Activation of the *Salmonella enterica* serovar Typhimurium translocated glycerophospholipid:cholesterol acyltransferase SseJ by the small GTPase RhoA

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2012

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Program Authorized to Offer Degree:

Microbiology

University of Washington

ABSTRACT

Activation of the *Salmonella enterica* serovar Typhimurium translocated glycerophospholipid:cholesterol acyltransferase SseJ by the small GTPase RhoA

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Salmonella enterica serovar Typhimurium translocates a glycerophospholipid:cholesterol acyltransferase (SseJ) into the host cytosol after its entry into mammalian cells. SseJ is recruited to the cytoplasmic face of the host cell phagosome membrane where it is activated and converts cholesterol to cholesterol ester upon binding the small GTPase, RhoA. This study shows that SseJ is regulated similarly to cognate eukaryotic effectors and only the GTP-bound form of RhoA, RhoB, RhoC, but not other Rho GTPases stimulates enzymatic activity. Using NMR and biochemistry this work demonstrates that SseJ competes effectively in binding with eukaryotic effectors and specifically competes for a similar RhoA binding surface as Rhotekin, ROCK, and PKN1. The RhoA surface that binds SseJ includes the regulatory switch regions that controls activation of mammalian effectors. These data

were used to create RhoA mutants with altered SseJ binding and activation. This structure-function analysis supports a model in which SseJ activation occurs predominantly through binding to residues within switch region II. We further defined the nature of the interaction between SseJ and RhoA by constructing SseJ mutants in the RhoA binding surface. We discovered that SseJ binding to RhoA is required for recruitment of SseJ to the endosomal network and for full *Salmonella* virulence for inbred susceptible mice, indicating that regulation of SseJ by small GTPases is an important virulence strategy of this bacterial pathogen. The dependence of a bacterial effector on regulation by a mammalian GTPase further defines how intimately host pathogen interactions have co-evolved through similar and divergent evolutionary strategies.

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LIST OF ABBREVIATIONS

CA	Constitutively active
CI	Competitive index
DN	Dominant negative
GAP	GTPase activating protein
GCAT	Glycerophospholipid:cholesterol acyltransferase
GDI	Guanosine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GTP	Guanosine triphosphate
GTPγS	Guanosine 5'[β,γ-imido] triphosphate
LAMP-1	Lysosomal associated membrane protein 1
mDia1	Diaphanous-related formin 1
PKN1	Protein kinase N1
PNPP	p-nitrophenyl palmitate
RBD	Rho-binding domain
ROCK	Rho-associated protein kinase
SCV	*Salmonella* containing vacuole
Sif	*Salmonella* induced filaments
SPI-1	*Salmonella* pathogenicity island 1
SPI-2	*Salmonella* pathogenicity island 2
T3SS	Type 3 secretion system

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ACKNOWLEDGEMENTS

I would like to express my sincerest appreciation to my mentor, Dr. Sam Miller, for his support and guidance. I am especially grateful for his enthusiasm, patience, advice, and knowledge. I would also like to express my gratitude to Dr. Carleen Collins, who was a great mentor, role model, and friend. I also thank my undergraduate mentor, Dr. Lisa-Anne Foster, who inspired me to pursue a career in science. I thank my committee members Drs. Celeste Berg, Jim Champoux, Rachel Klevit, John Leigh, and Nina Salama for their contributions to this project. I am grateful to those people who specifically contributed to this project especially Dr. Peter Brzovic, Dr. Itay Levin, Dr. Matthias Christen, Dr. Lisette Coye, Marie-Pierre Blanc, and Richard Pfuetzner. I would also like to thank all the members of the Collins and Miller labs for their help and friendship. I am grateful for my parents and siblings for their love and support through all these years of school. Finally, I must thank my husband Chris for his love, encouragement, and patience.

DEDICATION

To Carleen.

CHAPTER 1. INTRODUCTION

Salmonellae

Salmonellae are Gram-negative, motile bacteria of worldwide importance because of their capability of colonizing and causing disease in a wide range of animals. *Salmonella enterica* species are comprised of thousands of serovars, and include both typhoidal and non-typhoidal strains. *Salmonella enterica* serovar Typhi and Paratyphi are human-restricted and are the causative agents of the systemic disease known as typhoid (or enteric) fever, which is characterized by fever and abdominal pain. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is a broad host range pathogen that causes acute focal gastroenteritis in humans, cattle, swine, and poultry, but a systemic infection resembling typhoid fever in susceptible inbred mice (177).

Oral ingestion of contaminated food or water is the most common means of Salmonellae acquisition by a host. *S.* Typhimurium is able to survive gastric acidity to access the intestinal epithelium where it adheres to the apical surface of cells and promotes its uptake by induction of invasion-associated machinery. Additionally, *S.* Typhimurium elicits inflammatory changes to the intestinal epithelium that includes infiltration of neutrophils and fluid into the intestinal lumen, manifesting in the symptoms of diarrhea (99). The inflammatory reaction caused by intracellular bacteria provides nutrients that can be utilized by Salmonellae but not resident microflora in the intestinal lumen (14, 207, 231).

There is also extensive evidence suggesting that the ability of Salmonellae to survive and replicate within epithelial cells is essential for pathogenesis (80, 183, 208, 239). In immunocompetent adults *S.* Typhimurium-associated enteric disease is self-limiting and

resolves within 7 days, although individuals may continue to carry and shed bacteria for several months (16, 36). Salmonellae preferentially cross the epithelial barrier through specialized epithelial microfold (M) cells that overlay the Peyer's patches, or through uptake by dendritic cells (DCs), or CD18 expressing phagocytes (116, 221). From the basolateral side of epithelial cells Salmonellae can enter and replicate within intestinal epithelial cells, and turnover of these cells returns the bacteria into the intestinal lumen. Bacteria that cause systemic infection, including *S.* Typhimurium infection of susceptible Nramp- mice, may then infect macrophages in the Peyer's patches, and use the mononuclear phagocyte system to disseminate and reside predominantly within cells in the gallbladder, spleen, and liver (168, 187).

Intracellular niche

Salmonellae induce their uptake into epithelial cells and can be engulfed by phagocytes to access their intracellular niche. *S.* Typhimurium can invade and survive within dendritic cells and fibroblasts, but undergo limited replication (33, 108, 150, 171) and are rapidly cleared by neutrophils (228). In contrast, within epithelial cells and macrophages, *S.* Typhimurium can survive, replicate, and avoid neutrophil-mediated killing (33). Inside host cells *Salmonella* has the ability to resist and recognize components of the innate immune system, including antimicrobial peptides and low pH. Recognition of components of host innate immunity by *Salmonella* results in the upregulation of genes controlled by the two-component regulatory system, PhoP/PhoQ, important for bacterial intracellular survival (162).

Phagocytosis by macropinocytosis into macrophages or bacterial-mediated uptake into epithelial cells by *Salmonella* occurs rapidly, and initially bacteria reside in a spacious

phagosome (5, 72). This spacious phagosome undergoes a maturation process where it shrinks to the size of the enclosed bacteria and develops into a phagolysosomal compartment known as the *Salmonella*-containing vacuole (SCV) (172). Maturation of the SCV involves a transient interaction of this unique compartment with early endosomes and involves the gain of markers including the transferrin receptor, early endosomal antigen-1 and the small GTPase Rab5 (202, 211). By 30-60 minutes post invasion, early endosomal markers are replaced with late endosomal markers including the lysosome-associated membrane proteins (LAMPs), the GTPase Rab7, vacuolar ATPase, and cholesterol which accumulate on the SCV (34, 59, 82, 156, 211). Other late endocytic markers including mannose-6-phosphate receptor and lysobisphosphatidic acid are believed to be absent from the mature SCV (82).

SCV maturation also involves the trafficking of the membranous compartment via the microtubular network to the perinuclear region of host cells, a process dependent on the *Salmonella* pathogenicity island 2 (SPI-2) secreted effectors (185). The small GTPase Rab7, which in normal cells regulates the fusion of endosomes with lysosomes, is also involved in the movement of the SCV (91, 100, 101, 118). Rab7-interacting lysosomal protein (RILP) links active Rab7 to the motor protein dynein (40, 118), and this complex is important for the centripetal movement of the SCV at earlier stages of infection (91, 100). From the perinuclear location, *Salmonella* induce the dynamic formation of microtubule-dependent long tubular extensions from the SCV in cultured epithelial cells that are LAMP-1 positive, termed *Salmonella*-induced filaments (Sifs) (20, 32, 83, 212). Both Sif formation, and the juxtanuclear positioning of the SCV are dependent upon the eukaryotic microtubular motors kinesin and dynein (91). Dynein is a microtubule motor protein that transports cargo towards the minus end of microtubules which are normally pointed towards the cell center, while

kinesin is a plus end directed motor protein responsible for transporting cargo towards the cell periphery. At later stages of *Salmonella* infection (14-24 hours post infection) there is a displacement of the SCV from the perinuclear region of host cells to the cell periphery which is important for bacterial transfer between epithelial cells, a process also dependent on SPI-2 effectors (214).

Type III secretion system

Salmonella, like many other Gram-negative pathogens or symbionts, utilize a specialized protein-export apparatus known as the type III secretion system (T3SS). Phylogenetic analysis indicates T3SSs are related to flagella and were horizontally acquired (90, 175). The T3SSs from pathogenic bacteria cluster into three families, the *Yersinia* Ysc archetype; the *Salmonella* SPI-1 archetype; and the enteropathogenic and enterohaemorrhagic *Escherichia coli* (EPEC or EHEC) and *Salmonella* SPI-2 archetype (52). *S.* Typhimurium pathogenesis is dependent upon two distinct T3SSs encoded on *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2). The two T3SSs were canonically thought to function at distinct temporal points during infection, where the SPI-1 T3SS transports proteins across the eukaryotic plasma membrane upon contact with host cells to induce bacterial invasion into cells, and the SPI-2 T3SS transports proteins across the SCV membrane important for intracellular survival (95). However, it has been demonstrated that effectors from the SPI-1 T3SS persist within host cells after bacterial internalization and may be important for intracellular survival within the SCV (27, 86, 210). In addition, the SPI-2 T3SS is expressed in mice before intestinal cell colonization, suggesting SPI-2 effectors or the T3SS itself may have a role in host cell invasion (29).

The T3SS has a core supramolecular structure known as the needle complex (Figure 1) (120, 126). The needle complex comprises a cylindrical structure that has a narrow channel in the center, which likely serves as a passageway for secreted proteins. At the bottom of the needle complex is a multi-ring base that spans the inner and outer bacterial membranes and which anchors the structure to the bacterial cell. A needle-like projection extends past the bacterial surface and is anchored to the base by an inner rod. A different set of proteins form the translocon pore, connecting the needle tip to the host cell membrane to allow passage of proteins from the bacterial cell into the host cytosol (120). Needle complex assembly is a stepwise process where the base is assembled first. Once the base is fully assembled, it secretes proteins that comprise the inner rod and needle structures or that are necessary for their assembly. Upon completion of the supramolecular structure substrate switching occurs, and the complex is then able to secrete proteins that are delivered into host cells (49, 127, 148). The diverse array of proteins that are delivered into host cells are collectively known as effectors, which typically have biochemical activities involving modulation of host molecules to the benefit of bacteria. Type three secreted proteins possess a poorly characterized secretion signal in the first ~20-30 amino acids (119, 160). In addition to the secretion signal, many effectors have binding sites for specific chaperone proteins. These chaperones are small acidic proteins which binds a 50-100 amino acid domain of the secreted effector and are thought to target the secreted protein to the T3SS, facilitate the rapid unfolding of effectors prior to secretion, and prevent undesirable interactions of effectors within the bacterial cell (68, 195, 205, 232). *S.* Typhimurium possesses two sets of T3SS effectors that traverse from bacteria to host cell using the two distinct SPI-1 and SPI-2 encoded T3SSs, although there are a few effectors that appear to be substrates for both T3SSs.

