Roche Diagnostics), as described above. Samples were resuspended in SDS loading buffer containing 10 mM dithiothreitol and heated for 3 min at 80°C prior to PAGE separation. Subsequent processing was as described for prenylation analysis.

Sodium Carbonate Extraction—The pellet fraction from an S100 centrifugation was resuspended in fresh 0.1 M Na2CO3 for 15 min on ice. Samples were centrifuged at 100,000 × g for 60 min, 4°C. The soluble fraction was removed and sodium carbonate extraction of the pellet fraction repeated. The soluble fractions were pooled. P100 pellet and S100 soluble fractions were brought to the same volume, precipitated by addition of trichloroacetic acid to 10%, acetone washed, and resuspended to the same volume in sample buffer prior to analysis by SDS-PAGE.

RESULTS

To investigate the intracellular localization of SifA, two constructs were generated whereby GFP was fused to either the N (GFP-SifA-CLCCFL) or C terminus (SifA-GFP) of SifA. Following transfection into 293T cells, GFP-SifA-CLCCFL localized to the plasma membrane and to structures concentrated in the perinuclear region (Fig. 1). Nuclear localization was not observed. In contrast, SifA-GFP was distributed throughout the cell with no clear plasma membrane or perinuclear accumulation. Nuclear localization was also observed. This suggested...
that the location of the GFP tag could influence targeting of SifA, possibly by masking a localization signal. Sequence analysis suggested the presence of a potential C-terminal prenylation motif \(33^{\text{L}}\text{CLCCFL}\), as has previously been observed (23, 30). The importance of this sequence for intracellular localization was demonstrated in cells transfected with GFP-SifA-GFP. This confirmed that the C-terminal region was important for localization of SifA, possibly by functioning as a site for isoprenoid addition.

From sequence analysis alone it was not possible to establish whether SifA would be modified preferentially by PGGT, PFT, or RGGT (Fig. 2). The motif was homologous to prenylation signals associated with the Rab-GT-Pase family of proteins (23, 30). As the majority of Rab proteins are modified by the addition of two geranylgeranyl groups it would be reasonable to assume that this would also be the case for SifA. Such additions would be predicted to confer strong membrane binding properties to the modified protein. However, the SifA-encoded sequence could also be aligned with motifs present in proteins modified by either PGGT or PFT (Fig. 2), which modify proteins containing related CAXX motifs (31). CAXX motifs contain the critical cysteine residues onto which the isoprenoid group is covalently attached, generally followed by two aliphatic residues in position \(\alpha_1\) and \(\alpha_2\). A leucine in position \(\chi\) favors modification by PGGT, whereas methionine, serine, or glutamine favor modification by PFT (31). SifA aligns more closely with members possessing additional cysteine residues than those containing an associated polybasic region (Fig. 2). SifA also possesses a C-terminal leucine residue, the amino acid favored by PGTG, to determine if SifA was modified by isoprenoid addition and to gain insight into the nature of the prenyltransferase involved, each cysteine residue (Cys\(^{331}\), Cys\(^{333}\), Cys\(^{334}\)) in the GFP-SifA-CLCCFL motif was mutated to serine, individually and in combination. After transient transfection, the cellular localization of the mutated proteins was monitored by immunofluorescence microscopy (Fig. 1). Substitution of all three cysteine residues to serine (GFP-SifA-SLSSFL) resulted in an intracellular distribution pattern similar to GFP-SifA\(^{36}\) and SifA-GFP. Of the single cysteine to serine mutations, Cys\(^{333}\) was the most dramatic (GFP-SifA-SLCSFL) and was associated with substantial cytosolic and nuclear localization but with less clear plasma membrane and perinuclear accumulation. Modification of residues Cys\(^{331}\) (GFP-SifA-SLCCFL) or Cys\(^{334}\) (GFP-SifA-CLCSFL) increased the level of cytosolic fluorescence. However, plasma membrane and perinuclear accumulation were also seen, as for the wild-type construct. Mutation of two cysteine residues in combination Cys\(^{331}\)/Cys\(^{333}\) (GFP-SifA-SLSCFL) resulted in drastic localization patterns similar to the deletion mutant, whereas mutation of Cys\(^{331}/334\) (GFP-SifA-CLSSFL) resulted in predominant cytosolic and nuclear localization but with some apparent perinuclear localization. The immunofluorescence analysis suggested that Cys\(^{333}\) was the critical residue influencing localization but that residues Cys\(^{331}\) and Cys\(^{333}\) were also influential.

