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Science Signaling **2** (95), ra71. [DOI: 10.1126/scisignal.2000430]

HOST-PATHOGEN INTERACTIONS

Activation of a Bacterial Virulence Protein by the GTPase RhoA

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(Published 3 November 2009; Volume 2 Issue 95 ra71)

The Rho family of guanosine triphosphatases (GTPases) are essential eukaryotic signaling molecules that regulate cellular physiology. Virulence factors from various pathogens alter the signaling of GTPases by acting as GTPase activating factors, guanine nucleotide exchange factors, or direct covalent modifiers; however, bacterial virulence factors that sense rather than alter the signaling states of Rho GTPases have not been previously described. Here, we report that the translocated *Salmonellae* virulence factor SseJ binds to the guanosine triphosphate–bound form of RhoA. This interaction stimulates the lipase activity of SseJ, which results in the esterification of cholesterol in the host cell membrane. Our results suggest that the activation of molecules downstream of GTPases is not exclusive to eukaryotic proteins, and that a bacterial protein has evolved to recognize the activation state of RhoA, which regulates its enzymatic activity as part of the host-pathogen interaction.

INTRODUCTION

Salmonella enterica serovar Typhimurium (*S. typhimurium*) is a facultative intracellular pathogen that is a major cause of gastroenteritis and systemic infections in humans and animals; it invades host cells to replicate within a specialized vacuole referred to as the *Salmonella*-containing vacuole (SCV) (*1*). The SCV segregates from the normal phagosome maturation pathway to enable bacterial survival and replication. Invasion of animal cells and remodeling of the SCV depend on the translocation of proteins from the bacteria to the cytosol of the host cell by specific secretion systems (*2*–*4*). Several of these proteins are required for successful systemic infection of mice and intracellular replication within cultured cells (*5*). Among these proteins is SseJ, which localizes to the cytoplasmic face of the SCV after translocation (*6*–*8*). SseJ belongs to the GDSL-like lipolytic enzyme family and exhibits basal deacylase, phospholipase, and glycerophospholipid-cholesterol acyltransferase (GCAT) activity in vitro (*7*, *9*, *10*). *S. typhimurium* strains that express *sseJ* with mutations that encode residues in the active site have attenuated virulence for mice similar to that of *sseJ* null mutants (*7*). This indicates that the enzymatic activity of SseJ is important for bacterial pathogenesis within animals. The basal enzymatic activity of SseJ can be stimulated upon the addition of eukaryotic total cell extracts (*10*), which suggests that eukaryotic factors might be required for the activation of SseJ. From a yeast two-hybrid screen of a mammalian splenic complementary DNA (cDNA) library, we previously identified RhoA and RhoC as binding targets of SseJ (*11*). Based on these findings, we tested whether the enzymatic activity of SseJ was stimulated by a mammalian guanosine triphosphatase (GTPase).

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RESULTS

SseJ recruits activate RhoA to the SCV

Previously, we identified members of the Rho family of GTPases as potential mammalian binding targets of SseJ (*11*); therefore, we analyzed whether SseJ interacted with RhoA during infection with *S. typhimurium*. HeLa cells transfected with a plasmid encoding myc-tagged RhoA were infected with either an *S. typhimurium sseJ* null mutant strain or a strain expressing a gene encoding hemagglutinin (HA)-tagged SseJ (SseJ-HA). Myc-tagged RhoA remained evenly distributed in HeLa cells infected with the *sseJ* null mutant strain of *S. typhimurium* (Fig. 1A). Only a weak signal was detected on SCV membranes in the presence of secreted SseJ-HA which, as expected, localized to the SCV (*6*–*8*) (Fig. 1B). In contrast, when cells were transfected with a plasmid encoding a myc-tagged, constitutively active mutant RhoA, RhoA-G14V (CA RhoA), CA RhoA was not enriched on SCVs in HeLa cells infected with the *sseJ* null mutant strain of *S. typhimurium* (Fig. 1C), but was specifically recruited to the SCV of bacteria expressing SseJ-HA (Fig. 1D). Thus, recruitment of CA RhoA to the SCV was specifically associated with the presence of SseJ.

Formation of the SseJ-RhoA complex depends on the activation state of RhoA

We further investigated the interaction between SseJ and RhoA in vitro by gel filtration. We found that purified RhoA and SseJ formed a complex that was dependent on the activation state of RhoA (Fig. 2A). Gel filtration with equimolar ratios of SseJ and nucleotide-free RhoA (apo-RhoA) resulted in a minor shift of SseJ toward a higher molecular mass species; free RhoA was still observed under these conditions. In contrast, gel filtration of SseJ in the presence of activated RhoA, in which the GTPase was preloaded with a nonhydrolyzable analog of guanosine triphosphate (GTP), GTPγS, resulted in a strong shift of SseJ toward a higher molecular mass species, with free RhoA absent from the elution profile (Fig. 2A). Subsequent experiments with equimolar ratios of SseJ and various GTPγS-loaded GTPases (including RhoB, RhoC, Cdc42, H-Ras, and

Rac1) revealed that SseJ formed a complex exclusively with GTPγS-loaded RhoA and RhoC (Fig. 2B and fig. S1).