SPI-1 effectors

The SPI-1 T3SS orchestrates the uptake of bacteria into epithelial cells by delivering effectors that alter the host cell actin cytoskeleton and affects intestinal inflammatory responses. The activities of the SPI-1 effectors are summarized in Figure 2 and Table 1. The SPI-1 effectors SopE and SopE2 both activate the small GTPases Rac1 and Cdc42 with their guanine nucleotide exchange factor (GEF) activity, which leads to actin cytoskeletal reorganization that promotes bacterial internalization by non-phagocytic cells (76, 213). Through an indirect and not fully understood mechanism, SopB can also activate Cdc42 to promote bacterial entry (241). In addition, the phosphoinositide phosphatase activity of SopB facilitates bacterial entry by promoting host membrane fission and actin cytoskeleton remodeling mediated by the hydrolysis of phosphatidylinositol 4,5-bisphosphate (216, 241). Two SPI-1 effectors, SipA and SipC promote bacterial internalization by directly binding and modulating actin. SipA inhibits depolymerization and increases actin bundling at the site of entry (242, 243), while SipC bundles and nucleates actin (104, 167).

Many of the SPI-1 effectors including SopE, SopE2, SopB, SipA, SipC, SopA contribute to *Salmonella*-induced gut inflammation. These factors stimulate production of the proinflammatory cytokine IL-8 through the mitogen-activated protein kinase (MAPK) and NF-κB pathways, and destabilize tight junctions facilitating polymorphonuclear neutrophil (PMN) transepithelial migration into the intestinal lumen (152, 153, 177). The SPI-1 effector SipB additionally modulates the host inflammatory response by binding and activating caspase-1, which results in the release of the proinflammatory cytokines IL-18 and IL-1β by macrophages (107). The flagella protein FliC, and the PrgJ rod protein of the T3SS apparatus also activate caspase-1 and trigger the release of IL-18 and IL-1β (157, 159). This

inflammation may be important to provide a growth advantage for *Salmonella* that reside in the lumen for enhancing immediate transmission (209, 231).

After bacterial entry into host cells, the host actin cytoskeleton reverts back to its original architecture and host inflammatory responses are dampened. The SPI-1 effector SptP deactivates Rac1 and Cdc42 by its GTPase activating protein (GAP) activity, which reverses the activation of these GTPases by SopE, SopE2, and SopB to downregulate membrane ruffling (206). Through its tyrosine phosphatase domain, SptP, reverses the bacterial-induced MAPK associated inflammation and IL-8 secretion (96, 142, 166). Additionally, SptP persists late into infection (at least 8 hours post invasion) and dephosphorylates the AAA+ ATPase, valosin-containing protein, which is important for bacterial intracellular replication and Sif formation (110). The SPI-1 effectors SspH1 and AvrA also contribute to downregulation of IL-8 production by epithelial cells induced by *S.* Typhimurium. SspH1 is a leucine-rich-repeat protein with E3 ubiquitin ligase activity that localizes to the mammalian cell nucleus and inhibits NF-κB dependent gene expression possibly involving its ability to bind protein kinase N 1 (PKN1), a protein kinase involved in the NF-κB pathway (96, 97). AvrA is an acetyltransferase with activity toward MAPKs and inhibits inflammation mediated through the MAPK and NF-κB pathways (61, 117).

SPI-2 effectors

The SPI-2 T3SS translocates effectors across the SCV membrane that are important for intracellular replication and survival. After 4 hours post-internalization, the SPI-2 T3SS is expressed and translocates SPI-2 effectors to the cytoplasmic side of the SCV. From this location effectors are trafficked to different locations throughout the host cell, collectively

promoting intracellular survival of *Salmonella* through mechanisms that are not well understood. Overall, relatively little is known about the mammalian cell targets and the specific activities of the SPI-2 effectors, though many have been found to be involved in manipulating membrane trafficking (Figure 3 and Table 1). A handful of effectors result in virulence defects when deleted in the systemic mouse model of infection, although no single deletion causes attenuation equal to a SPI-2 defect, suggesting that effectors likely have overlapping and partially redundant functions. Of all the SPI-2 effectors tested to date, SifA has the most profound virulence defect when mutated (19). Additional SPI-2 effectors that confer virulence defects are SpvB, SseJ, SseF, SseG, SseI, SopD2 and PipB2 (19, 31, 55, 74, 112, 122, 154, 191).

A number of SPI-2 effectors control Sif formation in epithelial cells and macrophage-like cells. The mechanism of Sif formation and contribution of Sif formation to virulence is not well understood, and formation of Sifs has not been demonstrated *in vivo*. Although no physiological function has been demonstrated for Sif formation the mechanisms involved in tubulation are important for virulence because mutation of effectors required for their formation results in virulence attenuation. It is thought that the purpose of Sifs may be to collect membrane and nutrients important for *Salmonella* survival, to dilute lysosomal enzymes, or to promote the stability of the SCV membrane (60, 184). The SPI-2 effectors, SifA, PipB2, SseF, SseG, SseJ, SpvB and SopD2 all contribute to Sif dynamics.

SifA and its eukaryotic binding partner SifA kinesin interacting protein (SKIP) are essential for the formation of Sifs. SKIP binds SifA and kinesin, while SifA additionally associates with the SCV and Sifs through its carboxyl-terminus CaaX motif that is post-translationally lipidated (22, 23, 186). Together, the SifA-SKIP complex links the SCV to

the microtubular network, and may initiate the fission of periphery directed transport vesicles to trigger Sif formation along microtubules (23, 62). Also, SifA may bind to Rab7 which would displace the RILP-dynein complex from Sifs, which would promote the peripheral movement of Sifs (100). PipB2 directly binds kinesin and contributes to the peripheral movement of Sifs, and bacteria lacking PipB2 induce shorter Sifs (105, 123). SopD2 localizes to the SCV membrane and when deleted, impairs *Salmonella's* ability to form normal Sifs. An increase in the formation of tubules that lack continuous LAMP-1 distribution, pseudo-Sifs, is observed suggesting that SopD2 impairs the fusion of endocytic vesicles to the SCV (112, 197). Similarly, SseF and SseG localize to the SCV and Sifs, and mutations in *sseF* or *sseG* result in *Salmonella*-induced pseudo-Sifs (129). In addition to their role in modifying the endosomal compartment, SseF and SseG are necessary for bundling of microtubules in close proximity to the SCV, suggesting these proteins play a role in the fusion of vesicles into tubules along microtubules (130). There is evidence that SpvB and SseJ negatively modulate Sif formation, further suggesting the complexity of Sif formation and that *Salmonella* dynamically regulates Sif formation (20).

SseJ

SseJ is a SPI-2 effector belonging to the GDSL lipase family with glycerophospholipid:cholesterol acyltransferase (GCAT) activity. Bacteria lacking SseJ are attenuated for replication in cultured epithelial cells and macrophages (74, 191). Further, SseJ enzymatic activity is required for full virulence of *S.* Typhimurium in mice, as attempts to complement the Δ*sseJ* virulence defect with SseJ containing mutations in the catalytic residues (Ser 151, Asp 247, His 384) fail to restore virulence (74, 173, 191). SseJ localizes

to the SCV and Sifs upon infection, and during transient transfection localizes to LAMP-1 positive endosomes and induces the aggregation of this membranous compartment (191). SseJ increases accumulation of cholesterol esters in cells directly through its enzymatic activity (170). Cholesterol esters are neutral lipids that can associate with membranes and also accumulate in lipid droplets, which are lipid storage organelles. SseJ also increases lipid droplet production in cells upon infection, though specifically how lipid droplet accumulation is important for bacterial virulence remains unclear (170). A rare genetic disease, Niemann-Pick type C (NPC) disease, manifests in the accumulation of cholesterol in endosomes/lysosomes and leads to altered protein and lipid trafficking (45, 178). Although the NPC proteins normally function at the level of cholesterol efflux from the endosome/lysosome (41, 169), the accumulation of cholesterol in lipid droplets is morphologically similar to what is observed for SseJ. The accumulation of cholesterol by the action of SseJ may alter the recruitment of specific proteins and therefore change cell signaling pathways and membrane vesiculation to promote *Salmonella* survival.

SseJ cooperates with SifA to maintain the stability of the SCV. *S.* Typhimurium lacking *sifA* lose the vacuolar membrane and escape to the cytoplasm, while bacteria that lack both *sifA* and *sseJ* remain inside the vacuolar membrane, indicating that membrane disruption is dependent upon SseJ (191). SseJ, but not catalytically inactive SseJ, modifies the ability of coexpressed SifA to associate with LAMP-1 positive vesicles in HeLa cells. This observation suggests that SseJ activity antagonizes SifA association with endosomal membranes. However, SifA distribution on the endosome during infection is unaffected by the absence or presence of SseJ, indicating that the antagonism of SifA association with the endosomal membrane is only observed when these proteins are in excess (170). The

coexpression of SseJ and SifA induces the formation of endosomal tubules in HeLa cells, supporting the idea that these effectors cooperate to induce tubulation of endosomal compartments and possibly Sif formation (174). Intriguingly, SseJ coexpressed with GTP-bound RhoA, RhoB, or RhoC also induces endosomal tubulation, indicating that SseJ activation by RhoA, RhoB, or RhoC may be important for Sif formation, and that SifA may also function in this pathway (174). The carboxyl domain of SifA has a fold similar to SopE, a *Salmonella* effector with Rho GTPase guanine nucleotide exchange factor (GEF) activity (174). SifA also binds GDP-bound RhoA, suggesting that it may activate SseJ enzymatic activity by activating RhoA, although no SifA GEF activity for RhoA has been demonstrated to date (8, 174). Despite SifA's GEF-like fold, the important catalytic residues for GEF activity in SifA are divergent compared to other identified bacterial GEFs (109), supporting the idea that SifA does not have GEF activity but could instead act as a scaffold for assembly with other proteins.