The addition of isoprenoids to many proteins can be monitored indirectly by changes in membrane association, Triton X-114 partitioning and increased mobility in SDS-PAGE (32). To investigate potential prenylation of SifA, three of the SifA constructs, GFP-SifA-CLCCFL, GFP-SifA-SLSSFL, or GFP-SifA-GFP were harvested and split into three equal fractions. Results shown are representative of two experiments. A, cells from fraction 1 were lysed in 1% Nonidet P-40 and subjected to SDS 12% PAGE separation and Western blot analysis with the anti-GFP monoclonal antibody JL-8. All SifA constructs were expressed at a similar level but show no difference in PAGE mobility. B and C, cells in fraction 2 were subjected to S100 fractionation. Panel B represents Western blot analysis of material that pelleted after centrifugation at 500 \(\times\) 10 for 10 min. Positions of MHC class I and SifA-chimeric proteins are indicated. A greater proportion of GFP-SifA-CLCCFL is present in the pelleted material than either GFP-SifA-SLSSFL or GFP-SifA-GFP. The relative amounts of material pelleted from GFP-SifA-SLSSFL and GFP-SifA-GFP are shown as a percentage of the material pelletted from GFP-SifA-CLCCFL. C, supernatant fractions from B were centrifuged at 100,000 \(\times\) 10 for 60 min to generate pellet (p) and cytosolic (c) fractions. Western blot analysis demonstrates greater accumulation of GFP-SifA-CLCCFL in the pellet fraction than either GFP-SifA-SLSSFL or GFP-SifA-GFP. Results are representative of two experiments.
equal efficiency, but not as well as GFP alone (Fig. 3A). No difference in the mobility of GFP-SifA-CLCCFL and constructs lacking the CLCCFL motif was observed. To separate membrane from cytosolic components S100 fractionation of cell lysates was undertaken. After repeated freeze thaw cycles and passage through a 25-gauge needle, cell debris was removed by low speed centrifugation. More of the wild-type protein was observed in this fraction than either GFP-SifA-SLSSFL or GFP-SifAΔ6 (Fig. 3B). Similar levels of MHC class I and HSP70 (data not shown) were present in each fraction, demonstrating that equivalent quantities of cell lysate had been loaded. Upon subcellular fractionation (100,000 x g for 60 min) of GFP-SifA-CLCCFL-containing lysates, a greater proportion of the protein was observed in the pellet fraction (65%) than the supernatant (35%) (Fig. 3C). This distribution was reversed for both the GFP-SifA-SLSSFL (38%/62%) and GFP-SifAΔ6 (33%/67%) constructs (Fig. 3C). Again, similar levels of MHC class I were present in each of the pellet fractions confirming that equal quantities of lysate were loaded. This suggested that a greater proportion of GFP-SifA was associated with membrane than was observed for either GFP-SifA-SLSSFL or GFP-SifAΔ6.

However, even in the absence of the potential prenylation motif, ~30% of the SifA protein remained membrane associated. Fig. 3C also shows the presence of a slower migrating band in the pellet fraction of the wild-type protein. We are uncertain as to the nature of this minor band but do not think it represents the prenylated SifA protein as prenylation usually results in increased migration under SDS-PAGE conditions. In addition, as reported earlier, SifA lacking the residue critical for prenylation can be metabolically labeled and migrates at exactly the same position as SifA that is both prenylated and S-acylated.

