HHS Public Access

Author manuscript

Sci Signal. Author manuscript; available in PMC 2016 November 04.

Published in final edited form as:

Sci Signal. ; 2(95): ra71. doi:10.1126/scisignal.2000430.

Activation of a bacterial virulence protein by the GTPase RhoA

Matthias Christen[1,*], **Lisette H. Coye**[1,*], **Jill S. Hontz**[2], **Doris L. LaRock**[3], **Richard A. Pfuetzner**[1], **Megha**[2], and **Samuel I. Miller**[1,2,3,4,¶]

[1]Department of Immunology, University of Washington, Seattle, WA, 98195, USA

[2]Department of Genome Sciences, University of Washington, Seattle, WA, 98195, USA

[3]Department of Microbiology, University of Washington, Seattle, WA, 98195, USA

[4]Department of Medicine, University of Washington, Seattle, WA, 98195, USA

Abstract

Rho-family GTPases are essential eukaryotic signaling molecules that regulate cellular physiology. Virulence factors from various pathogens alter GTPase signaling by functioning as GTPase activating factors (GAPs), guanine exchange factors (GEFs) or direct covalent modifiers. Bacterial virulence factors that sense rather than alter Rho-family GTPase signaling states have not been previously described. Here, we report that the translocated Salmonellae virulence factor SseJ binds to the GTP bound form of RhoA with resultant stimulation of its enzymatic activity that results in host cell membrane cholesterol esterification. Therefore, GTPase mediated downstream activation is not exclusive to eukaryotic proteins, and a bacterial protein has evolved to recognize the GTPase signaling state of RhoA to regulate 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 as a pathogenic strategy (1). Invasion of animal cells as well as remodeling of the *Salmonella*- containing vacuole (SCV) depend on the translocation of proteins from bacteria to the animal cell cytosol 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 expressing SseJ with mutations in active

¶To whom correspondence should be addressed. millersi@u.washington.edu.

*These authors contributed equally to this report.

Correspondence and requests for materials should be addressed to (millersi@u.washington.edu).

Author Contributions

S.I.M. supervised the project. M.C. conducted together with L.H.C., J.S.H., D.L., R.A.P. and M. the experiments in this study with the exception of the infection assays and microscopy, which were conducted by L.C and M.C., M.C, L.H.C and S.I.M. contributed to the writing of the manuscript.

site residues are attenuated for virulence for mice similar to *sseJ* null mutants (7). This indicates that SseJ enzymatic activity is important for bacterial pathogenesis within animals.

Results

Previously, our group identified Rho family GTPase members as potential mammalian binding targets of SseJ (11). Based on this finding, we analyzed whether SseJ interacts with RhoA during *S. typhimurium* infection. HeLa cells transfected with myc-tagged RhoA were infected with either a *S. typhimurium sseJ* null mutant strain or a strain expressing hemaglutinin (HA) tagged SseJ (SseJ-HA). Myc-tagged RhoA remained evenly distributed in HeLa cells infected with an *sseJ* mutant (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 constitutively active myc-tagged RhoA-G14V (CA RhoA) was expressed, it was specifically recruited to the SCV of bacteria expressing SseJ-HA (Fig. 1D) and was not enriched on SCVs in HeLa cells infected with the *sseJ* deletion mutant (Fig. 1C). Thus, recruitment of CA RhoA to the SCV was specifically associated with the presence of SseJ.