That the bacterial virulence factor SseJ specifically interacted with the GTPases RhoA and RhoC in the presence of GTPγS was of interest because Rho GTPases play pivotal roles in eukaryotic signal transduction cascades (*12*). Small GTPases act as molecular switches, cycling between inactive, guanosine diphosphate (GDP)–bound and active, GTP-bound forms, and exert their function by binding to and activating their cognate downstream effectors in a GTP-dependent manner (*13*). Many bacterial virulence factors have evolved to manipulate GTPase signaling by mimicking regulators of these proteins (*14*–*16*) or by covalently modifying them (*17*–*21*). Among these virulence factors are SopE and SptP, which act as a guanine nucleotide exchange factor (GEF) and GTPase activating factor (GAP) for Cdc42 and Rac-1, respectively (*15*). Therefore, we investigated whether SseJ affected the GTP-GDP cycling of RhoA or interfered with the performance of endogenous Rho GEFs and GAPs. We found that when in excess (2 μM SseJ versus 1 μM RhoA), SseJ affected neither the intrinsic activity of RhoA nor the ability of the GTPase activity of RhoA to be stimulated by 50 nM p50RhoGAP (Fig. 2C). In addition, GEF filter binding assays that measured the incorporation of [^{33}P]GTP into 2.5 μM RhoA in the presence or absence of an equimolar concentration of SseJ with or without 0.5 μM Dbs (a eukaryotic GEF) did not reveal either a GEF activity for SseJ or evidence of its competition with Dbs (Fig. 2D), indicating that SseJ did not function as a GAP or GEF for RhoA and that its binding to RhoA did not alter the functions of endogenous regulatory proteins.

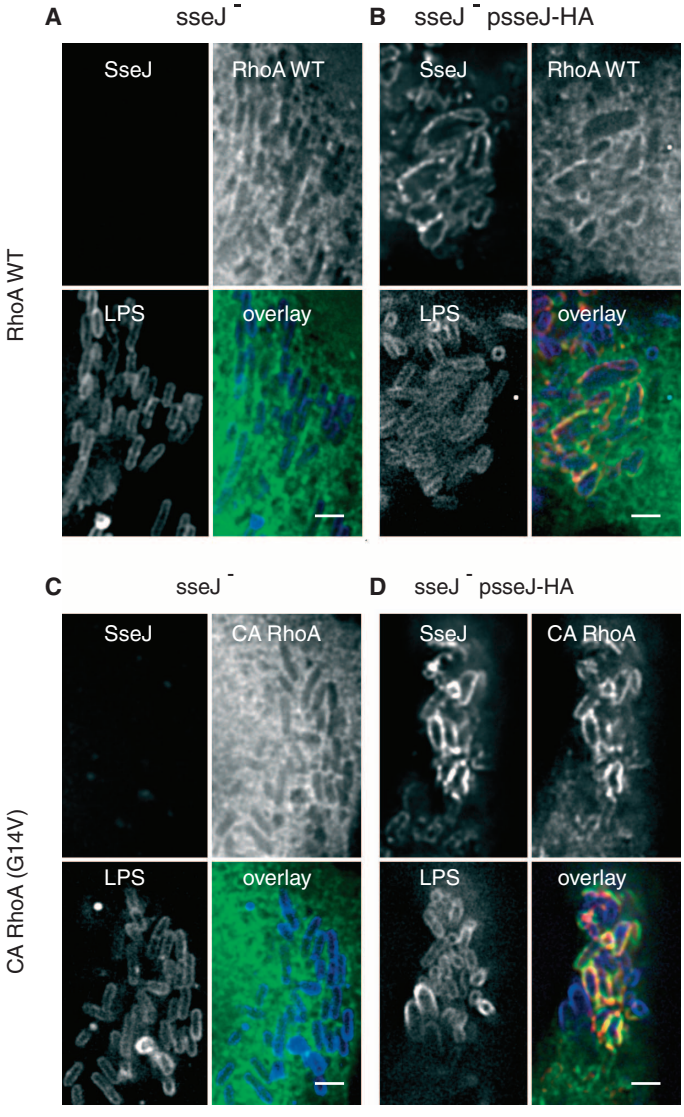

Fig. 1. SseJ recruits constitutively active, myc-tagged RhoA to the SCV. HeLa cells transfected for 4 hours with plasmids encoding wild-type (WT) myc-RhoA (A and B) or constitutively active (CA) myc-RhoA (C and D) were infected for 15 hours with either an *sseJ* mutant strain of *S. typhimurium* (A and C) or an *S. typhimurium* strain secreting SseJ-HA (*sseJ* $^{-}$, psseJ-HA) (B and D) and were immunostained with antibodies against myc-RhoA (green), SseJ-HA (red), and lipopolysaccharide (LPS) (blue). Scale bar, 2 μm. This experiment was performed three times, and the images shown are representative of more than 10 samples examined.

SseJ is a modular protein that consists of two discrete domains. The N-terminal region contains a translocation signal, similar to those found in other *Salmonella* effector proteins secreted across the phagosome membrane, which targets the protein to the mammalian endocytic compartment. The C-terminal domain of SseJ shares sequence similarity with members of the GDSL-like lipolytic enzyme family, which harbors phospholipase (PLA) and glycerophospholipid-cholesterol acyltransferase (GCAT) activity. However, only weak deacylase and transferase activities have been detected in vitro in experiments with purified recombinant proteins (*7*, *9*, *10*). The basal PLA and GCAT activities of SseJ are stimulated by HeLa cell extracts, which suggests that a eukaryotic factor is required for the activation of SseJ (*10*). Therefore, gel filtration was performed with SseJ and GTPγS-loaded RhoA and the eluted fractions were tested for lipase activity with the chromogenic substrate *p*-nitrophenylpalmitate (PNPP). Lipase activity was strongly stimulated in the presence of RhoA with an activation profile that corresponded to fractions containing the SseJ-RhoA complex (Fig. 2A). Subsequent PNPP lipase assays with 200 nM SseJ and 200 nM GTPγS-loaded RhoA, RhoB, RhoC, H-Ras, Rac1, or Cdc42 confirmed that only RhoA acted as a potent activator of the lipase activity of SseJ in vitro (Fig. 2, E and F). We observed the partial stimulation of the lipase activity of SseJ by RhoC, but not by RhoB, Cdc42, H-Ras, or Rac1 (Fig. 2, E and F). These observations underline the specificity of SseJ for a single GTPase and also correlated the physical binding of SseJ to RhoA with the activation of SseJ.