Changes in hydrophobicity because of addition of isoprenoids can be detected by altered partitioning in the detergent Triton X-114. The final aliquot of transfected cells was therefore subjected to lysis and partitioning in this detergent. As shown in Fig. 4, control proteins MHC class I and HSP70 partitioned exclusively into the detergent and aqueous phases respectively.

GFP alone also partitioned exclusively into the aqueous phase. In contrast, all three SifA constructs distributed between both aqueous and detergent phases, and in each case most of the protein (63–73%) was in the aqueous phase. Although not dramatically different, wild-type GFP-SifA-CLCCFL showed greater accumulation in the detergent phase than either of the two mutant proteins (37% as compared with 27%). This suggested that, irrespective of the putative prenylation motif, SifA possessed hydrophobic properties that could enable it to partition into the detergent phase. In support of this, upon repartitioning of the initial aqueous fractions (Fig. 4A) ~30% of the proteins again partitioned into the detergent fraction (Fig. 4B). Distribution of the HSP70 control in this repartitioning was restricted to the aqueous phase.

To eliminate possible influences of GFP on our biochemical analysis and to enhance detection of small differences in mobility upon PAGE, the GFP tag was replaced with the smaller HA tag. No difference in mobility was observed between proteins segregating in the P100 or S100 fractions or between HA-SifA and constructs in which the various cysteine residues had been mutated (Fig. 5, MHC class I and HSP70 controls not shown). Thus, even with the small HA tag we were unable to discriminate between potentially prenylated and unprenylated proteins by virtue of changes in mobility upon SDS-PAGE. From subcellular fractionation of the panel of mutants (Fig. 5), it was evident that in situations where residue Cys333 was substituted for serine a greater proportion of the protein was present in the cytosolic (65–81%) as compared with pellet fraction (19–35%). This suggested that residue Cys333 was the key residue influencing membrane association. Membrane association was also influenced by Cys331 and Cys334, although to a lesser extent, as shown by the greater proportion of HA-SifA-CLCCFL and HA-SifA-CLCSFL in the cytosol than observed for HA-SifA-CLCCFL.
Prenylation and S-Acylation of SifA

The studies described above utilized indirect means of assessing the hydrophobic status of SifA. To directly investigate prenylation of SifA, the HA-SifA-CLCCFL, HA-SifA-SLSSFL, and HA-SifA-CLSCFL constructs were subjected to \textit{in vitro} translation in the presence of \[^{3}H\]mevalonic acid. As shown in Fig. 7A (top panel), after immunoprecipitation and fluorography only the HA-SifA-CLCCFL product incorporated the labeled isoprenoid precursor into the mature protein. All three transcripts were translated to a similar extent, as shown by Western analysis of an aliquot of the immunoprecipitate (Fig. 7A, bottom panel). Thus Cys\(^{333}\) is an essential residue for isoprenoid incorporation, consistent with modification by a prenyltransferase.

To investigate the nature of the prenyltransferase involved, the \textit{in vitro} \[^{3}H\]mevalonic acid labeling was repeated in the presence of various concentrations of FTI 227 or GGTI 298, specific inhibitors of PFT and PGGT, respectively. As shown in Fig. 7B (top panel), isoprenoid attachment to HA-SifA-CLCCFL was inhibited in the presence of 1 \(\mu\)M GGTI-298, a concentration below that required to inhibit PGGT processing of Rap 1A (3 \(\mu\)M IC\(_{50}\)). In contrast, substantial processing of HA-SifA-CLCCFL was observed in the presence of 10 \(\mu\)M FTI-277, a concentration far in excess of that required to inhibit PFT processing of H-Ras (100 nM IC\(_{50}\)). This strongly suggests that residue Cys\(^{333}\) is subject to geranylgeranyl addition through the action of PGGT. \[^{3}H\]mevalonic acid labeling was also undertaken on HA-SifA-transfected cells but we were unable to demonstrate incorporation of label into HA-SifA even after 3 months exposure to film (data not shown). This may reflect limited uptake of the \[^{3}H\]mevalonic acid by the transfected 293T cells as we were also unable to detect labeling of an endogenous lamin control. The inability to label transfected SifA with \[^{3}H\]mevalonic acid and the long exposure times required to detect endogenous proteins precluded the use of this technique for the study of endogenous SifA secreted from bacterial cells.