SseJ exhibits phospholipase A1, deacylase, and GCAT activity; these activities are all dependent on the same catalytic residues and in cells results in the enzymatic cleavage of phospholipids at the sn-1 position and the transfer of this acyl chain onto cholesterol (145, 170, 173). These SseJ activities are dependent upon interaction with the activated form of the eukaryotic small GTPase, RhoA (47). SseJ alone has minimal lipase activity, while the SseJ-RhoA complex has potent lipase activity, which is measurable with the chromogenic lipase substrate, p-nitrophenyl palmitate (PNPP). GTP-bound RhoA, the activated form of the GTPase, enhances SseJ lipase activity preferentially over the apo-RhoA (unbound) or GDP-bound RhoA. Additionally, the binding of SseJ to RhoA does not affect the ability of RhoA to cycle between the GDP- and GTP- bound states (47). SseJ senses the activation

state of RhoA in a manner similar to RhoA activated eukaryotic downstream effector molecules such as ROCK. The requirement of sensing RhoA in regulating SseJ enzymatic activity suggests that tight regulation of SseJ activity may be important for pathogenesis.

Cholesterol acyltransferases

In humans there are two major cholesterol acyltransferases, lecithin:cholesterol acyltransferase (LCAT), and acyl-coenzyme A (CoA):cholesterol acyltransferase (ACAT) that are important in cholesterol transport and homeostasis. LCAT is a plasma protein that specifically transfers the sn-2 acyl chain of phosphatidylcholine (PC) to cholesterol on high-density lipoproteins (HDLs) when activated by apolipoprotein A-I (ApoA-1) (69, 70, 73, 87). Esterified cholesterol is then sequestered in the lipoprotein core, and the elimination of cholesterol from HDL surface creates a concentration gradient for additional cholesterol from cells to migrate to HDLs to be transported through the bloodstream for excretion (88). LCAT can hydrolyze short soluble substrates in the absence of ApoA-1 (114), and it is not fully understood how ApoA-1 activates LCAT. In addition to a role in binding to LCAT, ApoA-1 it is believed to be important for the presentation of the acyl chain of long chain PC substrates to LCAT (115, 236).

ACAT is an intracellular enzyme primarily localized to the endoplasmic reticulum that uses the acyl chain from acyl coenzyme A to esterify cholesterol within mammalian cells (89, 102). ACAT activity promotes accumulation and sequestration of cholesterol in lipid droplets within the cytoplasm. Cholesterol and some oxysterols activate ACAT, possibly through a ligand-induced conformational change (44, 163). Overall ACAT is believed to play an important role in regulating the cholesterol content of membranes within cells.

Cholesterol is a critical component of eukaryotic membranes, but is not present in most prokaryotic membranes, therefore the ability to acylate cholesterol is primarily a pathogenic strategy used by bacteria to target host cells. Several bacteria make enzymes with glycerophospholipid:cholesterol acyltransferase (GCAT) activity, and these enzymes are members of the GDSL lipase family that are characterized by a conserved GDSL motif and catalytic triad (S-D-H) (3). GCATs transfer an acyl chain from a phospholipid onto cholesterol, to form cholesterol ester and a lysophospholipid. In the absence of the cholesterol acceptor, these enzymes will exhibit phospholipase activity. *Aeromonas* is a fish pathogen that secretes a cytotoxic GCAT (SatA) that is potentiated by binding lipopolysaccharide (38, 139). SatA possesses phospholipase A2 and lysophospholipase A activity (38), and has a preference for phospholipids with short-chain or unsaturated fatty acids although all of the most common glycerophospholipids can be used (37). SatA is activated by proteolytic processing of the 38 kDa proform to a 26 kDa active form by a secreted *Aeromonas* serine protease, AspA (65, 222). Although these enzymes are major secreted products of *Aeromonas* species, they appear to have little effect on pathogenesis (222).

The intracellular pathogen *Legionella pneumophila* encodes multiple phospholipases, one of which is a GCAT (PlaC) which is activated by proteolytic processing by the *Legionella* zinc metalloprotease (ProA) (13, 134). PlaC prefers short acyl chains, has phospholipase, lysophospholipase, and GCAT activity, can acylate ergosterol, and is thought to be secreted by the type 2 secretion system (13). The ability of PlaC to acylate ergosterol may reflect *L. pneumophila*'s primary host, amoebae, which do not possess cholesterol but use ergosterol as the major sterol group (182, 203). The final characterized bacterial GCAT

is SseJ, which secreted by the SPI-2 T3SS of *Salmonella*. As mentioned in an earlier section, SseJ requires binding to the eukaryotic GTPase RhoA for activation of its enzymatic activity (47). GCAT enzymes can deacylate different glycerophospholipids including those commonly found in eukaryotic and prokaryotic membranes. Therefore, the regulation of bacterial GCATs must be essential to prevent unintended deacylation and damage to the bacterial membrane. Although SseJ is not proteolytically processed by a separately secreted bacterial factor like SatA or PlaC, it has evolved to sense the activation state of a mammalian protein for potentiation of GCAT activity.

Membrane tubulation

Membrane trafficking is dependent upon membrane fission and fusion events. Fission is the formation of a vesicle from a lipid membrane, and involves the stretching of the membrane and curvature stress to facilitate these events (179). There are many proteins that regulate the process of membrane fission, and the GTPase dynamin can induce membrane tubulation and fission in vitro (15, 180). A number of other mammalian proteins are important for membrane fission, including the coat-proteins which form a scaffold structure to contribute to membrane curvature (128, 189), and BAR-domain containing proteins such as amphiphysin and endophilin, which deform membranes and induce tubulation (66, 215). Membranes of many organelles involved in membrane trafficking are known to tubulate under different conditions and by various mechanisms. Interestingly, Brefeldin A (BFA) - stimulated endosomal and Golgi tubulation events are dependent upon the activity of phospholipases (30, 54). It is not well understood how the action of phospholipases promotes membrane tubulation, but it may involve inherent curvature stress evoked by the

enzymatic removal of an acyl chain from a phospholipid and the production of a lysophospholipid. The uneven distribution of the differentially shaped cylindrical and cone shaped phospholipids compared to the inverted cone shaped lysophospholipids can promote spontaneous membrane curvature stress, promoting fission and possibly tubulation (124). This suggests that SseJ phospholipase activity could promote membrane fission and tubulation. Membrane tubulation of the endosomal compartment is important for exchange of components within the endocytic pathway. Interestingly, the accumulation of cholesterol in the endosomal compartment inhibits the exchange of membrane components through the endocytic trafficking pathway (238). This suggests that the recruitment of cholesterol by *Salmonella* to the SCV likely alters endocytic trafficking within mammalian cells.

Salmonella induce the formation of Sifs during infection of cultured cells, which involves the formation of membrane tubules from a LAMP-1 positive compartment. How *Salmonella* induces Sifs is not well understood, though it in part involves the recruitment of mammalian proteins involved in membrane tubulation and trafficking in addition to the enzymatic activities of the SPI-2 effectors. Initial perinuclear movement of the SCV and the eventual extension of Sifs requires the mammalian cell microtubular network and associated proteins. Sif formation is important for the eventual movement of the SCV towards the cell periphery for dissemination (214). Sif formation may also be important for bacteria to gain access to vesicles that contain nutrients for replication and membranes to expand the SCV. Apart from Sifs, *Salmonella* also induce tubulation of membranes that are secretory carrier membrane protein 3 (SCAMP3) positive but lack late endosomal markers (164). SCAMP3 is a protein normally found in the trans-Golgi network, suggesting that the tubular network surrounding *Salmonella* includes membrane tubules of Golgi and endosomal origin. Finally,

tubules that are effector-positive but that lack endosomal markers (pseudo-Sifs) are also induced upon *Salmonella* infection, and these are thought to be precursors to Sifs (112, 197).

Rho GTPases

RhoA is a member of the Rho GTPase family, which function as major molecular switches that cycle between the GDP-bound inactive state, and the GTP-bound active state (Figure 4). The Rho GTPase family is comprised of around 20 signaling molecules, including the most well studied proteins RhoA, Cdc42 and Rac. The activity of Rho GTPases is regulated by three distinct groups of proteins, guanosine nucleotide dissociation inhibitors (GDIs), which interact with the GDP-bound form of Rho GTPases and sequester Rho from the membrane, guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP, and GTPase activating proteins (GAPs), which stimulate the hydrolysis of GTP to GDP. The study of Rho GTPases has been facilitated by mutations that force RhoA to behave either constitutively active (CA) (mimicking the GTP-bound state) or dominant negatively (DN) (mimicking the GDP-bound state) (67, 224). Rho GTPases regulate a large number of cellular processes such as actin reorganization, microtubule dynamics, and cell cycle progression, and mediates these effects through proteins known as effectors, which adopt an active conformational state on binding the activated form of Rho GTPases. There are three Rho isoforms that share 85% amino acid identity, known as RhoA, RhoB and RhoC. When overexpressed in fibroblasts, all three isoforms induce stress-fiber formation (176). RhoA regulates actin polymerization (6), RhoB localizes to endosomes and regulates endocytic trafficking (1, 81), and RhoC may be involved in cell movement (235).

Two eukaryotic effector binding domains, protein kinase N 1 (PKN1-Hr1a) and Rho-associated protein kinase (ROCK-RBD), use coiled-coil motifs to bind RhoA, and have been crystalized in complex with RhoA-GTP (64, 147). Additionally, the binding domain of the scaffold protein mammalian Diaphanous 1 (mDia1-GBD/FH3) has been crystallized in complex with RhoC-GTP and it is an effector that also binds RhoA in vitro (188). All three effectors bind to Rho in the switch regions. The most common mechanism by which mammalian Rho GTPase effectors are activated, is by binding to Rho GTPases through an autoinhibitory domain. The binding of the effector autoinhibitory domain to active Rho GTPase exposes the functional domain within the effector. PKN1, ROCK, and mDia1 all have autoinhibitory domains that bind to RhoA. When these autoinhibitory domains are bound to RhoA, their enzymatic kinase activities (PKN1 and ROCK), or scaffolding activity that connects RhoA to actin (mDia1) are exposed (7, 121, 125, 226, 227).

Overall, the structurally diverse spectrum of proteins that bind Rho GTPases do so through the Rho switch regions, and the switch regions are surfaces of Rho that change conformation when Rho GTPases are bound to either GDP or GTP (Figure 5). Often Rho GTPase binding proteins achieve binding specificity through critical interaction with residues in the switch regions that are divergent between Rho, Cdc42, and Rac1. The switch regions of RhoA, RhoB and RhoC are highly conserved, with only a single conservative amino acid substitution between switch I of RhoB compared to RhoA and RhoC, and no differences in switch II (Figure 6). Therefore, it is not surprising that it has been difficult to identify which of these three Rho proteins, if any, is the true target of either proteins that regulate Rho GTPases or the effectors. The insert helices of RhoA, RhoB, and RhoC are less conserved, is present only in Rho family GTPases (a 13 amino-acid helix that replaces a loop in Ras family

GTPases), and may be one way that effector binding and specificity is conferred (Figure 6) (132, 223, 245). Relatively little is known about how specificity for RhoA, RhoB, or RhoC is achieved in vivo, but it is thought that the spatial distribution of these GTPases, the regulatory proteins, and effector proteins may play an important role (229).