We further investigated the interaction between SseJ and RhoA *in vitro* using gel filtration. We found that purified RhoA and SseJ formed a complex dependent on the activation state of RhoA (Fig. 2A). Gel filtration with equimolar ratios of SseJ and apo-RhoA (nucleotide free RhoA) resulted in a minor shift of SseJ toward a higher molecular mass, and 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 GTP (GTPγS), resulted in a strong shift of SseJ towards a higher molecular mass, and free RhoA was absent in 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 S1). The observation that the bacterial virulence factor SseJ specifically interacted with the GTPase subfamily members RhoA and RhoC in the presence of GTPγS was significant because Rho GTPases are known to play a pivotal role in eukaryotic signal transduction cascades (12). Small GTPases act as molecular switches, cycling between inactive GDP-bound and activated GTP-bound forms, and exert their function by binding and activating their cognate downstream effectors in a GTP-dependent manner (13). Many bacterial virulence factors have evolved to manipulate GTPase signaling by mimicking their regulators (14–16) or by acting as covalent modifiers of the GTPases (17–21). Among them are SopE and SptP, which act as guanine exchange factor (GEF) and GTPase activating factor (GAP) for Cdc42 and Rac-1 respectively (15). Therefore, we investigated if SseJ affected the GTP/GDP cycling of RhoA or interfered with the performance of endogenous Rho GEFs and GAPs. We found that an excess of 2 μM SseJ versus 1 μM RhoA affected neither intrinsic RhoA activity nor RhoA GTPase stimulation by 50 nM p50RhoGAP (Fig. 2C). In addition, GEF filter binding assays measuring ^{33}P-GTP incorporation of 2.5 μM RhoA in the presence or absence of equimolar SseJ ± 0.5 μM Dbs (eukaryotic GEF) did not reveal GEF activity for SseJ or competition with Dbs (Fig. 2D), indicating that SseJ does not function as a GAP or GEF for RhoA and that its binding does not alter endogenous regulatory proteins from functioning.

SseJ is a modular protein consisting of two discrete domains. The amino-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 carboxyl-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 activity has been detected *in vitro* using purified recombinant protein (7, 9, 10). Recently, it has been demonstrated that basal PLA and GCAT activities of SseJ are stimulated by HeLa cell extracts, suggesting that a eukaryotic factor is required for SseJ activation (10). Therefore, gel filtration was performed with SseJ and GTPγS-loaded RhoA, and the eluted fractions were tested for lipase activity using the chromogenic substrate para-Nitrophenylpalmitate (PNPP). Lipase activity was strongly stimulated in the presence of RhoA with an activation profile corresponding to fractions containing the SseJ-RhoA complex (Fig. 2A). Subsequent PNPP lipase assays with 200 nM SseJ and 200nM GTPγS-loaded RhoA, RhoB, RhoC, H-Ras, Rac1 or Cdc42 confirmed that only RhoA acts as a potent activator of SseJ lipase activity *in vitro* (Fig. 2E,F). Partial lipase stimulation was observed with RhoC, and no lipase stimulation was seen with RhoB, Cdc42, H-Ras or Rac1 (Fig. 2E,F). These observations underline the specificity of SseJ for a single GTPase subset and also correlated physical binding with activation.

Since our findings from microscopy (Fig. 1) and the results from gel filtration (Fig. 2) suggested that the interaction between RhoA and SseJ was dependent on the RhoA activation state, we further investigated if lipase stimulation was affected by the RhoA guanosine nucleotide loading state. PNPP lipase assays with 200 nM SseJ and 200 nM Apo-RhoA, RhoA-GDP or RhoA-GTPγS resulted in a factor of five increase in SseJ lipase stimulation by Rho-GTPγS (1.2 mAU/min) compared to Rho-GDP (0.21mAU/min)(Fig. 3A,B). Titration experiments with 200 nM SseJ and increasing concentrations of Apo-RhoA, RhoA-GDP or RhoA-GTPγS also revealed a concentration-dependent activation curve that reached a saturation plateau with maximal stimulation in the presence of the GTPγS complex (Fig. 3C). Although partial stimulation was observed in the presence of RhoA-GDP or Apo-RhoA, neither reached a maximal level comparable to the activation seen with RhoA-GTPγS, even at elevated RhoA concentration (Fig. 3C). This dependence of SseJ activation upon the GTP loading state of RhoA is similar to the conformation-dependent stimulation of eukaryotic downstream effectors by their cognate GTP-bound GTPases.

The SCV is a major site of intracellular cholesterol accumulation during late stages of *S. typhimurium* infection (22). SseJ has been shown to possess weak GCAT activity and to esterify cholesterol *in vivo* when expressed within HeLa cells and macrophages (9, 10). To test whether the activated SseJ-RhoA complex exhibited GCAT activity *in vitro*, we analyzed enzyme performance and substrate specificity of the activated SseJ-RhoA complex using the physiological substrates dioleolylphosphocholine (DOPC) and cholesterol. Liposomes containing 60:30:10 molar ratios 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 lyso-phosphatidylcholine (lyso-PC) and cholesterol ester (CE) in the presence of RhoA-GTPγS, while no activity was detected for SseJ alone (Fig. 3D). To

determine if CA RhoA enhanced SseJ GCAT activity *in vivo*, we coexpressed both proteins within HeLa cells. Ectopic expression of SseJ in HeLa cells resulted in its localization to the late endosomal compartments which displays morphological changes (6) (Fig. S2). As in the SCV there is an accumulation of cholesterol to the endosomal compartments and an overall cellular decrease in free cholesterol due to cholesterol ester formation by SseJ (9). We found that when cotransfected, CA RhoA and SseJ colocalized as expected, and the endosomal compartments appeared enlarged (Fig. S2) as compared to cells expressing SseJ alone. These observations suggested that activated RhoA also stimulated the GCAT activity of SseJ in mammalian cells.