Subsequent to isoprenoid attachment many proteins are subject to S-acylation on cysteine residues that reside in close proximity to the site of prenylation. To investigate whether Cys\(^{333}\) was subject to such a modification, HA-SifA-CLCCFL and HA-SifA-CLSCFL-transfected cells were metabolically labeled for 7 h with \[^{3}H\]palmitic acid. After immunoprecipitation and fluorography a strong signal of the expected molecular

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**Fig. 6.** Isolated cytosolic SifA associates with freshly supplied membrane but is resistant to membrane extraction with sodium carbonate. A, P100 membrane fraction, derived from an HA-SifA-CLCCFL-transfected cell lysate, was resuspended in sodium carbonate. After centrifugation the supernatant was recovered and the pellet resuspended in fresh sodium carbonate. After centrifugation the pellet fraction \((p)\) and pooled supernatant fractions \((c)\) were subjected to SDS-PAGE separation and Western blot analysis with anti-HA antibody. The majority of HA-SifA-CLCCFL and HA-SifA-CLSCFL was recovered in the supernatant fraction, implying that it is directly associated with the membrane but remained associated with the pellet fraction \((p)\). Results shown are representative of three experiments. B, post-nuclear cell lysates from HA-SifA-CLCCFL- and HA-SifA-SLSSFL-transfected cells were centrifuged at 100,000 \(\times\) g for 60 min to generate P100 pellet \((p)\) and S100 cytosol \((c)\) fractions. The cytosolic fractions were resuspended together with an excess of membrane derived from untransfected cells. After recentrifugation the pellet \((p)\) and supernatant fractions \((c)\) were recovered and all fractions analyzed by SDS-PAGE and Western blotting with anti-HA, anti-HSP70 and anti-MHC I primary reagents. HA-SifA-CLCCFL partitions largely into the pellet fraction and upon supply of fresh membrane a proportion of the cytosolic protein is found associated with the membrane fraction. HA-SifA-SLSSFL partitions largely with the cytosolic fraction but associates with freshly supplied membrane components to pellet with the membrane fraction \((p)\). MHC and HSP controls suggest relatively even loading. Results shown are representative of two experiments.

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30 min and only a single phase separation was performed. Using these conditions aqueous and detergent controls still separated efficiently into their respective phases. Substitution of all three cysteine residues for serine reduced the ability of HA-SifA-SLSSFL to partition exclusively in the detergent phase. The majority of HA-SifA-CLCCFL was inhibited by low concentrations of GGTI 298 but not FTI 277, consistent with a requirement for processing by protein farnesyl transferase I. Lower panel shows Western blotting of an aliquot of the immunoprecipitated material to confirm comparable protein translation. 