Rho GTPases are major targets of bacterial pathogens. Many of the modifications of Rho GTPases by bacterial molecules affect the regulatory functions of GTPases to benefit the pathogen. Two major types of bacterial toxins affect Rho GTPases, the first being those that non-covalently modify GTPases by mimicking endogenous regulators, and the second are toxins that covalently modify GTPases to affect function. A number of bacterial pathogens encode effectors that behave as either GEFs or GAPs for different Rho GTPases. In *Salmonella*, SopE and SopE2 which are translocated by the SPI-1 T3SS behave as GEFs, activating Rac and Cdc42 (98). The *Salmonella* SPI-2 effectors SifA and SifB may also function as GEFs since they bear structural resemblance to SopE's catalytic domain (174). *Salmonella* encodes the SPI-1 effector SptP, that behaves as a GAP, reversing the activation of Rac and Cdc42 by SopE and SopE2 once bacterial internalization has taken place (77). Covalent modifications of Rho GTPases by *Salmonella* proteins have not been demonstrated to date. Many bacterial pathogens do secrete covalently modifying toxins that affect Rho GTPases to either activate or inhibit their function. There are a diverse array of modifications that can take place, including ADP-ribosylation, adenylylation, glucosylation, deamidation and proteolytic cleavage. Depending on the amino acid that is targeted by the toxin, activation or inhibition of the GTPase can occur. For example, ADP-ribosylation by the C3 toxin of *Clostridium botulinum* covalently modifies RhoA at Asn41, leading to RhoA inhibition (4, 190). ADP-ribosylation by the TccC5 toxin of *Photorhabdus luminescens* at

Gln63 of RhoA leads to persistent RhoA activation (133). SseJ is the first example of a bacterial protein that behaves similarly to an effector of RhoA, where SseJ binding to RhoA activates SseJ enzymatic activity. This work sought to identify whether SseJ binds to a similar region of RhoA as its mammalian effectors, and further characterizes the interaction between SseJ and RhoA.

Table 1. Effectors of the *Salmonella* T3SSs

SPI-1				
Effector	***Target***	***Activity***	***Function***	***Reference***
AvrA	MAP kinase kinases, IκB, β-catenin	Acetyltransferase; deubiquitinase?	Acetylates MAPKK and IκB, (and deubiquitinates β-catenin?) to inhibit inflammation; inhibits apoptosis	(50, 61, 117, 234, 237)
SipA (SspA)	Actin, T-plastin	Binds and stabilizes F-actin	Binds actin and the actin-bundling protein T-plastin, to inhibit depolymerization and increase acting bundling respectively to facilitate invasion; promotes PMN transepithelial migration; disrupts tight junctions; influences localization and maturation of the SCV	(2, 24, 27, 28, 138, 242, 243)
SipB (SspB)	Cholesterol; caspase-1	Translocon	Binds cholesterol; important for bacterial attachment to non-phagocytic cells; binds and activates caspase-1 to induce pyroptosis; induces autophagy	(103, 106, 107, 135)
SipC (SspC)	Membrane; actin; Syntaxin6	Translocon; bundles and nucleates actin; binds Syntaxin6	Important for bacterial attachment to and invasion of non-phagocytic cells; binding of Syntaxin6 important for LAMP-1 recruitment to the SCV	(104, 135, 146, 167, 194)
SopA	Unknown	HECT E3 ubiquitin ligase	Induces PMN transepithelial migration	(240)
SopB (SigD)	Cdc42; inositol phosphates	GDI; phosphoinositide phosphatase	Modulates actin by altering inositol phosphate levels to facilitate invasion; inhibits Cdc42 nucleotide exchange; disrupts tight junctions; controls SCV membrane surface charge to inhibit SCV maturation	(12, 24, 39, 216)
SopD	Unknown	Unknown; binds InvC (SPI-1) ATPase	Contributes to gastroenteritis and systemic virulence	(112, 183)
SopE	Rac1, Cdc42	GEF	Activates Cdc42 and Rac1 to regulate actin; disrupts tight junctions; activates caspase-1 to elicit gut inflammation	(24, 76, 98, 165)
SopE2	Rac1, Cdc42	GEF	Activates Cdc42 and Rac1 to regulate actin; disrupts tight junctions	(24, 76, 213)
SptP	Rac1, Cdc42; VCP	GAP; tyrosine phosphatase	Inhibits Cdc42 and Rac1 to restore epithelial cell morphology after invasion; inhibits IL-8 production by epithelial cells; inhibits MAPK pathway by inhibiting Raf; dephosphorylates VCP to promote intracellular replication and Sif formation	(77, 96, 110, 142, 166)
SPI-1 &2				
Effector	***Target***	***Activity***	***Function***	***Reference***
SlrP	Thioredoxin; ERdj3 (chaperone)	E3 ubiquitin ligase	Ubiquitinates thioredoxin to reduce redox-related signaling activity and trigger cell death; interferes with ERdj3 function; inhibits T-cell proliferation	(17, 18, 93)
SspH1	PKN1	E3 ubiquitin ligase	Inhibits NF-κB dependent gene expression and IL-8 secretion	(96, 97)
SteA	Unknown	Unknown	Required for mouse spleen colonization, localizes to the Golgi network	(85)
SteB	Unknown	Unknown	Unknown	(85)

Table 1 continued. Effectors of the *Salmonella* T3SSs

SPI-2				
Effector	***Target***	***Activity***	***Function***	***Reference***
GogB	Unknown	Unknown	Unknown	(51)
PipB	Unknown	Unknown	Unknown	(122)
PipB2	Kinesin-1	Binds kinesin-1	Recruits kinesin to SCV; contributes to Sif formation; important for SCV integrity; important for peripheral movement of SCV at later stages of infection for bacterial cell-to-cell transfer; inhibits T-cell proliferation	(93, 105, 122, 123, 214)
SifA	SKIP; Rab7?; RhoA?	Binds SKIP; GEF?	Induces Sif formation; important for SCV integrity; may link SKIP and kinesin to the SCV and microtubule network to promote endosomal tubulation; role in fission of LAMP-1-positive vesicles from the SCV; inhibits T-cell proliferation	(8, 19, 23, 32, 58, 62, 93, 100, 174, 191)
SifB	Unknown	Unknown	Unknown	(74)
SopD2	Unknown	Unknown	Regulates SCV membrane dynamics; contributes to Sif formation; inhibits T-cell proliferation	(93, 112, 197)
SpiC	Hook3; TassC	Unknown; component of the secretion apparatus	Interferes with cellular trafficking; promotes flagella gene expression	(75, 137, 198, 218, 219)
SpvB	Actin	ADP-ribosylates actin	Destabilizes the actin cytoskeleton	(31, 140, 158, 217)
SpvC	pErk	Phosphothreonine lyase	Inactivates pErk; attenuates intestinal inflammation	(94, 151)
SseF	Unknown	Unknown	Important for microtubule bundling and perinuclear positioning of the SCV	(55, 92, 129)
SseG	Unknown	Unknown	Important for microtubule bundling and perinuclear positioning of the SCV	(55, 92, 129)
SseI	IQGAP1; TRIP6; filamin	Binds IQGAP1; binds TRIP6; binds filamin	Inhibits directed migration of macrophages and dendritic cells; contributes to host cell dissemination	(154, 158, 233)
SseJ	Cholesterol, phospholipids, RhoABC	Deacylase, phospholipase A1, glycerophospholipid:cholesterol acyltransferase	Esterifies cholesterol on the SCV; important for SCV integrity; negatively regulates Sif formation	(20, 47, 145, 173, 174, 191)
SseK1	Unknown	Unknown	Unknown	(131)
SseK2	Unknown	Unknown	Unknown	(131)
SseL	Ubiquitin; IκBα; oxysterol-binding protein (OSBP)	Deubiquitinase; binds OSBP	Suppresses NFκB activation and impairs IκBα ubiquitination and degradation; prevents accumulation of lipid droplets; manipulates host lipid metabolism	(9, 10, 136, 192)
SspH2	Filamin; profilin	E3 ubiquitin ligase	Inhibits T-cell proliferation; slows the rate of actin polymerization	(93, 141, 158, 161, 181)
SteC	Unknown	Unknown	Unknown	(85)

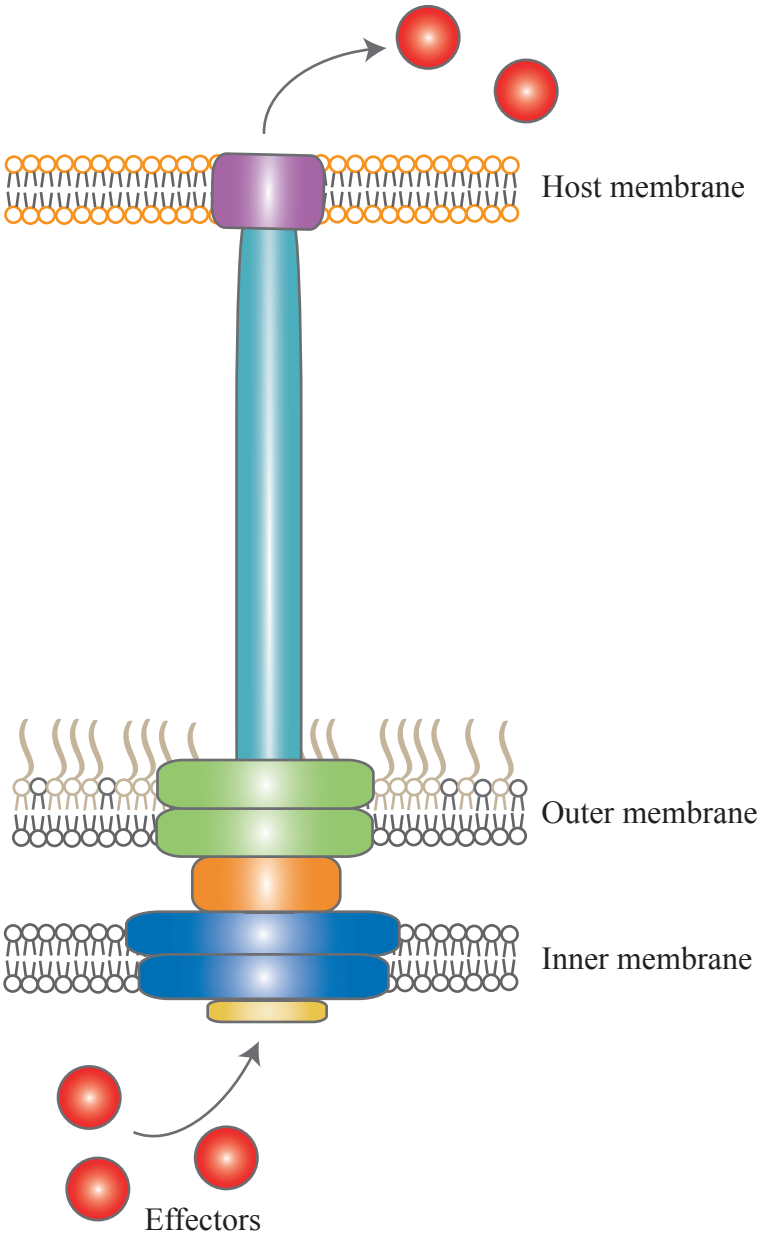

Figure 1. T3SS needle complex schematic. The base of the needle complex is made up of the inner membrane ring (blue), outer membrane ring (green), neck (orange), and ATPase (yellow). The needle (teal) extends from the base in the bacterial membrane to the translocon pore (purple) in the mammalian membrane. Effector proteins (red) are translocated from the bacterial cytosol to the mammalian cytosol through the needle complex.