The activation state of RhoA determines the activity of SseJ

Because our findings from microscopic analysis (Fig. 1) and the results from the gel filtration experiments (Fig. 2) suggested that the interaction between RhoA and SseJ was dependent on the activation state of RhoA, we investigated whether the stimulation of the lipase activity of SseJ was affected by the guanosine nucleotide–loading state of RhoA. PNPP lipase assays were performed with 200 nM SseJ incubated with 200 nM apo-RhoA, RhoA-GDP, or RhoA-GTPγS. Rho-GTPγS increased the lipase activity of SseJ fivefold (1.2 mAU/min) compared to that of Rho-GDP (0.21 mAU/min) (Fig. 3, A and B). Titration experiments were performed with 200 nM SseJ and increasing concentrations of apo-RhoA, RhoA-GDP, or RhoA-GTPγS. Although the lipase activity of SseJ was partially stimulated by RhoA-GDP and by apo-RhoA, neither could match the maximal stimulation caused by RhoA-GTPγS, not even at the highest concentrations of RhoA (Fig. 3C). This dependence of the activation of SseJ on the GTP-loading state of RhoA is similar to the conformation-dependent stimulation of eukaryotic downstream effectors by their cognate GTP-bound GTPases.

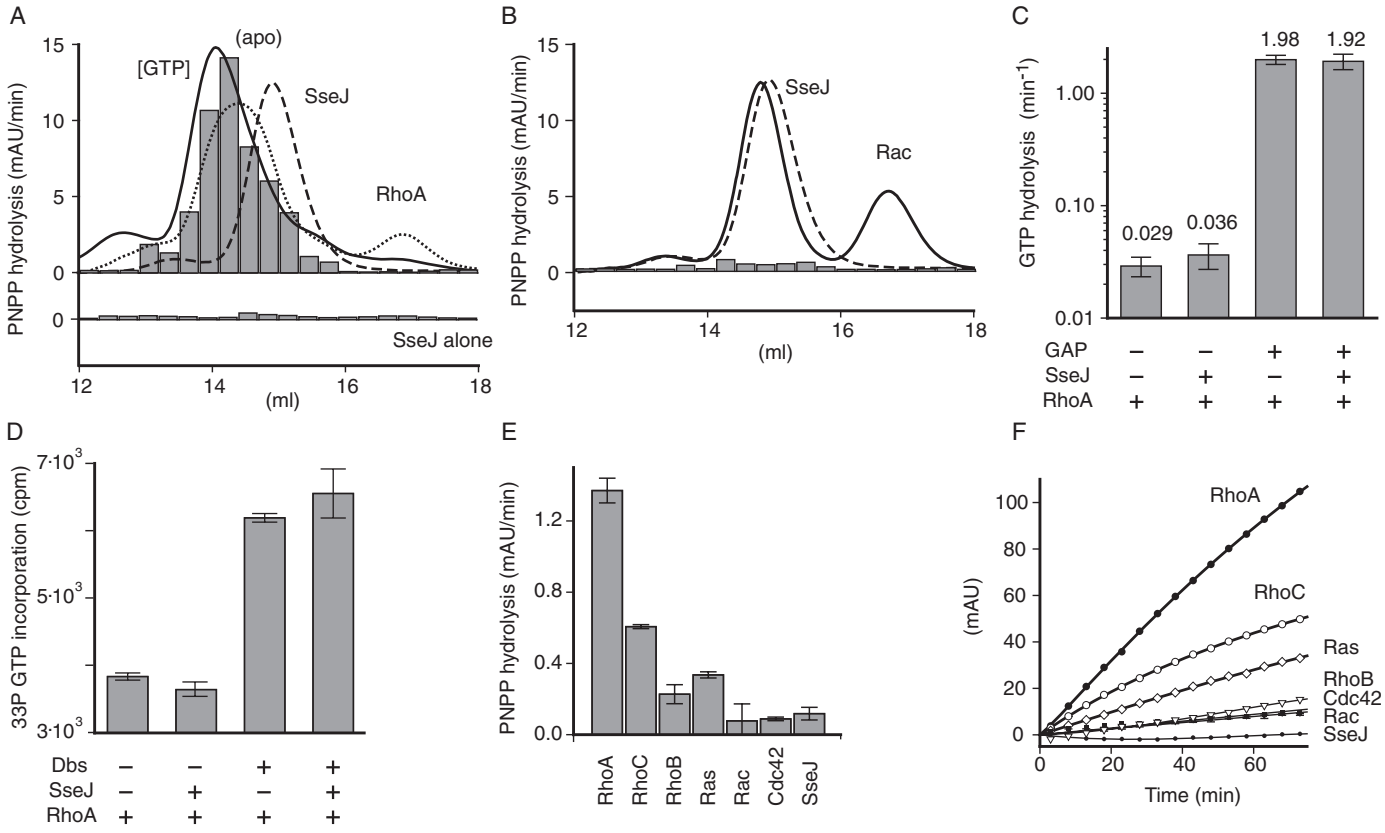

Fig. 2. SseJ forms a complex with RhoA that is dependent on the activation state of RhoA. (**A** and **B**) Gel filtration analyses of SseJ alone (dashed line), in the presence of apo-RhoA (dotted line), or in the presence of GTPγS-loaded RhoA (black line) revealed the formation of an enzymatically active complex with RhoA-GTPγS (A) but not with Rac-GTPγS (B). The upper bar graph shows the lipase activity of eluted fractions from the gel filtration of SseJ and RhoA-GTPγS. The lower bar graph shows the basal lipase activity of eluted fractions that contained SseJ alone. The gel filtrations shown are representative of three independent experiments. The formation of the SseJ-RhoA complex does not impair RhoA GDP-GTP cycling in vitro. (**C**) Binding of SseJ does not alter either the intrinsic or the p50RhoGAP-stimulated GTPase activity of RhoA. (**D**) The presence of SseJ does not impair the H-Dbs-mediated GEF activity of RhoA. Bar graphs (**E**) and the corresponding enzyme kinetics (**F**) of the stimulation of the lipase activity of SseJ in the presence of GTPγS-loaded RhoA, RhoB, RhoC, H-Ras, Rac-1, or Cdc42 indicate the specific stimulation of the lipase activity of SseJ by the Rho GTPases subfamily members RhoA and RhoC. Each bar graph shows the averages of three individual experiments. Error bars indicate the SD.