We then tested the substrate specificity of activated SseJ. Liposomal assays were performed with two sn-1 or sn-2 specific fluorogenic substrates (PEDA1 and PED6), composed of a quencher (dinitrophenyl) on the glycerophosphoethanolamine head group and BODIPY® acyl chains at either position sn-1 or sn-2. We found that the GCAT activity of activated SseJ specifically cleaved the sn-1 position as demonstrated by a two order of magnitude faster fluorescence release in the presence of PEDA1 ($1.52*10^4$ RFU/min) compared to 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 cotransfected HeLa cells expressing SseJ and CA RhoA with 3.5 μM PEDA1 for 2 h revealed an increase in fluorescent cholesterol ester formation in the presence of SseJ and its activator CA RhoA (Fig. 5B), confirming our biochemical results using purified proteins (Fig. 4A,B). No fluorescence increase was detected either in cells cotransfected with CA RhoA and the SseJ3x catalytic mutant (Fig. 5A,B) or in cells transfected with SseJ3x or CA RhoA alone (Fig. 5B and Fig S3).

Discussion

Taken together, our results indicate that the *S. typhimurium* protein SseJ modulates cellular cholesterol levels 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 for which activity is weak *in vitro*. In contrast, with many other bacterial effector proteins, alteration of RhoA GDP/GTP signaling state appears not to be the function of the effector protein SseJ. Instead, SseJ defines a new class of bacterial effector proteins that link GTPase signaling state to pathogen-controlled output functions. The only other known bacterial virulence protein to be enzymatically regulated by a GTPase is cholera toxin whose ADP-ribosylation activity of G proteins is moderately activated by the GTP bound form of the small GTPase Arf. However, cholera toxin has been shown *in vitro* to modify several protein including Arf (19), and it is unknown whether this modification alters the signaling state of Arf. The identification of RhoA as a potent GTP-dependent regulator of SseJ activity provides a key to understanding the molecular mechanism of SseJ action. These findings suggest that during *S. typhimurium* infection, SseJ specifically alters membrane cholesterol composition of vesicular

compartments including the SCV, dependent on the RhoA signaling state. In order to alter SCV membane cholesterol composition and to coordinate SCV membrane dynamics, it may be crucial to tightly regulate SseJ activity in a spatio-temporal manner to promote bacterial pathogenesis. Thus, GTPase mediated activation is not exclusive to eukaryotic proteins and is an intimate part of the host-Salmonellae interaction because SseJ and perhaps other proteins have evolved to similarly use the GTPase signaling state to regulate their enzymatic activity.

Materials and Methods

Co-transfections and infections

HeLa cells (ATCC) were transfected with vectors (Supplementary Table 1) using Fugene 6 (Roche) as recommended by the manufacturer and left for 24 h. For the PEDA1 (Invitrogen) experiment, cells were incubated for 2 h with 3.5 μM of the substrate. HeLa cells were infected at a MOI of 1:100 with *S. typhimurium* (Supplementary Table 1) grown from a back dilution from an overnight culture. Cells were washed with 1XPBS and treated with 0.15mg/ml gentamicin for an hour followed by incubation for 14 h with 0.015 mg/ml of gentamicin. The cells were fixed and permeabilized using BD cytofix/cytoperm kit (BD-Biosciences) Immunostaining and 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 using the NIH-elements image analysis software.

Protein purification

The purified proteins of the GTPases RhoA, RhoC, Cdc42, Ras and Rac were purchased from Cytoskeleton. In addition his-RhoA, his-RhoB and his-SseJ were expressed in bacteria, purified over a nickel column followed by cleavage of the his-tag with thrombin and further purification over a gel filtration column. Purified proteins were stored in TBS buffer supplemented with 10% glycerol and 2 mM DTT at −80 °C.