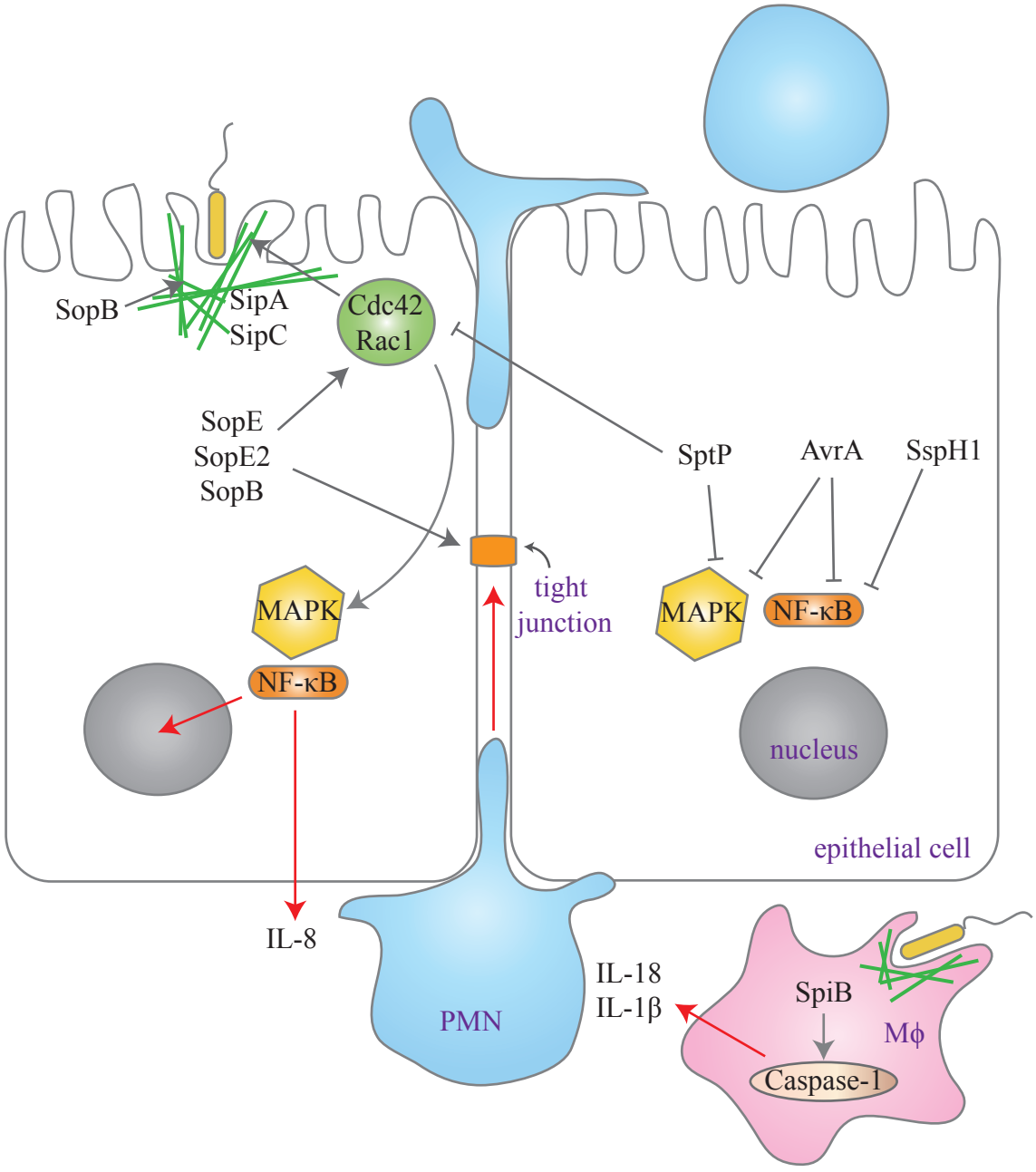

Figure 2. SPI-1 effectors induce bacterial uptake. Upon contact with host cells, *Salmonella* translocates effectors through the SPI-1 T3SS. SopE, SopE2, and SopB activate the small GTPases Cdc42 and Rac1 to promote actin rearrangements. SopB also promotes actin rearrangement and membrane alterations independently of GTPases. SipA and SipC bind and modulate actin directly. These SPI-1 effectors activate the proinflammatory response through the MAPK and NF-κB pathways, resulting in the release of IL-8, destabilization of tight junctions, and the migration of PMNs into the intestinal lumen. SpiB also contributes to the inflammatory response by activating Caspase-1 to release IL-18 and IL-1β by macrophages (MΦ). Dampening of the inflammatory response and reversal of cytoskeletal rearrangements is mediated through the SPI-1 effectors SptP, AvrA and SspH1.

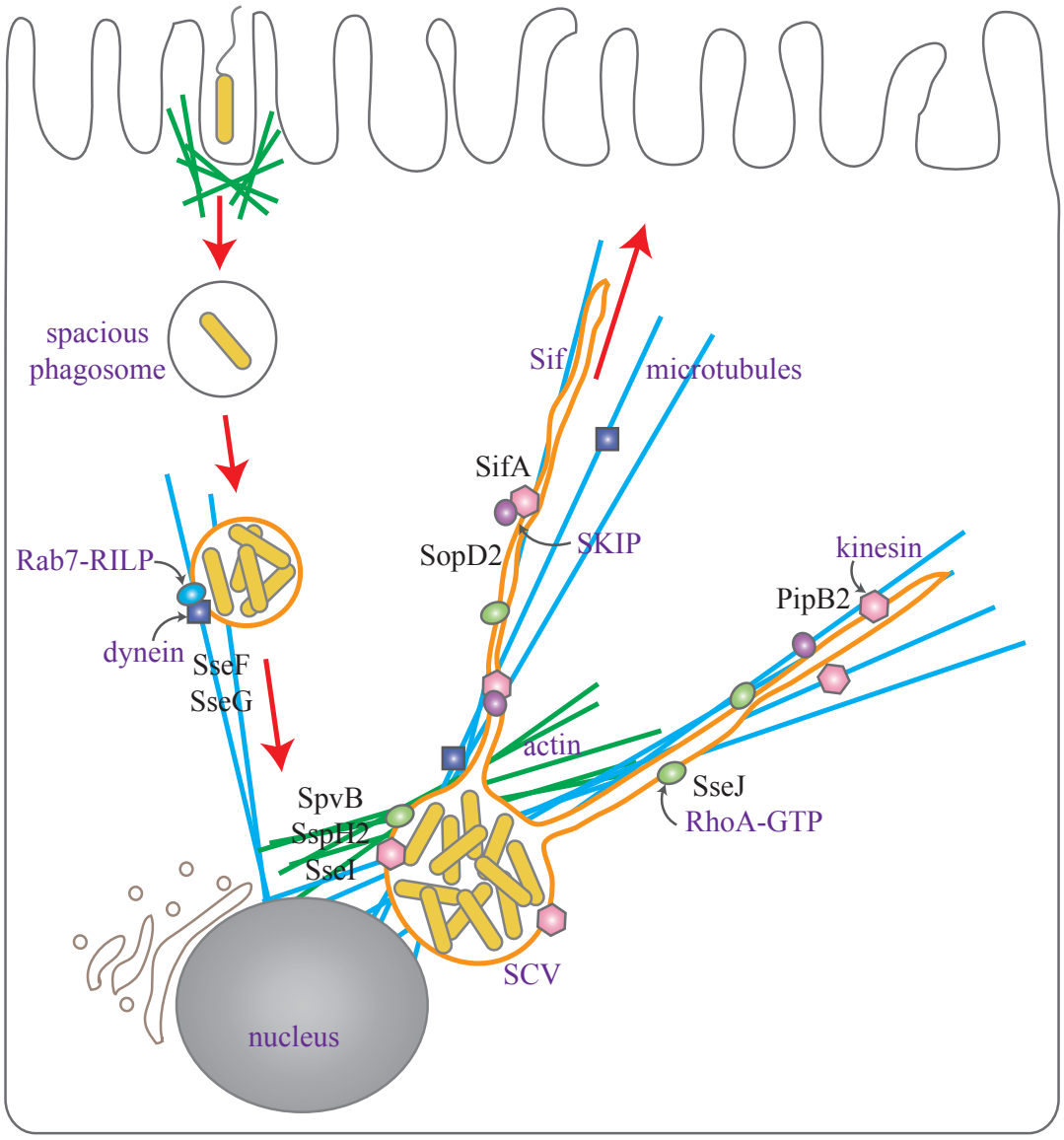

Figure 3. SPI-2 effectors regulate membrane dynamics. Immediately after uptake *Salmonella* are enclosed in a spacious phagosome. The phagosome matures into the SCV, which acquires markers of late endosomes, including LAMP-1. The early SCV traffics towards the cell nucleus along the microtubular network by the mammalian Rab7-RILP complex that binds dynein. The SPI-2 effectors SseF and SseG play a role in microtubule bundling and are involved in modifying the SCV membrane. SifA induces the formation of Sifs along the microtubular network, possibly linking the SCV to the microtubular network through its interaction with SKIP. PipB2 binds the microtubular motor protein kinesin, and is important for the extension of Sifs towards the cell periphery. SopD2 is important for Sif formation, and is involved in modifying the SCV membrane. SpvB, SspH2, and SseI modulate actin, which is found to accumulate near the SCV. SseJ is a GCAT that is activated by binding RhoA-GTP, and whose activity results in the conversion of cholesterol to cholesterol ester on the SCV.