The SCV is a major site of intracellular cholesterol accumulation during the late stages of infection by *S. typhimurium* (*22*). SseJ has weak GCAT activity and esterifies cholesterol in HeLa cells and macrophages (*9*, *10*). To test whether the activated SseJ-RhoA complex exhibited GCAT activity in vitro, we analyzed the enzymatic activity and substrate specificity of the activated SseJ-RhoA complex in experiments with the physiological substrates dioleolylphosphocholine (DOPC) and cholesterol. Liposomes containing a 60:30:10 molar ratio of DOPC, cholesterol, and oleic acid were incubated with SseJ in the presence or absence of GTPγS-bound RhoA, and product formation was analyzed by thin-layer chromatography (TLC). SseJ efficiently converted DOPC and cholesterol to lysophosphatidylcholine (lyso-PC) and cholesterol ester (CE) in the presence of RhoA-GTPγS, whereas no activity was detected for SseJ alone (Fig. 3D). To determine whether CA RhoA enhanced the GCAT activity of SseJ in cells, we co-transfected HeLa cells with plasmids encoding both proteins. Ectopic expression of SseJ in HeLa cells resulted in its localization to late endosomal compartments, which aggregated, resulting in globular structures (*6*) (fig. S2). As in the SCV, there was an accumulation of cholesterol in the endosomal compartments and an overall decrease in the amount of cellular free cholesterol due to the formation of cholesterol esters by SseJ (*9*). CA RhoA and SseJ colocalized as expected, and the endosomal compartments appeared enlarged (fig. S2) compared to those in cells transfected with the SseJ-expressing plasmid alone. These observations suggested that activated RhoA also stimulated the GCAT activity of SseJ in mammalian cells.

The SseJ-RhoA complex preferentially acts on sn-1 chains

We then tested the substrate specificity of activated SseJ. Liposomal assays were performed with the sn-1– and sn-2–specific fluorogenic substrates PEDA1 and PED6, respectively, which are composed of a quencher (dinitrophenyl) on the glycerophosphoethanolamine head group and 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) fluorophore acyl chains at either position sn-1 or position sn-2. We found that the GCAT activity of activated SseJ caused specific cleavage at the sn-1 position, as shown by the two orders of magnitude faster release of fluorescent product in the

presence of PEDA1 [1.52×10^4 relative fluorescence units (RFU)/min] compared to that in the presence of PED6 (1.62×10^2 RFU/min) (Fig. 4A). Analysis of the fluorescent reaction products by TLC confirmed the formation of a fluorescent BODIPY-modified cholesterol ester species in the presence of PEDA1 (Fig. 4B).

We further used the sn-1–specific fluorogenic substrate PEDA1 as a probe to visualize the spatial distribution of SseJ activity in transfected HeLa cells by fluorescence microcopy. Incubation of HeLa cells cotransfected with plasmids expressing SseJ and CA RhoA with 3.5 μM PEDA1 for 2 hours revealed an increase in the formation of fluorescent cholesterol esters in the presence of SseJ and its activator CA RhoA (Fig. 5), confirming our biochemical results with purified proteins (Fig. 4, A and B). No increase in fluorescence was detected in cells cotransfected with CA RhoA and the SseJ3x, a catalytically inactive mutant of SseJ (Fig. 5, A and B), or in cells transfected with SseJ3x or CA RhoA alone (Fig. 5B and fig. S3).

DISCUSSION

Together, our results indicate that the *S. typhimurium* protein SseJ modulates the abundance of cellular cholesterol by acting as a RhoA-dependent GCAT enzyme. A similar mechanism may account for the activation of the related toxin YspM from *Yersinia enterocolitica* (*23*) and other effector proteins and toxins of diverse enzymatic functions whose activities are weak in vitro. In contrast to many other bacterial effector proteins, alteration of the activation state of RhoA does not appear to be the function of the effector protein SseJ. Instead, SseJ defines a previously unidentified class of bacterial effector proteins that link the activation state of GTPases to pathogen-controlled functions. The only other bacterial virulence protein that is enzymatically regulated by a GTPase is cholera toxin, whose ADP-ribosylation on the α-subunit of heterotrimeric guanine nucleotide–binding proteins (G proteins) is activated by the GTP-bound form of the small GTPase Arf. However, Arf is also covalently modified by cholera toxin (*19*), and whether this modification alters the signaling state of Arf is unknown.

As part of lipid rafts, the cholesterol content of the membrane dictates the types of Rab proteins, motor proteins, and lipids present, which makes cholesterol important in endocytic trafficking events (*24*). By altering the cholesterol content, it is possible that SseJ is controlling the membrane dynamics of the SCV, the maintenance of which is essential for the intracellular survival of the bacteria.This alteration in cholesterol content may also explain the formation of endosomal tubules when SseJ and CA RhoA are coexpressed in HeLa cells (*11*).

The identification of RhoA as a potent GTP-dependent regulator of SseJ activity provides a key to understanding the molecular mechanism of the action of SseJ. These findings suggest that during *S. typhimurium* infection, SseJ specifically alters the composition of membrane cholesterol in vesicular compartments, including the SCV, in a manner that is dependent on the signaling state of RhoA. To alter the composition of membrane cholesterol in the SCV and to coordinate SCV membrane dynamics, it may be crucial to tightly regulate the activity of SseJ in a spatiotemporal manner to promote bacterial pathogenesis. Thus, GTPase-mediated activation is not exclusive to eukaryotic proteins and is an intimate part of the interaction between the host and *Salmonellae* because SseJ and perhaps other proteins have evolved to similarly use the activation state of a host GTPase to regulate their enzymatic activity.