Size exclusion chromatography

The GTPases RhoA, RhoB, RhoC, Cdc42, Ras and Rac were loaded prior to gelfiltration with GTPγS by adding 100 μM GTPγS to 10 μM GTPase in TBS buffer containing 2 mM EDTA and incubated on ice for 10 min. The GTPγS-GTPase complex was stabilized by adding 10 mM $MgCl_2$. Samples containing 100 μl of 4 μM SseJ alone, or equimolar ratios of 4 μM SseJ and GTPγS bound GTPase in TBS buffer containing 10 mM $MgCl_2$ and 50 μM GTPγS were injected onto a sephadex-200 size exclusion column on a Acta FPLC (Amersham). Protein complexes were separated at a flow rate of 0.5 ml/min using TBS buffer supplemented with 10 mM $MgCl_2$. Fractions of 300 μl were collected and lipase activity was determined by a PNPP lipase assay (see below).

PNPP lipase assays

Lipase activity was determined by hydrolysis of the substrate para-nitrophenyl palmitate (PNPP) and subsequent release of para-nitrophenol, which is detected by measuring absorbance at 405 nm. Assays were performed as triplicates in a 384 well format. In a

standard assay, 0.4 μl of 10 mM PNPP dissolved in DMSO was added to 40 μl of 200 nM SseJ in the presence or absence of 200 nM GTPase and 5 μM GTPγS or GDP. After mixing, plates were incubated at 37°C and increase in absorption at 405 nm was measured on a EnVision Multilabel Reader (Perkin Elmer, MA) at 2 min intervals.

Liposomal Phospholipase Cholesterol esterase assays

Liposomes containing 60:30:10 molar ratio DOPC:Cholesterol:Oleic acid were prepared by adding to a 2 ml eppendorf tube chloroform dissolved stock solutions of 120 μl 10 mM cholesterol, 60 μl 10 mM DOPC and 20 μl 10 mM oleic acid. Lipids were dried under N2 gas and dry lipids were completely resolved in 20 μl ethanol by incubation for 30 seconds at 55 °C. Liposomes were prepared by rapid dilution in 2 ml TBS buffer containing 10 mM $MgCl_2$. Aliquots of 100 μl were supplemented with 200 nM SseJ in presence or absence of 200 nM GTPγS loaded RhoA and incubated at 37°C for 30 min for phospholipase cholesterol esterase assays. The reaction was stopped by the addition of 100 μl chloroform: methanol (2:1 vol/vol) and reaction products were separated by TLC on silica gel (Whatman, Clifton, NJ) using the solvent hexane: ethylether: acetic acid (80:15:5 vol/vol) as mobile phase and 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, CA). Aliquots of 100 μl were supplemented with 200 nM SseJ in presence or absence of 200 nM GTPγS loaded RhoA and incubated at 37°C. Cleavage at the sn-1 or sn-2 position was followed by measuring fluorescence increase (Ex = 488 nm, Em =530 nm) on an EnVision Multilabel Reader (Perkin Elmer, MA).

GAP assays

Purified RhoA (1 μM) was GTP loaded in presence of 5μM GTP containing 5 μCi [α-^{33}P]GTP in 100 μl GTP-loading buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 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 2x GAP-buffer (20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT) and split into four aliquots of 50 μl. Intrinsic and p50RhoGAP-mediated GTP hydrolysis was measured in absence or presence of 2 μM SseJ and 50nM p50RhoGAP. At individual time points a 5 μl aliquot of each assay was stopped in 5 μl running buffer containing 1:1.5 (v/v) saturated $(NH_4)_2SO_4$ and 1.5 M KH_2PO_4, pH 3.60, and blotted on 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 3.60, dried, and exposed on a Storage PhosphorScreen (Amersham Biosciences). GTP hydrolysis was quantified by measuring the intensity of GTP and GDP using ImageJ software version 1.33 and by curve fitting with the proFit software 4.6 (Quantumsoft).