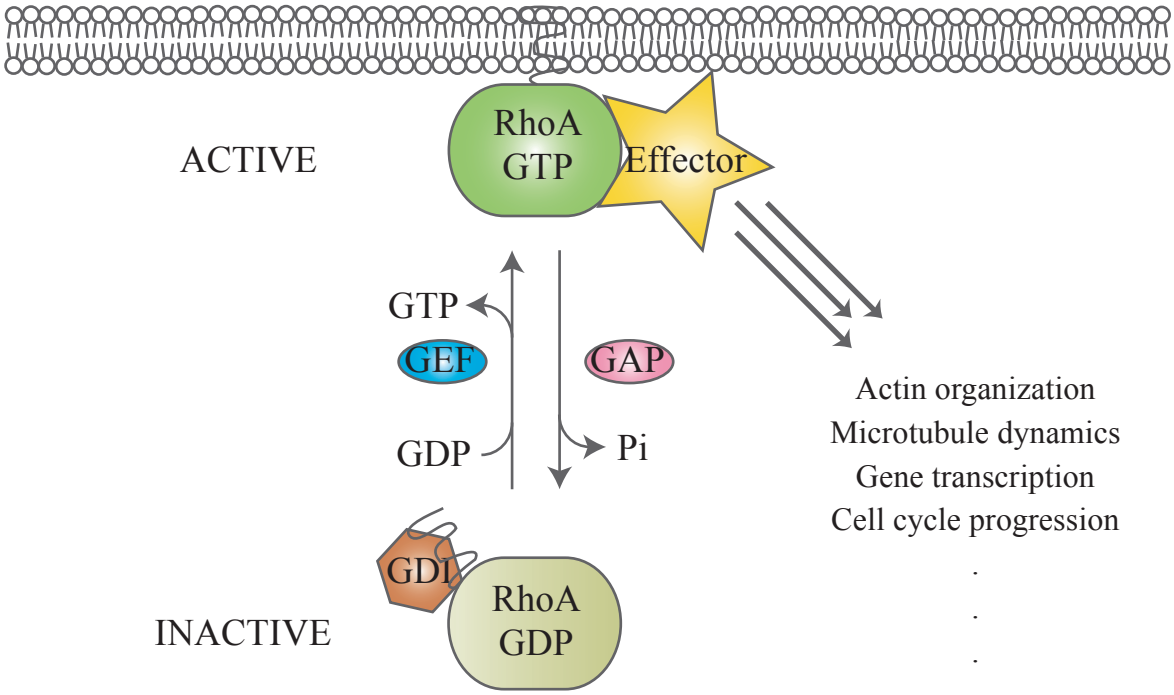

Figure 4. Rho GTPase regulation. Rho GTPases such as RhoA are signaling molecules that are active when bound to GTP and anchored to membranes by the prenylated carboxyl terminus. When active, Rho GTPases interact with downstream effectors, which are in turn activated and have functions that include reorganization of actin, changes to microtubule dynamics, alterations to gene transcription and cell cycle progression among others. Rho GTPases are inactive when bound to GDIs which sequester Rho from the membrane in its inactive, GDP-bound state. GEFs activate Rho by promoting the exchange of GDP for GTP, while GAPs inactivate Rho by promoting GTP hydrolysis.

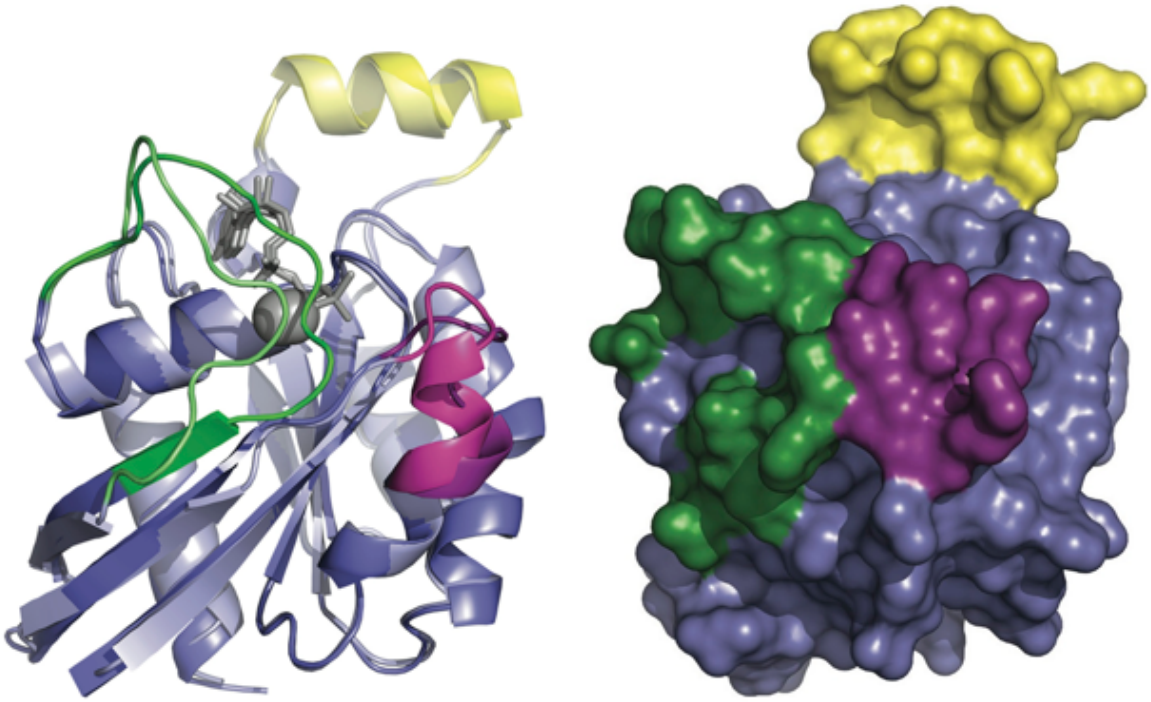

Figure 5. Conformation change on the RhoA structure as a result of binding either GDP or GTP. (Left) The crystal structures of RhoA bound to GDP (PDB: 1FTN, duller), or bound to GTPγS (PDB: 1A2B; brighter) are overlaid. The switch region I is highlighted in light green/green, switch region II in pink/purple, and the insert helix is shown in light yellow/yellow. The magnesium ion that facilitates nucleotide binding and the nucleotides (GDP/GTPγS) are shown in grey. (Right) The surface of the crystal structure of RhoA bound to GTPγS with switch I (green), switch II (purple), and insert helix (yellow) highlighted.

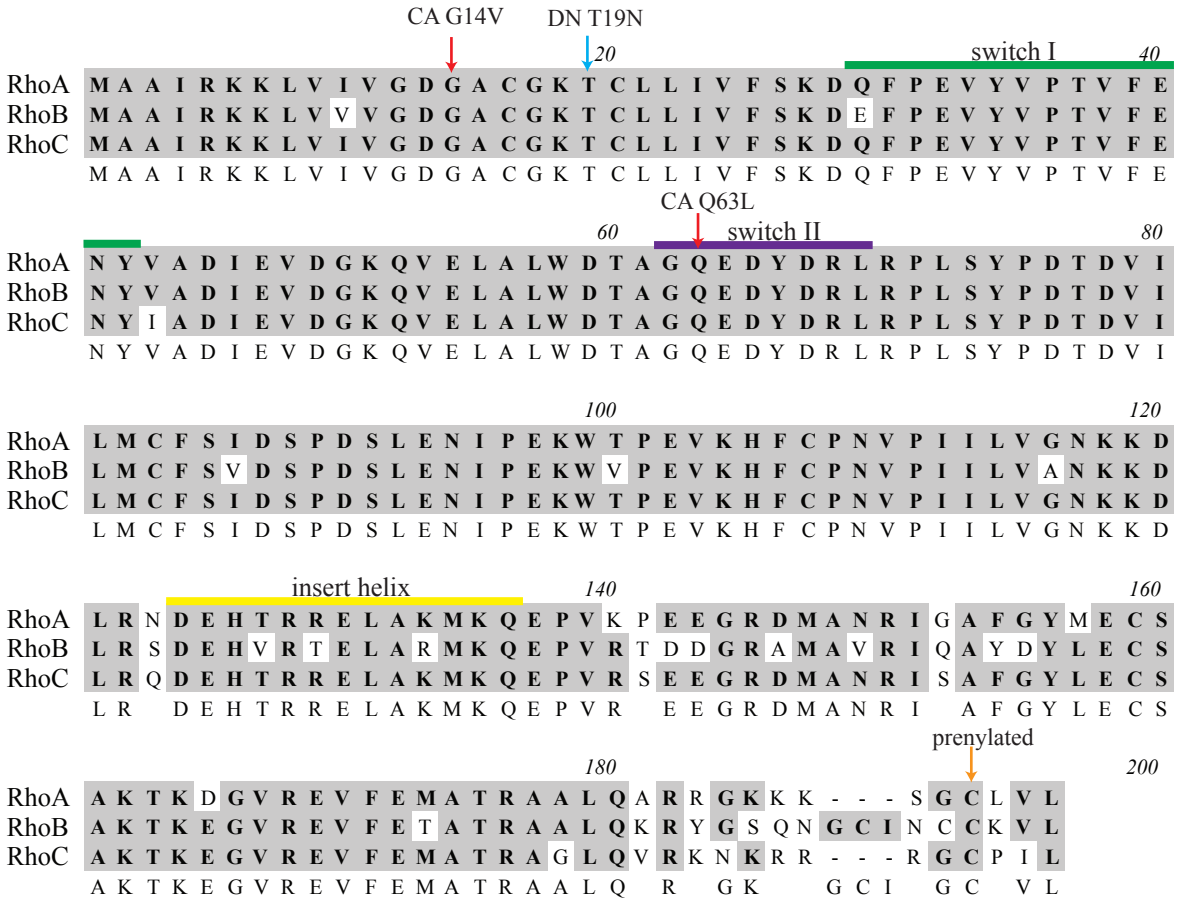

Figure 6. Alignment of RhoA, RhoB, and RhoC. The amino-acid sequences of RhoA, RhoB, and RhoC were aligned using MacVector. The Rho isoforms share 85% amino acid identity, with the majority of differences occurring at the carboxyl termini. Residues that are identical between the three Rho isoforms are highlighted in grey, while non-conserved residues are not highlighted. Switch I is labeled in green, switch II in purple, and the insert helix in yellow. Residues that when mutated cause RhoA to be either constitutively active (CA) or dominantly negative (DN) are indicated. The prenylated carboxyl terminus cysteine is also shown.

CHAPTER 2. MATERIALS AND METHODS

Strains, plasmids and molecular techniques

All plasmids used in this study are listed in Table 2, and all *S.* Typhimurium strains used in this study are listed in Table 3. *Escherichia coli* and *S.* Typhimurium strains used in this study were grown and maintained in Luria-Bertani (LB) broth or agar plates at 37°C. Human epithelial HeLa cell line from ATCC was maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% FBS and penicillin/streptomycin incubated at 37°C with 5% CO_2. HeLa cells were seeded on 12mm glass coverslips in 24 well plates at 5 x 10^4 cells and in 6 well plates at 3 x 10^5 cells.

Plasmids used for protein expression of His-RhoA and His-SseJ were previously described (47). The constructs for protein expression of His-RhoA-G14V$_{1-181}$, His-ROCK-RBD, and His-PKN1-Hr1a were engineered using standard cloning techniques into pET28a. Site-directed mutagenesis was carried out using the QuikChange Lightning Kit (Stratagene). To produce the expression construct for binding experiments, HA-tagged SseJ was inserted in-frame with an amino-terminal glutathione-s-transferase (GST) into pGEX-5x(1) with EcoRI and XhoI. The chromosomal point mutants in *sseJ* were created using the λ Red Recombinase system (53). Initially, *tetRA* encoding tetracycline resistance was recombined in the chromosome deleting amino acids 67-342 of *sseJ* in CS093 (DLL109). The point mutations in *sseJ* were originally generated in the pWSK plasmid system in frame with a carboxyl terminus HA tag, and followed immediately by a FRT-Kan-FRT from pKD4. The SseJ-HA-Kan and mutants were recombined into DLL109 selected with kanamycin. The mutations were transduced into a clean background using standard P22 transduction

techniques (244). The Kan^R cassette was then flipped out by introduction of pCP20 to yield strains used for mouse experiments. All plasmid constructs and chromosomal mutations were verified by DNA sequencing.