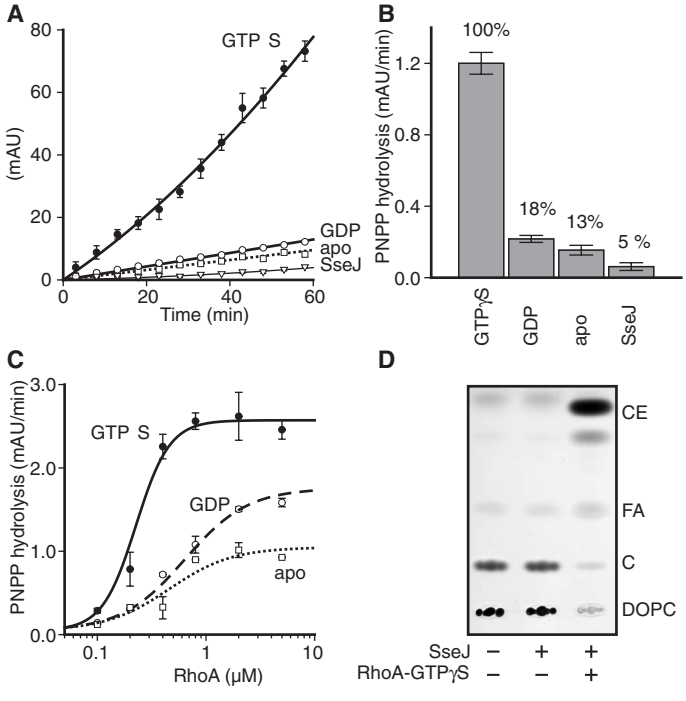

Fig. 3. The activation of SseJ is dependent on the guanosine nucleotide–loading state of RhoA. (**A** and **B**) Lipase activation assays with 200 nM SseJ and 200 nM RhoA-GTPγS, RhoA-GDP, or apo-RhoA revealed that maximal stimulation of the lipase activity of SseJ occurs with RhoA-GTPγS. (**C**) Lipase assays in the presence of 200 nM SseJ and increasing concentrations of RhoA-GTPγS, RhoA-GDP, or apo-RhoA revealed a higher binding affinity and stronger activation of SseJ by RhoA-GTPγS than by RhoA-GDP or apo-RhoA. (**D**) TLC analysis of the reaction products indicating the RhoA-dependent glycerophospholipid-cholesterol acyltransferase activation in presence of the biological substrates DOPC and cholesterol. Averages of three individual experiments are plotted in (A) to (C). Error bars indicate the SD.

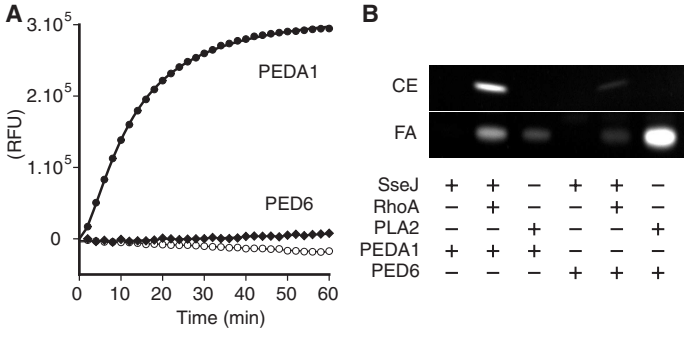

Fig. 4. The activated SseJ-RhoA complex preferentially transfers sn-1 acyl chains from glycerophospholipids to cholesterol. (**A**) Enzymatic assays with liposomes containing the sn-1–selective fluorescent probe PEDA1 (filled circle) and the sn-2–selective fluorescent probe PED6 (diamond). SseJ does not cleave PEDA1 in the absence of GTPγS-loaded RhoA (open circle). For each condition, a representative curve is shown from three independent kinetic measurements. (**B**) TLC analysis of the fluorescent reaction products after a 60-min incubation with the enzyme mix confirms the formation of a BODIPY-modified cholesterol ester (CE) in the presence of PEDA1. Bee venom phospholipase A_2 (PLA_2), which generates free fatty acids (FA), was used as a control.