**Fig. 7.** HA-SifA-CLCCFL is modified by prenylation through the action of protein geranylgeranyl transferase I. A, protein was translated from 1 \(\mu\)g of HA-SifA-CLCCFL, -SLSSFL, or CLSCFL mRNA using nucleas ether-treated rabbit reticulocyte lysate in the presence of \[^{3}H\]mevalonic acid. After immunoprecipitation with the anti-HA antibody, proteins were separated by SDS-PAGE and gels processed for fluorography. Only HA-SifA-CLCCFL incorporated the labeled isoprenoid precursor into the mature protein demonstrating that residue Cys\(^{333}\) is required for isoprenoid addition. Lower panel shows Western blotting of an aliquot of the immunoprecipitated material to confirm comparable protein translation. B, \textit{in vitro} translation was performed as above but in the presence of 1, 10, or 20 \(\mu\)M GGTI 298 or FTI 277, specific inhibitors of protein geranylgeranyl transferase I and protein farnesyl transferase respectively. Isoprenoid addition to HA-SifA-CLCCFL was inhibited by low concentrations of GGTI 298 but not FTI 277, consistent with a requirement for processing by protein geranylgeranyl transferase I. Lower panel shows Western blotting of an aliquot of the immunoprecipitated material as a measure of protein translation efficiency. FTI 277 caused inhibition of translation at 10 and 20 \(\mu\)M.
weight was associated with HA-SifA-CLCCFL but not HA-SifA-SLCCFL precipitated material (Fig. 8A). Similar levels of SifA protein were present in each of the lysates, as demonstrated by Western blotting of an aliquot of each precipitate (Fig. 8B). The simplest interpretation of this in vitro labeling is that SifA is modified by S-acylation on residue Cys331. To investigate if geranylgeranyl addition on residue Cys333 was required prior to S-acylation of residue Cys331, cells expressing HA-SifA-CLCCFL, HA-SifA-SLCCFL, and HA-SifA-CLSCFL were subjected to S-acylation analysis (data not shown). In this case the labeling period was increased to 12 h. Weak but comparable signals were associated with both HA-SifA-CLCCFL and HA-SifA-CLSCLF and a strong signal similar to that seen in Fig. 8B was observed for HA-SifA-CLCCFL. We interpreted this as evidence that geranylgeranyl addition on residue Cys333 was required prior to S-acylation of residue Cys331. The incorporation of 3H into HA-SifA-CLCCFL and HA-SifA-CLSCFL precipitated material is consistent with the longer labeling period and consequent metabolism of the 3H-palmitic acid and reincorporation as amino acids. This extended labeling period was used to allow comparison of the electrophoretic properties of modified and unmodified SifA as discussed earlier.

To investigate the potential importance of prenylation and S-acylation of SifA, mutant bacterial strains were generated in which Cys331 (sifA::331S) and Cys333 (sifA::333S) were individually substituted for serine residues. Balb/c mice were injected intravenously with 100 cfu of S. Typhimurium (12023), S. Typhi mutant strain sifA::mTn5 (12023), or sifA::331S or sifA::333S, the strains deficient in S-acylation and prenylation (and S-acylation) respectively. Bacterial viable counts performed on spleen and liver homogenates on day 5 are shown in Fig. 9. At the 95% confidence interval the mutant strain sifA::mTn5 shows clear attenuation of survival in both liver (t = 3.29, p < 0.02) and spleen (t = 3.35, p < 0.022), unlike sifA::331S which was comparable to the wild-type strain 12023. Interestingly sifA::333S was attenuated for growth in liver (t = 6.25, p < 0.002) but not spleen. Replication of the various strains was examined more directly for full function, in addition to isoprenoid attachment, many proteins to interact with cellular membranes (35, 36). However, for full function, in addition to isoprenoid attachment, many CAAX-containing substrates require either an associated polybasic region or an additional modification on closely situated cysteine residues. By labeling with 3H-palmitic acid we demonstrated modification of residue Cys331 by S-acylation, an event that required prior prenylation of residue Cys331. This is consistent with a requirement for cysteine residues to be in close proximity to the membrane for S-acylation to occur. Although palmitoylation is the most likely modification to residue Cys331, palmitic acid serves as a metabolic precursor for other fatty acids such as stearic acid and so the exact nature of the added fatty acid remains to be determined.