Expression and purification of recombinant proteins

The effector fragment, GST-Rhotekin-RBD, and the GTPases RhoA, RhoB, RhoC, Cdc42, H-Ras, and Rac1 were purchased from Cytoskeleton. His-RhoA, His-RhoB, and His-SseJ were expressed from pET vectors in *E. coli* BL21 (DE3) or BL21 Rosetta (DE3) cells. *E. coli* was grown up in LB (SseJ and RhoA-codon optimized) or terrific broth (TB – RhoA and RhoB) to OD_{600} 0.4, and induced with 0.2 mM IPTG overnight at 18°C. Cells were lysed with a French press or cell homogenizer (Avestin), spun to remove cell debris in Beckman ultracentrifuge at 40K rpm at 4°C for 40 minutes in a Ti 45 rotor. The supernatant was purified over a 5 mL His-Trap HP column with successive washes containing TBS, TBS with 25 mM imidazole, TBS with 50 mM imidazole, and eluted with TBS with 300 mM imidazole. The His-tags were cleaved with thrombin (His-RhoA and His-SseJ) or TEV protease (His-RhoB) while dialyzed using 10K MWCO SnakeSkin dialysis tubing (Pierce) into TBS with 1 mM DTT overnight. The proteins were further purified by gel filtration (Hi-load 16/60 Superdex 200) using an Acta FPLC system (Amersham) as previously described (47).

Triple labeled RhoA for NMR experiments was expressed from BL21 (DE3) for 36 hours in M9 minimal media supplemented with 1 g per liter $^{15}NH_4Cl$, 3 g per liter ^{13}C glucose, made up in D_2O, and purification steps were performed as above. His-ROCK-RBD, His-PKN1-Hr1a, His-RhoA-G14V$_{1-181}$ point mutants, and His-RhoAΔRas were expressed in *E. coli* BL21 (DE3) cells and purified over a 1 mL spin nickel column. His-SseJ point

mutants were expressed in *E. coli* BL21 Rosetta (DE3) cells. Columns were washed 3 times with 10 mL of TBS, then 3 times 5 mL of TBS with 25 mM imidazole, and 3 times 5 mL of TBS with 50 mM imidazole. Proteins were eluted with successive washes of 1 mL of TBS with 300 mM imidazole. Elution fractions containing protein (generally first 3 elutions) were pooled and were dialyzed into TBS pH 7.6, supplemented with 5 mM $MgCl_2$, and 1 mM DTT. GST-HA-SseJ was expressed in *E. coli* BL21 and purified by gravity-flow over a glutathione Sepharose column. Glutathione Sepharose was loaded with protein by end-over-end mixing with lysate supernatants for 1 hour at 4°C, the columns were washed with TBS, and eluted with 10 mM reduced glutathione in TBS. Eluted protein was dialyzed into TBS pH 7.6, supplemented with 5 mM $MgCl_2$ and 1 mM DTT. All purified proteins were stored in TBS, pH 7.6, supplemented with 10% glycerol, 5 mM $MgCl_2$ and 1 mM DTT at -80°C.

Size exclusion binding assays

The GTPases RhoA, RhoB, RhoC, Cdc42, H-Ras, and Rac1 were loaded with GTPγS by the addition of 100 mM GTPγS to 10 mM GTPase in TBS containing 2 mM EDTA and incubation on ice for 10 min. Samples containing 100 μL of 4 mM SseJ alone or 4 mM SseJ and excess GTPγS-bound GTPase 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 in TBS pH 7.6 supplemented with 10 mM $MgCl_2$.

To examine how pH affected the SseJ-RhoA complex formation, the Superdex 200 column was equilibrated with TBS pH 6.5, 7.5, or 8.5 supplemented with 5 mM $MgCl_2$ and 1 mM DTT. RhoA (200 μL of 100 μM) was loaded with GTPγS (28 μL of 7 mM) in TBS with 10 mM EDTA on the benchtop for 10 min. Samples containing 200 μL 28.5 mM SseJ

with 21.5 mM RhoA- GTPγS were applied to the Superdex 200 column at each pH at 0.5 mL/min.

PNPP lipase assays

SseJ lipase activity was determined by hydrolysis of the substrate p-nitrophenyl palmitate (PNPP). The SseJ dependent hydrolysis and release of p-nitrophenol was detected by measuring the absorbance of the reaction solution at 405 nm. Assays for determining GTPase specificity were performed in triplicate in 384-well format. 40 μL of 200 nM SseJ in the presence of 200 nM GTPase bound to GTPγS was aliquoted into a 384-well plate and 0.4 μL of 10 mM PNPP dissolved in dimethyl sulfoxide (DMSO) was added. Plates were incubated at 37°C, and increases in absorption at 405 nm were measured on an EnVision Multilabel Reader (Perkin Elmer) at 1-min intervals.

All subsequent lipase assays were performed in duplicate in a 96-well plate and repeated at least 3 separate times. To measure SseJ activity in the presence of effector binding fragments, in binding buffer (TBS pH 7.6, 5 mM $MgCl_2$, 1 mM DTT), 2.5 μL of 10 mM PNPP dissolved in DMSO was added to 200 μL of 250 nM SseJ and 250 nM RhoA-GTPγS with varying concentrations of GST-Rhotekin-RBD, His-ROCK-RBD, and His-PKN1-Hr1a. To measure SseJ activation by RhoA mutants, 2.5 μL of 10 mM PNPP dissolved in DMSO was added to 200 μL of 250 nM SseJ with varying concentrations of His-RhoA mutants. Plates were incubated at 37°C, and increases in absorption at 405 nm were measured on an EnVision Multilabel Reader (Perkin Elmer) at 1-min intervals.

Native Gel analysis

SseJ and eukaryotic effectors ROCK, or Rhotekin were mixed first, followed by addition of RhoA, all at a 1:1:1 ratio unless specified. A 6% non-denaturing gel was loaded with 2.5 μg protein per lane containing 1x sample buffer without SDS (90 mM Tris pH 6.8, 10% glycerol, 0.02% bromophenol blue) at 50V for 4 hours in the cold room. Gels were stained with Coomassie stain, and interaction between proteins was assessed by changes to protein migration patterns.

NMR spectroscopy

All NMR samples were prepared in 25 mM Tris pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT at concentrations of 0.3–0.5 mM and data were collected at 25 °C. Samples for backbone assignments and titration experiments used ^{2}H,^{13}C,^{15}N-labeled RhoA-GDP and RhoA-GTPγS. Assignment of RhoA backbone resonances was accomplished by analysis of standard triple-resonance experiments (193). All NMR data were collected on Bruker 500 MHz DMX (University of Washington) or Varian INOVA 600, 800, and 900 MHz spectrometers (Pacific Northwest National Labs). Data were processed and analyzed using NMRPipe (56) and NMRView (113).

Protein binding

Purified GST-SseJ or GST protein (10 μg) was mixed with 10 μg His-RhoA in 200 μL TBS, 5 mM $MgCl_2$, 1 mM DTT and incubated with 20 μL glutathione Sepharose beads at 4°C with rotation. After 3 hours the beads were pelleted, washed five times with PBS, and resuspended in 40 μL 1x sample buffer (90 mM Tris pH 6.8, 10% glycerol, 2% SDS, 5% β-

mercaptoethanol, 0.02% bromophenol blue). Samples were boiled, separated by SDS-page, transferred to nitrocellulose, and His-RhoA was detected using nickel-HRP (KPL). GST and GST-SseJ were detected by staining for proteins directly on the nitrocellulose membranes with Ponceau S.

Circular Dichroism Spectroscopy

Secondary structure of His-SseJ and mutants was assessed by far-UV CD spectroscopy (Avi Biomedical Model 420). Recombinant proteins were prepared in 25 mM Tris pH 7.5, 50 mM NaCl, 1 mM DTT. Data was collected in a 0.1 cm cuvette at 1 nm intervals, at 25°C, over a wavelength range of 197-260 nm. The buffer alone spectrum was subtracted from each sample spectrum and the mean residue molar ellipticity was calculated to correct for small differences in protein concentration.

Transfections, immunofluorescence microscopy, and Western blotting

Plasmids were purified using Endo-free Maxi kits (QIAGEN). HeLa cells (American Type Culture Collection) were transfected with plasmids using Fugene 6 (Roche) as recommended and were cultured for 24 hours. For the PEDA1 (Invitrogen) experiment, cells were transfected for 22 hours then incubated for an additional 2 hours with 3.5 μM PEDA1 substrate in an additional 200 μL DMEM media. If the fluorogenic PEDA1 substrate is cleaved at the sn-1 position, the BODIPY group will exhibit green fluorescence (ex/em = 488/530). The cells were washed with PBS, fixed for 20 minutes and permeabilized with the BD cytofix/ cytoperm kit (BD Biosciences), and immunostaining was performed. All antibodies were used at 1:200 in BD cytoperm buffer. Widefield deconvolution microscopy

was performed on an Eclipse TE2000-E or Ti microscope (Nikon) equipped with a Cascade II 1024 EM-CCD camera (Photometrics) or spinning disk confocal microscopy performed on an Eclipse Ti microscope with CCD camera (Nikon). Images were analyzed with the NIS-elements image analysis software.

Transfected myc-SseJ and myc-SseJ mutants were tested for stability by Western blot. HeLa cells transfected as above for 24 hours in 6 well plates were washed 3 times with PBS, and cells scraped and lysed into 100 μL sample buffer. Lysates were boiled for 5 minutes, 20 μL of samples were separated by SDS-PAGE, and transferred to nitrocellulose using the semi-dry system (Biorad) for 20 minutes at 20 Volts. After blocking with 1% BSA TBS-T, membranes were probed with anti-myc antibody followed by an HRP-conjugated secondary, and developed with ECL Plus detection reagent (GE Biosciences).

Infections

S. Typhimurium was diluted 1:100 and grown for 3 hours from an overnight culture. HeLa cells were infected at an MOI (multiplicity of infection) of 100:1 with *S.* Typhimurium for 1 hour. HeLa cells were washed with PBS and treated with gentamicin (150 μg/ml) for 1 hour followed by incubation for 14 hours with gentamicin (15 μg/ml). For fluorescent phospholipid experiments, HeLa cells were infected as above for 17.5 hours then incubated for an additional 30 minutes with 5 μM NBD-phospholipid in an additional 200 μL of media. The cells were fixed and permeabilized with BD cytofix/ cytoperm kit and immunostaining was performed. Antibodies were used at 1:200 in BD cytoperm buffer unless otherwise noted. Hoechst 33342 nuclear stain (200 ug/mL) was used at 1:50, and anti-Salmonella antibody used at 1:500. Deconvolution microscopy was performed on an Eclipse TE2000-E

or Ti microscope (Nikon) equipped with a Cascade II 1024 EM-CCD camera (Photometrics) or spinning disk confocal microscopy performed on an Eclipse Ti microscope with CCD camera (Nikon). Images were analyzed with the NIS-elements image analysis software.