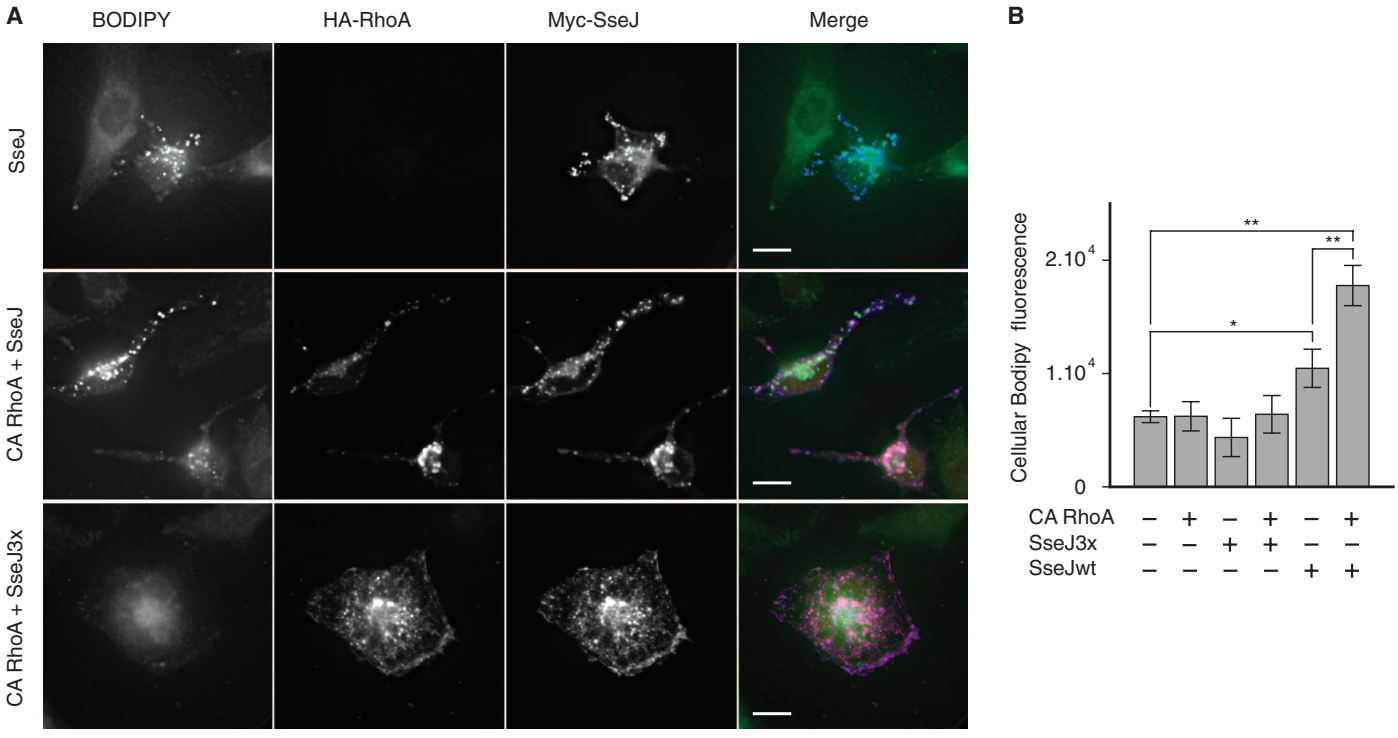

Fig. 5. SseJ and CA RhoA colocalize on globular structures containing fluorescent BODIPY-labeled cholesterol in transfected HeLa cells. (**A**) HeLa cells were transfected with a plasmid encoding myc-SseJ alone or with plasmids encoding CA HA-RhoA and myc-SseJ or the catalytic mutant SseJ3x for 24 hours and were then incubated for 2 hours with 3.5 μM PEDA1 followed by incubation with antibodies against myc (blue) and HA (red). Scale bar, 10 μm. (**B**) Mean intensity of the cellular BODIPY fluorescence was measured with the NIS-Elements image analysis software. For each condition, 10 individual fields were analyzed. Statistical comparison among groups by one-way analysis of variance followed by Tukey's post hoc tests revealed that cotransfection of SseJ and CA RhoA resulted in a statistically significant increase in cellular BODIPY fluorescence compared to that in cells transfected with SseJ alone or that in nontransfected control cells. *$P < 0.05$; **$P < 0.01$; error bars indicate the SD.

MATERIALS AND METHODS

Transfections and infections

HeLa cells (American Type Culture Collection) were transfected with the appropriate plasmids (table S1) with Fugene 6 (Roche) as recommended by the manufacturer and were cultured for 24 hours. For the PEDA1 (Invitrogen) experiment, cells were incubated for 2 hours with 3.5 μM substrate. HeLa cells were infected at an MOI (multiplicity of infection) of 1:100 with *S. typhimurium* (table S1) grown from a back dilution from an overnight culture. Cells were washed with 1× phosphate-buffered saline (pH 7.4) and treated with gentamicin (0.15 mg/ml) for 1 hour followed by incubation for 14 hours with gentamicin (0.015 mg/ml). The cells were fixed and permeabilized with the BD cytofix/cytoperm kit (BD-Biosciences), and immunostaining was performed as described previously (*11*). Deconvolution microscopy was performed on an Eclipse TE2000-E microscope (Nikon) equipped with a Cascade II 1024 EM-CCD camera (Photometrics). Images were deconvolved and analyzed with the NIS-elements image analysis software.

Protein purification

The purified proteins of the GTPases RhoA, RhoC, Cdc42, H-Ras, and Rac were purchased from Cytoskeleton. In addition, histidine (His)-tagged RhoA (His-RhoA), His-RhoB, and His-SseJ were expressed in bacteria and purified over a nickel column, after which the His-tag was cleaved with thrombin and the proteins were further purified by gel filtration. Purified proteins were stored in tris-buffered saline (TBS), pH 7.6, supplemented with 10% glycerol and 2 mM dithiothreitol (DTT) and stored at −80°C.

Size exclusion chromatography

The GTPases RhoA, RhoB, RhoC, Cdc42, H-Ras, and Rac were loaded with GTPγS before gel filtration by the addition of 100 μM GTPγS to 10 μM GTPase in TBS containing 2 mM EDTA and incubation on ice for 10 min. The GTPγS-GTPase complex was stabilized by the addition of 10 mM $MgCl_2$. Samples containing 100 μl of 4 μM SseJ alone or equimolar ratios of 4 μM SseJ and the appropriate GTPγS-bound GTPase in TBS containing 10 mM $MgCl_2$ and 50 μM GTPγS were injected onto a Superdex-200 size exclusion column on an Acta FPLC system (Amersham). Protein complexes were separated at a flow rate of 0.5 ml/min by TBS supplemented with 10 mM $MgCl_2$. Fractions of 300 μl were collected and were analyzed for lipase activity as determined by the PNPP lipase assay.

PNPP lipase assays

Lipase activity was determined by hydrolysis of the substrate PNPP and subsequent release of *p*-nitrophenol, which was detected by measuring the absorbance of the reaction solution at 405 nm. Assays were performed in triplicate in a 384-well format. In a standard assay, 0.4 μl of

10 mM PNPP dissolved in dimethyl sulfoxide was added to 40 μl of 200 nM SseJ in the presence or absence of 200 nM of the appropriate GTPase and 5 μM GTPγS or GDP. After mixing, plates were incubated at 37°C, and increases in absorption at 405 nm were measured on an EnVision Multilabel Reader (Perkin Elmer) at 2-min intervals.