GEF filter binding assay

2.5 μM RhoA alone or 2.5 μM RhoA and 0.5 μM 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 GEF buffer (20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 50

μg/ml bovine serum albumin). The reaction was terminated by adding $MgCl_2$ 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). GTP incorporation was quantified with the ImageJ program. Backround signal from samples containing SseJ or DBS alone were substracted from RhoA GTP incorporation assays

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Declan A. Doyle from the Structural Genomics Consortium at University of Oxford for the generous gift of the RhoB expression plasmid. We also thank L. Hoffman, H. Kulasekara, I. Levin, B. Kulasekara, and the Miller lab for discussions and critical reading of the manuscript. M.C. is supported by grants from Novartis Foundation and Swiss National Foundation PBBSA-120489 and PA00P3-124140. D. L. is supported by PHS NRSA T32 GM07270 from NIGMS and S.I.M by the NIH/NIAID grants R01 AI048683 and U54AI057141 for the Northwest Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research.

References

1. Steele-Mortimer O. The Salmonella-containing vacuole: moving with the times. Curr Opin Microbiol. 2008; 11:38. [PubMed: 18304858]
2. Haraga A, Ohlson MB, Miller SI. Salmonellae interplay with host cells. Nat Rev Microbiol. 2008; 6:53. [PubMed: 18026123]
3. Hensel M, Shea JE, Waterman SR, Mundy R, Nikolaus T, Banks G, Vazquez-Torres A, Gleeson C, Fang FC, Holden DW. 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. 1998; 30:163. [PubMed: 9786193]
4. Zhou D, Galán J. Salmonella entry into host cells: the work in concert of type III secreted effector proteins. Microbes Infect. 2001; 3:1293. [PubMed: 11755417]
5. Lawley TD, Chan K, Thompson LJ, Kim CC, Govoni GR, Monack DM. Genome-wide screen for Salmonella genes required for long-term systemic infection of the mouse. PLoS Pathog. 2006; 2:e11. [PubMed: 16518469]
6. Ruiz-Albert J, Yu XJ, Beuzón CR, Blakey AN, Galyov EE, Holden DW. Complementary activities of SseJ and SifA regulate dynamics of the Salmonella typhimurium vacuolar membrane. Mol Microbiol. 2002; 44:645. [PubMed: 11994148]
7. Ohlson MB, Fluhr K, Birmingham CL, Brumell JH, Miller SI. SseJ deacylase activity by Salmonella enterica serovar Typhimurium promotes virulence in mice. Infect Immun. 2005; 73:6249. [PubMed: 16177296]
8. Freeman JA, Ohl ME, Miller SI. The Salmonella enterica serovar typhimurium translocated effectors SseJ and SifB are targeted to the Salmonella-containing vacuole. Infect Immun. 2003; 71:418. [PubMed: 12496192]
9. Nawabi P, Catron DM, Haldar K. Esterification of cholesterol by a type III secretion effector during intracellular Salmonella infection. Mol Microbiol. 2008; 68:173. [PubMed: 18333886]
10. Lossi NS, Rolhion N, Magee AI, Boyle C, Holden DW. The Salmonella SPI-2 effector SseJ exhibits eukaryotic activator-dependent phospholipase A and glycerophospholipid: cholesterol acyltransferase activity. Microbiology (Reading, Engl). 2008; 154:2680.
11. Ohlson MB, Huang Z, Alto NM, Blanc MP, Dixon JE, Chai J, Miller SI. Structure and Function of Salmonella SifA Indicate that Its Interactions with SKIP, SseJ, and RhoA. Cell Host Microbe. 2008; 4:434. [PubMed: 18996344]