Our results showing modification of SifA by prenylation and S-acylation are consistent with the sequence composition of the CLCCFL motif. The leucine residue in the C-terminal position...
favors modification by PGGT and the phenylalanine in position α2 of SifA would be unlikely to favor processing by PFT because a tetrapeptide with a substituted aromatic group (phenylalanine) in the α2 position (CVFM) has been shown to act as a competitive inhibitor of PFT (37). Although we cannot rule out the possibility that SifA is also a substrate for RGGT this seems unlikely as the activity of RGGT is restricted to proteins capable of association with Rab escort protein and the only RGGT substrates known to date are Rab proteins themselves.

From what is generally understood to happen to prenylated proteins it is likely that, after modification of residue Cys333 by PGGT, the last three residues (CFL) would be cleaved from the protein by a CAAX protease. The carboxyl group of the prenylated cysteine residue would then be methylylated by a methyltransferase, which, together with the S-acylation of Cys333, would greatly influence the membrane association properties of SifA. As both the CAAX protease and methyltransferase are enriched on membranes of the endoplasmic reticulum this suggests that SifA may traffic to the endoplasmic reticulum (ER) for processing. Our studies showing that residues Cys333 and Cys338 are subject to direct modification provide an explanation for their role in membrane association and help to explain the immunofluorescence data. The influence of residue Cys334 is probably indirect and not linked to additional modifications. It is likely that substitution of Cys334 for serine in some way influences the recognition of the CLCCFL motif by either the PGGT or more likely the CAAX protease. In support of this substitution of cysteine to serine in the α1 position has been shown to prevent processing of Afc1p by the CAAX protease (38).

The outcome of S-acylation is varied but can influence membrane association and sorting of peripheral membrane and cytosolic proteins (36). It can influence sorting of integral membrane proteins from the ER to plasma membrane and affect endocytosis, recycling and protein stability (39). S-acylation can also play a role in localization to lipid rafts and is known to regulate signaling of various proteins, such as Src family kinases, and can be reversible in vivo (40). We investigated this latter role but were unable to demonstrate association of any of our SifA constructs with lipid rafts (data not shown).

We have recently reported the down-regulation of surface MHC class II in Salmonella-infected cells, and shown that this fails to occur in cells harboring the sifA::mTn5 mutant strain (19). We investigated this property in cells infected with sifA::331S and sifA::333S and found that in both cases surface MHC class II levels were reduced similar to the parental Salmonella strain (data not shown).

S-acylation of Cys331 may be an important reversible modification which together with prenylation of Cys333 could provide a mechanism for regulating SifA function. We therefore examined the significance of these modifications in macrophage survival assays and in a mouse model of systemic infection. In contrast to the replication defect clearly evident in the SifA mutant strain sifA::mTn5 (12032), strains sifA::331S and sifA::333S were not attenuated for replication, although they would be deficient in S-acylation and prenylation (and S-acylation), respectively. This was surprising given previous reports showing that sifA::mTn5 and sifA::Δ6, a mutant strain lacking the last 6 amino acids (CLCCFL), show clear and comparable defects in macrophage survival (29). It is possible that deletions within the CLCCFL motif result in a more extensive defect in the SifA protein compared with the cysteine to serine substitutions generated in this study. This seems likely given the progressive loss of SifA function seen with sequential removal of C-terminal residues (23). Had loss of function been due only to prevention of prenylation then removal of a single residue would lead to complete inactivation of the CAAX motif.

In our mouse infection studies the sifA::mTn5 mutant strain was clearly attenuated for virulence and we also observed a significant reduction in recovery of bacteria from the liver, but not spleen, of sifA::333S-infected mice. In contrast the infection titer with sifA::331S was not significantly different from infection with wild-type 12023 bacteria. It is possible that a more sensitive assay such as the competitive index method used to demonstrate the defect in the SifAΔ6 mutant would be more revealing (23). At present the functional significance of the S-acylation of SifA is unclear, and further work will be required to establish the full significance of this modification.

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A Salmonella typhimurium Effector Protein SifA Is Modified by Host Cell Prenylation and S-Acylation Machinery
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