Liposomal PLA cholesterol esterase assays

Liposomes containing a 60:30:10 molar ratio of DOPC/cholesterol/oleic acid were prepared by adding chloroform-dissolved stock solutions of 120 μl of 10 mM cholesterol, 60 μl of 10 mM DOPC, and 20 μl of 10 mM oleic acid to a 2-ml tube. Lipids were dried under N_2 gas and dry lipids were completely resolved in 20 μl of ethanol by incubation for 30 s at 55°C. Liposomes were prepared by rapid dilution in 2 ml of TBS containing 10 mM $MgCl_2$. Aliquots of 100 μl were supplemented with 200 nM SseJ in the presence or absence of 200 nM GTPγS-loaded RhoA and incubated at 37°C for 30 min for PLA cholesterol esterase assays. The reaction was stopped by the addition of 100 μl of chloroform/methanol (2:1 vol/vol) and reaction products were separated by TLC on silica gel (Whatman) with the solvent hexane/ethyl ether/acetic acid (80:15:5 vol/vol) as the mobile phase and were visualized by amido black staining (*9*). Selectivity of SseJ for acyltransfer from the sn-1 or sn-2 position was determined by lipase assays with liposomes containing 120 μM cholesterol, 60 μM DOPC, and 10 μM oleic acid supplemented with either 5 μM PEDA1 or 5 μM PED6 (Invitrogen). Aliquots of 100 μl were supplemented with 200 nM SseJ in the presence or absence of 200 nM GTPγS-loaded RhoA and incubated at 37°C. Cleavage at the sn-1 or sn-2 position was detected by measuring the fluorescence increase (excitation wavelength, 488 nm; emission wavelength, 530 nm) on an EnVision Multilabel Reader (Perkin Elmer).

GAP assays

Purified RhoA (1 μM) was loaded with GTP by incubating it with 5 μM GTP containing 5 μCi [α-^{33}P]GTP in 100 μl of GTP-loading buffer [20 mM tris-HCl (pH 7.5), 2 mM EDTA, and 2 mM DTT] at room temperature for 15 min. $MgCl_2$ was added to a final concentration of 10 mM, and GTP-bound RhoA was separated from unbound GTP on a Sephadex G-25 desalting column. The 100-μl elution fraction containing GTP-bound RhoA was supplemented with 100 μl of 2 × GAP-buffer [20 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 2 mM DTT] and split into four aliquots of 50 μl. Intrinsic and p50RhoGAP-mediated GTP hydrolysis was measured in the absence or presence of 2 μM SseJ and 50 nM p50RhoGAP. At individual time points, reactions in 5 μl aliquots of each assay solution were stopped by the addition of 5 μl of running buffer containing 1:1.5 (v/v) saturated $(NH_4)_2SO_4$ and 1.5 M KH_2PO_4 (pH 3.6). Samples were blotted onto Polygram CEL 300 polyethyleneimine cellulose thin-layer chromatography plates (Macherey-Nagel). Plates were developed in 1:1.5 (v/v) saturated $(NH_4)_2SO_4$ and 1.5 M KH_2PO_4 (pH 3.6), dried, and exposed on a Storage PhosphorScreen (Amersham Biosciences). Hydrolysis of GTP was quantified by measuring the intensity of GTP and GDP with ImageJ software version 1.33 and by curve fitting with the proFit software 4.6 (Quantumsoft).

GEF filter binding assay

RhoA (2.5 μM) alone or 2.5 μM RhoA and 0.5 μM H-Dbs (Cytoskeleton) in the presence or absence of 2.5 μM SseJ were incubated at 30°C for 10 min with 5 μM GTP containing 1 μCi [α-^{33}P]GTP in 50 μl of GEF buffer [20 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, and bovine serum albumin (50 μg/ml)]. The reaction was terminated by addition of $MgCl_2$ to a final concentration of 16 mM, followed by filtration through a nitrocellulose membrane on a 96-well microfiltration apparatus. The sample was then washed with 3 ml of buffer B [50 mM tris-HCl (pH 7.4), 50 mM NaCl, and 2 mM $MgCl_2$]. The membrane was then dried and exposed on a Storage PhosphorScreen (Amersham Biosciences). The incorporation of GTP was quantified with the ImageJ program. The background signals from samples containing SseJ or H-Dbs alone were subtracted from RhoA GTP incorporation assays.

SUPPLEMENTARY MATERIALS

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Fig. S1. Assessment of the interaction of SseJ with various GTPγS-loaded GTPases and their ability to stimulate its lipase activity.

Fig. S2. Cotransfection of HeLa cells with plasmids encoding CA RhoA and SseJ results in the accumulation of cholesterol and neutral lipids in the enlarged endosomal compartment.

Fig. S3. The expression of SseJ3x or RhoA alone does not induce the formation of BODIPY-labeled cholesterol esters.

Table S1. Descriptions of the strains of *S. typhimurium* and the plasmids used in this study.