12. Heasman SJ, Ridley AJ. Mammalian Rho GTPases: new insights into their functions from in vivo studies. Nat Rev Mol Cell Biol. 2008; 9:690. [PubMed: 18719708]
13. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. Nature. 2002; 420:629. [PubMed: 12478284]
14. Aktories K, Barbieri JT. Bacterial cytotoxins: targeting eukaryotic switches. Nat Rev Microbiol. 2005; 3:397. [PubMed: 15821726]
15. Fu Y, Galán JE. A salmonella protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. Nature. 1999; 401:293. [PubMed: 10499590]
16. Hardt WD, Chen LM, Schuebel KE, Bustelo XR, Galán JE. S. typhimurium encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. Cell. 1998; 93:815. [PubMed: 9630225]
17. Yarbrough ML, Li Y, Kinch LN, Grishin NV, Ball HL, Orth K. AMPylation of Rho GTPases by Vibrio VopS disrupts effector binding and downstream signaling. Science. 2009; 323:269. [PubMed: 19039103]
18. Flatau G, Lemichez E, Gauthier M, Chardin P, Paris S, Fiorentini C, Boquet P. Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine. Nature. 1997; 387:729. [PubMed: 9192901]
19. Tsai SC, Noda M, Adamik R, Moss J, Vaughan M. Enhancement of choleragen ADP-ribosyltransferase activities by guanyl nucleotides and a 19-kDa membrane protein. Proc Natl Acad Sci USA. 1987; 84:5139. [PubMed: 3110784]
20. Schmidt G, Sehr P, Wilm M, Selzer J, Mann M, Aktories K. Gln 63 of Rho is deamidated by Escherichia coli cytotoxic necrotizing factor-1. Nature. 1997; 387:725. [PubMed: 9192900]
21. Just I, Selzer J, Wilm M, von Eichel-Streiber C, Mann M, Aktories K. Glucosylation of Rho proteins by Clostridium difficile toxin B. Nature. 1995; 375:500. [PubMed: 7777059]
22. Catron DM, Sylvester MD, Lange Y, Kadekoppala M, Jones BD, Monack DM, Falkow S, Haldar K. The Salmonella-containing vacuole is a major site of intracellular cholesterol accumulation and recruits the GPI-anchored protein CD55. Cell Microbiol. 2002; 4:315. [PubMed: 12067317]
23. Witowski SE, Walker KA, Miller VL. YspM, a newly identified Ysa type III secreted protein of Yersinia enterocolitica. J Bacteriol. 2008; 190:7315. [PubMed: 18805975]

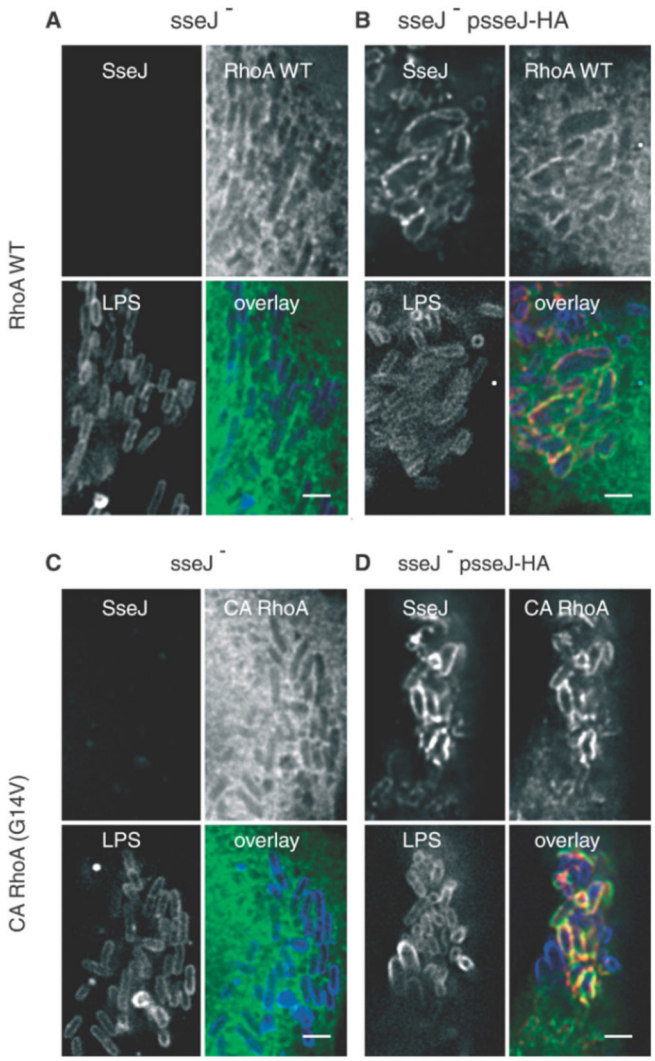

Fig. 1.

SseJ recruits constitutive active myc-RhoA to the SCV. HeLa cells transfected for 4 hours with wild type myc-RhoA (**A**,**B**) or constitutive active myc-RhoA (**C**,**D**) were infected for 15 hours with either a *sseJ*– mutant strain (**A**,**C**) or *S. typhimurium* strain secreting SseJ-HA (*sseJ*-, psseJ-HA) (**B**,**D**) and were immunostained for myc-RhoA (green), SseJ-HA (red), and lipopolysaccharide (LPS) (blue). Scale bar, 2 μm.