References

REFERENCES AND NOTES

1. O. Steele-Mortimer, The *Salmonella*-containing vacuole: Moving with the times. *Curr. Opin. Microbiol.* **11**, 38–45 (2008).
2. A. Haraga, M. B. Ohlson, S. I. Miller, Salmonellae interplay with host cells. *Nat. Rev. Microbiol.* **6**, 53–66 (2008).
3. M. Hensel, J. E. Shea, S. R. Waterman, R. Mundy, T. Nikolaus, G. Banks, A. Vazquez-Torres, C. Gleeson, F. C. Fang, D. W. Holden, Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. *Mol. Microbiol.* **30**, 163–174 (1998).
4. D. Zhou, J. Galán, *Salmonella* entry into host cells: The work in concert of type III secreted effector proteins. *Microbes Infect.* **3**, 1293–1298 (2001).
5. T. D. Lawley, K. Chan, L. J. Thompson, C. C. Kim, G. R. Govoni, D. M. Monack, Genome-wide screen for *Salmonella* genes required for long-term systemic infection of the mouse. *PLoS Pathog.* **2**, e11 (2006).
6. J. Ruiz-Albert, X. J. Yu, C. R. Beuzón, A. N. Blakey, E. E. Galyov, D. W. Holden, Complementary activities of SseJ and SifA regulate dynamics of the *Salmonella typhimurium* vacuolar membrane. *Mol. Microbiol.* **44**, 645–661 (2002).
7. M. B. Ohlson, K. Fluhr, C. L. Birmingham, J. H. Brumell, S. I. Miller, SseJ deacylase activity by *Salmonella enterica* serovar Typhimurium promotes virulence in mice. *Infect. Immun.* **73**, 6249–6259 (2005).
8. J. A. Freeman, M. E. Ohl, S. I. Miller, The *Salmonella enterica* serovar Typhimurium translocated effectors SseJ and SifB are targeted to the *Salmonella*-containing vacuole. *Infect. Immun.* **71**, 418–427 (2003).
9. P. Nawabi, D. M. Catron, K. Haldar, Esterification of cholesterol by a type III secretion effector during intracellular *Salmonella* infection. *Mol. Microbiol.* **68**, 173–185 (2008).
10. N. S. Lossi, N. Rolhion, A. I. Magee, C. Boyle, D. W. Holden, The *Salmonella* SPI-2 effector SseJ exhibits eukaryotic activator-dependent phospholipase A and glycerophospholipid: Cholesterol acyltransferase activity. *Microbiology (Reading, England)* **154**, 2680–2688 (2008).
11. M. B. Ohlson, Z. Huang, N. M. Alto, M. P. Blanc, J. E. Dixon, J. Chai, S. I. Miller, Structure and function of *Salmonella* SifA indicate that its interactions with SKIP, SseJ, and RhoA induce endosomal tubulation. *Cell Host Microbe* **4**, 434–446 (2008).
12. S. J. Heasman, A. J. Ridley, Mammalian Rho GTPases: New insights into their functions from in vivo studies. *Nat. Rev. Mol. Cell Biol.* **9**, 690–701 (2008).
13. S. Etienne-Manneville, A. Hall, Rho GTPases in cell biology. *Nature* **420**, 629–635 (2002).
14. K. Aktories, J. T. Barbieri, Bacterial cytotoxins: Targeting eukaryotic switches. *Nat. Rev. Microbiol.* **3**, 397–410 (2005).
15. Y. Fu, J. E. Galán, A *Salmonella* protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. *Nature* **401**, 293–297 (1999).
16. W. D. Hardt, L. M. Chen, K. E. Schuebel, X. R. Bustelo, J. E. Galán, *S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell* **93**, 815–826 (1998).
17. M. L. Yarbrough, Y. Li, L. N. Kinch, N. V. Grishin, H. L. Ball, K. Orth, AMPylation of Rho GTPases by *Vibrio* VopS disrupts effector binding and downstream signaling. *Science* **323**, 269–272 (2009).
18. G. Flatau, E. Lemichez, M. Gauthier, P. Chardin, S. Paris, C. Fiorentini, P. Boquet, Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine. *Nature* **387**, 729–733 (1997).

19. S. C. Tsai, M. Noda, R. Adamik, J. Moss, M. Vaughan, Enhancement of choleragen ADP-ribosyltransferase activities by guanyl nucleotides and a 19-kDa membrane protein. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5139–5142 (1987).
20. G. Schmidt, P. Sehr, M. Wilm, J. Selzer, M. Mann, K. Aktories, Gln 63 of Rho is deamidated by *Escherichia coli* cytotoxic necrotizing factor-1. *Nature* **387**, 725–729 (1997).
21. I. Just, J. Selzer, M. Wilm, C. von Eichel-Streiber, M. Mann, K. Aktories, Glucosylation of Rho proteins by Clostridium difficile toxin B. *Nature* **375**, 500–503 (1995).
22. D. M. Catron, M. D. Sylvester, Y. Lange, M. Kadekoppala, B. D. Jones, D. M. Monack, S. Falkow, K. Haldar, The *Salmonella*-containing vacuole is a major site of intracellular cholesterol accumulation and recruits the GPI-anchored protein CD55. *Cell. Microbiol.* **4**, 315–328 (2002).
23. S. E. Witowski, K. A. Walker, V. L. Miller, YspM, a newly identified Ysa type III secreted protein of *Yersinia enterocolitica*. *J. Bacteriol.* **190**, 7315–7325 (2008).
24. E. Ikonen, Cellular cholesterol trafficking and compartmentalization. *Nat. Rev. Mol. Cell Biol.* **9**, 125–138 (2008).
25. Acknowledgments: We thank D. A. Doyle from the Structural Genomics Consortium at the University of Oxford for the gift of the RhoB expression plasmid. We also thank L. Hoffman, H. Kulasekara, I. Levin, B. Kulasekara, and the Miller laboratory for discussions and critical reading of the manuscript. M.C. is supported by grants PBBSA-120489 and PA00P3-124140 from the Novartis Foundation and Swiss National Foundation. D.L. is supported by U.S. Public Health Service National Research Service Award T32 GM07270 from the National Institute of General Medical Sciences and S.I.M is supported by the NIH–National Institute of Allergy and Infectious Diseases grants R01 AI048683 and U54AI057141 for the Northwest Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research.
Author contributions: S.I.M. supervised the project. M.C., L.H.C., J.S.H., D.L.L., R.A.P., and S.I.M. conducted the experiments in this study with the exception of the infection assays and microscopy, which were conducted by L.H.C. and M.C. M.C, L.H.C., and S.I.M. contributed to the writing of the manuscript.

Submitted 14 May 2009
Accepted 16 October 2009
Final Publication 3 November 2009
10.1126/scisignal.2000430
Citation: M. Christen, L. H. Coye, J. S. Hontz, D. L. LaRock, R. A. Pfuetzner, Megha, S. I. Miller, Activation of a bacterial virulence protein by the GTPase RhoA. *Sci. Signal.* **2**, ra71 (2009).