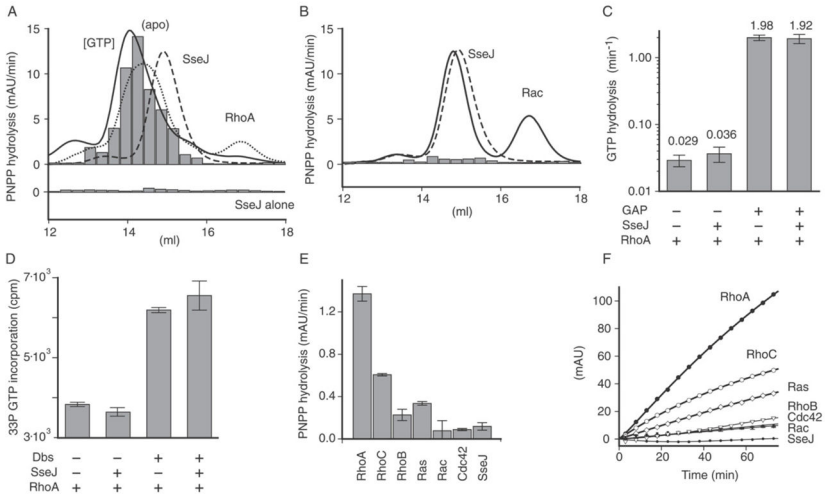

Fig. 2.

SseJ forms a complex with RhoA dependent on the activation state of RhoA. (**A**,**B**) Gel filtrations of SseJ alone (dashed line), in the presence of apo-RhoA (dotted line) or 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 lipase activity of eluted fractions from gel filtration of SseJ and RhoA-GTPγS. The lower bar graph shows basal lipase activity of eluted fractions containing SseJ alone. The SseJ-RhoA complex formation does not impair RhoA GDP/GTP cycling in vitro. (**C**) Binding of SseJ does not alter intrinsic or p50RhoGAP-stimulated GTPase activity of RhoA. (**D**) Presence of SseJ does not impair Dbs mediated GEF activity of RhoA. Bar graphs (**E**) and corresponding enzyme kinetics (**F**) of SseJ lipase stimulation in presence of GTPγS loaded RhoA, RhoB, RhoC, H-Ras, Rac-1 or Cdc42 indicate specific activation by the Rho GTPases subfamily member RhoA and RhoC.

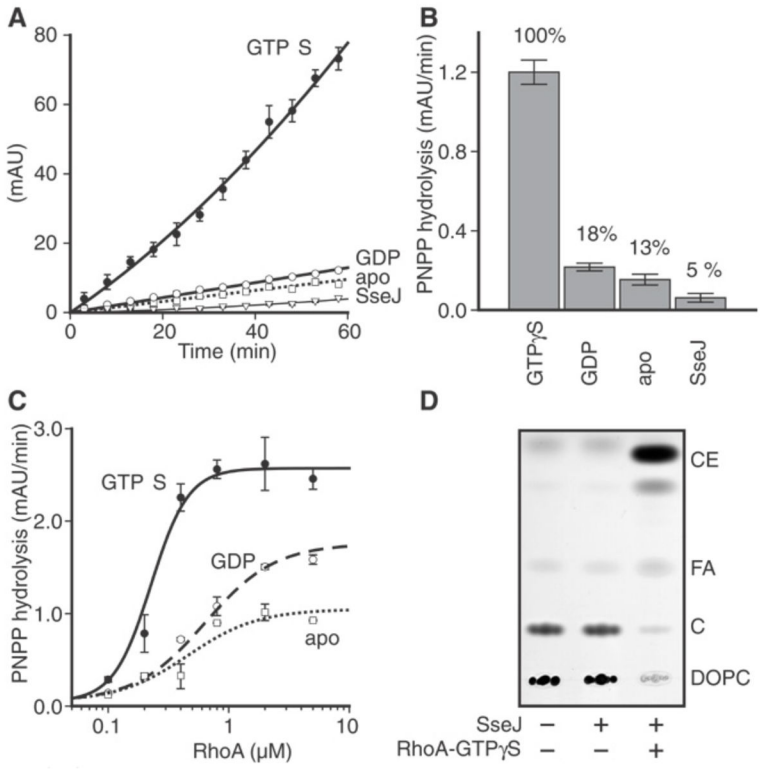

Fig. 3.

SseJ activation is dependent on the guanosine nucleotide loading state of RhoA. (**A**, **B**) Lipase activation assays with 200 nM SseJ and 200 nM RhoA-GTPγS, RhoA-GDP or Apo-RhoA reveal maximal stimulation by RhoA-GTPγS. (**C**) Lipase stimulation assays in presence of 200nM SseJ and increasing concentrations of RhoA-GTPγS, RhoA-GDP or apo-RhoA reveal a higher binding affinity and stronger activation by RhoA-GTPγS than GDP bound or apo RhoA. (**D**) TLC analysis of the reaction products indicating RhoA dependent glycerophospholipid-cholesterol acyltransferase activation in presence of the biological substrates DOPC and Cholesterol.

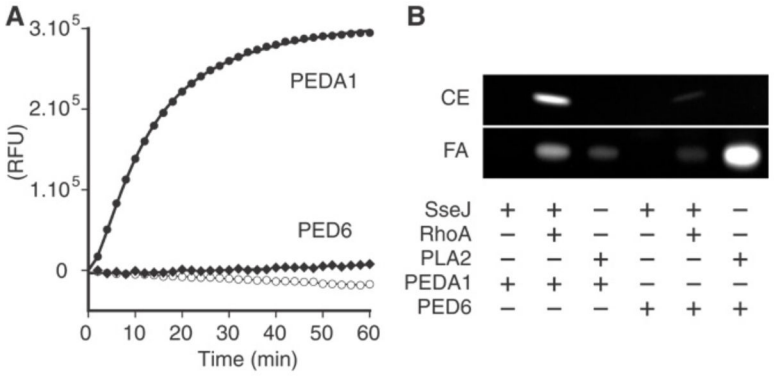

Fig. 4.

The activated complex preferentially transfers sn-1 acyl chains from glycerophospholipids to cholesterol. (**A**) Enzymatic assays with liposomes containing two sn-1 and sn-2 selective fluorescent probes PEDA1 (●) and PED6 (◆). SseJ does not cleave PEDA1 in absence of GTPgS loaded RhoA (❍). (**B**) TLC analysis of the fluorescent reaction products confirming the formation of a BODIPY® modified cholesterol ester (CE) in presence of PEDA1. Bee venom phospholipase A2 (PLA2), which generates free fatty acids (FA) was used as a control.

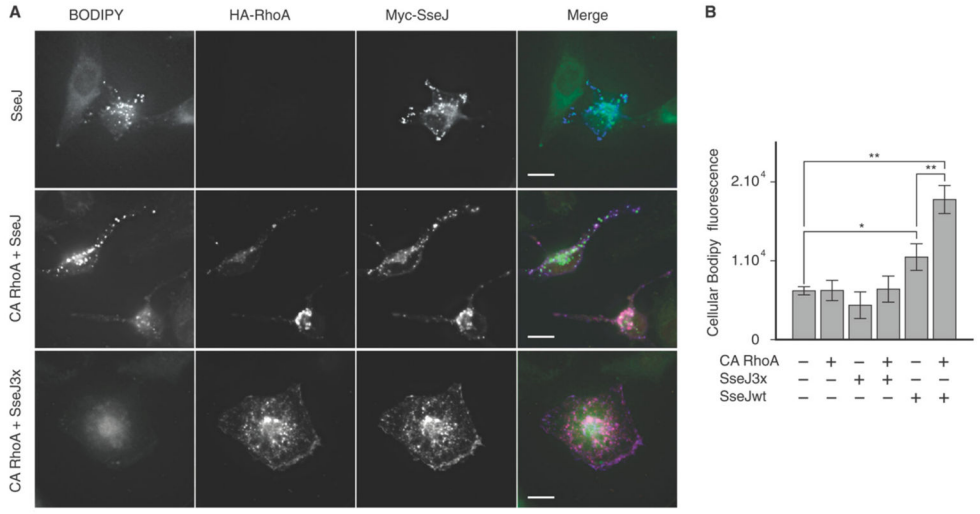

Fig. 5.

SseJ and CA RhoA localize in cotransfected HeLa cells on GMCs containing fluorescent BODIPY® labeled cholesterol. (**A**) HeLa cells were transfected with myc-SseJ alone or cotransfected with CA HA-RhoA and myc-SseJ or the catalytic mutant SseJ3x for 24 hours and were then incubated for 2h with 3.5 μM PEDA1 substrate followed by immunostaining for 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 analysed.