Competitive index assay

S. Typhimurium strains were generated using the lambda red recombinase system (53). Mice were ordered from Charles River Laboratories, Inc., and virulence phenotypes were tested by competitive index assay as described previously (74, 173). 6- to 8-week-old female BALB/c mice were inoculated intraperitoneally with a mixture of $5x10^4$ organisms each of two serovar Typhimurium strains for a total of 10^5 bacteria in a 0.2 ml volume. Each strain was diluted from cultures grown overnight containing a stable plasmid-based antibiotic marker to allow the strains to be differentiated. The bacterial inoculum contained approximately equal concentrations of both strains, and the ratio of the strains was confirmed by plating dilutions of the inoculum onto selective media. Forty-eight hours after infection, mice were euthanized by CO_2 asphyxiation, the spleens were dissected, and each spleen was homogenized in sterile PBS. Ratios of each strain in each spleen were calculated from bacterial counts produced by plating aliquots of 1:10 dilutions of homogenized spleen on selective media. The CI was calculated by dividing the ratio of bacteria isolated from the spleen by the ratio of bacteria inoculated into the mouse. CI results were presented as the means with standard deviations for 10 mice. Statistical significances were determined using PRISM 5 (GraphPad).

Liposome assays

Liposomes containing a 60:30:10 molar ratio of cholesterol/DOPS/oleic acid were prepared by 120 µL of 10 mM cholesterol, 60 µL of 10 mM DOPS (or DOPS/DOPC combinations), and 20 µL of 10 mM oleic acid. Lipids were dried under N_2 gas, then in a speed vacuum for 30 minutes, and dry lipids were completely resuspended 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 5 mM $MgCl_2$. Aliquots of 100 µL were supplemented with 250 nM SseJ in the presence or absence of 250 nM GTPγS-loaded RhoA and incubated at 37°C for 30 min for deacylase 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) and visualized with amido black staining (170).

Table 2. Plasmids used in this study.

Name	Description	Reference
pCMV-HA	HA tag mammalian expression vector, Amp^R	Clontech
pCMV-myc	myc tag mammalian expression vector, Amp^R	Clontech
pWSK29	low copy expression vector, Amp^R	(225)
pWSK129	low copy expression vector, Kan^R	(225)
pET28a	6x-His tag protein expression vector, Kan^R	Novagen
pGEX-Cdc42Hs	GST-Cdc42 in pGEX protein expression vector, Amp^R	(11)
pET28a-SseJ	His-SseJ in pET28a, Kan^R	This study
pMBO102	myc-SseJ in pCMV mammalian expression vector, Amp^R	(174)
pMBO103	myc-SseJ in pCMV mammalian expression vector, Amp^R	(174)
pJAF111	SseJ-HA in pWSK29, Amp^R	(74)
pMBO76	SseJ 3X-HA (SseJ S151A D247N H384N – HA) in pWSK29, Amp^R	(173)
pNIC28-Bsa4-RhoB	His-RhoB in pNIC28-Bsa4	(204)
pDL18	His-ROCK RBD (947-1015) in pET28a, Kan^R	This study
pDL19	His-PKN1 HR1a (13-98) in pET28a, Kan^R	This study
pDL23	His-RhoA1-181 G14V (CA) in pET28a, Kan^R	This study
pDL32	His-SseJ CT (121-408) in pET28a, Kan^R	This study
pDL33	myc-SseJ CT (121-408) in pCMV mammalian expression vector, Amp^R	This study
pDL38	myc-SseJ D368L in pCMV mammalian expression vector, Amp^R	This study
pDL44	myc-SseJ Q172L in pCMV mammalian expression vector, Amp^R	This study
pDL47	HA-RhoA Q63L (CA) in pCMV mammalian expression vector, Amp^R	This study
pDL68	His-RhoA1-181 G14V D76A in pET28a, Kan^R	This study
pDL75	His-RhoA1-181 G14V R68A in pET28a, Kan^R	This study
pDL77	myc-SseJ R177A in pCMV mammalian expression vector, Amp^R	This study
pDL78	myc-SseJ I373D in pCMV mammalian expression vector, Amp^R	This study
pDL79	myc-SseJ Q387L in pCMV mammalian expression vector, Amp^R	This study
pDL80	myc-SseJ F121D in pCMV mammalian expression vector, Amp^R	This study
pDL82	His-RhoA1-181 G14V V38A in pET28a, Kan^R	This study
pDL83	His-RhoA1-181 G14V E40A in pET28a, Kan^R	This study
pDL86	His-RhoA1-181 G14V D65A in pET28a, Kan^R	This study
pDL88	pGEX5x GST-HA-SseJ, Amp^R	This study
pDL89	His-RhoA1-181 G14V H105A in pET28a, Kan^R	This study
pDL91	His-RhoA1-181 G14V F39A in pET28a, Kan^R	This study
pDL92	His-RhoA1-181 G14V Y66A in pET28a, Kan^R	This study
pDL93	His-RhoA1-181 G14V Δras in pET28a, Kan^R	This study
pDL94	His-RhoA1-181 G14V L72A in pET28a, Kan^R	This study
pDL95	His-RhoA1-181 G14V F39A L72A in pET28a, Kan^R	This study
pDL96	His-RhoA1-181 G14V P71A in pET28a, Kan^R	This study
pDL98	His-RhoA1-181 G14V L69A in pET28a, Kan^R	This study
pDL99	His-RhoA1-181 G14V P75A in pET28a, Kan^R	This study
pDL100	His-RhoA1-181 G14V F106A in pET28a, Kan^R	This study
pDL115	SseJ F121D-HA in pWSK29, Amp^R	This study
pDL116	SseJ R177A-HA in pWSK29, Amp^R	This study

Table 3. *S.* Typhimurium strains used in this study.

Name	Description	Reference
CS093	14028s	ATCC
MBO87	CS093 ΔsseJ, Cam^R	(173)
MBO107	MBO87 with pJAF111, Cam^R Amp^R	(173)
MBO106	MBO87 with pMBO76, Cam^R Amp^R	(173)
MBO111	CS093 SseJ-HA + FRT-Kan-FRT, Kan^R	Ohlson, M. B.
MBO118	CS093 SseJ 3X-HA+ FRT-Kan-FRT, Kan^R	Ohlson, M. B.
DLL109	CS093 ΔsseJ (67-342) with pKD46, Tet^R Amp^R	This study
DLL129	CS093 F121D SseJ-HA + FRT-Kan-FRT, Kan^R	This study
DLL130	CS093 R177A SseJ-HA + FRT-Kan-FRT, Kan^R	This study
DLL140	CS093 SseJ-HA + FRT scar on chromosome with pWSK129, Kan^R	This study
DLL141	CS093 SseJ-HA + FRT scar on chromosome with pWSK29, Amp^R	This study
DLL142	CS093 SseJ 3X-HA (SseJ S151A D247N H384N – HA) + FRT scar on chromosome with pWSK29, Amp^R	This study
DLL143	CS093 F121D SseJ-HA + FRT scar on chromosome with pWSK29, Amp^R	This study
DLL144	CS093 R177A SseJ-HA + FRT scar on chromosome with pWSK29, Amp^R	This study
DLL162	MBO87 with pDL115, Cam^R Amp^R	This study
DLL163	MBO87 with pDL116, Cam^R Amp^R	This study

CHAPTER 3. SSEJ IS ACTIVATED BY THE SMALL GTPASES RHOA, RHOB, RHOC

Results

SseJ binds to RhoA, RhoB, RhoC, Cdc42 but not Rac, or Ras

Previous experiments suggested that SseJ and RhoA formed a protein complex and SseJ cotransfected with constitutively active (CA) RhoA, RhoB, RhoC induces endosomal tubulation. Cotransfection of CA Cdc42, Rac1 or Rab7 did not induce endosomal tubulation, but CA Cdc42 was recruited to the endosome when cotransfected with SseJ (174). We tested whether SseJ could bind directly Rho family GTPases using size exclusion chromatography. SseJ was purified from *E. coli*, and tested for binding to commercially purified Rho GTPases that were preloaded with GTPγS. A strong shift in apparent mass occurs when RhoA is added to SseJ as previously determined, and is indicative of complex formation (Figure 7) (174). Also a strong shift in elution time occurred when RhoC was added to SseJ. A smaller shift in elution time occurs when RhoB or Cdc42 is added to SseJ. No shift in SseJ elution time occurs when Rac1 or Ras are added. These data demonstrate that SseJ forms a protein complex with RhoA, RhoC, possibly RhoB and Cdc42, but not Rac or Ras. Further, these results are consistent with the published data that demonstrated SseJ interaction with specific Rho GTPases in cells (174).

SseJ is activated upon binding activated RhoA, RhoB, RhoC

Since SseJ binds the specific Rho GTPases RhoA, RhoB, RhoC and Cdc42, and endosomal tubulation is induced in HeLa cells when SseJ was expressed with RhoA, RhoB, and RhoC,

we believed that RhoA, RhoB and RhoC might activate SseJ enzymatic activity. To test this, SseJ purified from *E. coli*, and commercially purified Rho GTPases loaded with GTPγS were mixed and tested for SseJ enzymatic activation, assessed by the measuring the lipase activity of SseJ using the chromogenic substrate PNPP. Lipase activity of SseJ increased strongly in the presence of RhoA, and more weakly by RhoC (Figure 8 top). Surprisingly, RhoB did not activate SseJ lipase activity in these studies. The other Rho GTPases, Cdc42, Rac, and Ras also did not activate SseJ lipase activity. Our initial studies suggesting that RhoB did not activate SseJ used commercially available RhoB. Since overexpression of CA RhoA, CA RhoB and CA RhoC with SseJ can induce endosomal tubulation within HeLa cells (174), we reassessed SseJ-RhoB binding using RhoB that we purified, and determined that SseJ is able to bind to and be activated by RhoB (Figure 8 bottom). These data demonstrate that SseJ enzymatic activity is stimulated by binding activated RhoA, RhoB, and RhoC, but is not activated by Cdc42, Rac, or Ras.

SseJ carboxyl domain has deacylase activity in the presence of RhoA

Studies performed previously attempting to truncate SseJ to define the region of SseJ sufficient for catalytic activity have been unsuccessful. Information provided by Dr. Jijie Chai, Tsinghua University regarding the SseJ-RhoA complex crystal structure, suggested that the carboxyl terminus of SseJ (SseJ CT; amino-acids 121-408) is sufficient for binding RhoA. Since SseJ CT contains the catalytic residues of SseJ, we purified from *E. coli* the SseJ CT and tested it for lipase activity in the presence of RhoA. Lipase activity of SseJ and SseJ CT demonstrated no lipase activity without RhoA, and were equally activated in the presence of

CA RhoA (Figure 9). These results demonstrate that the SseJ CT behaves enzymatically like full length SseJ.

SseJ carboxyl domain colocalizes with $RhoA_{CA}$ in HeLa cells

Full length SseJ localizes to a LAMP-1 positive endosomal compartment when transiently transfected into HeLa cells (191). Although SseJ CT enzymatically behaves like full length SseJ when purified, it is possible that SseJ may require its amino terminus to localize to RhoA or the membrane inside mammalian cells. To test this, plasmids expressing full length SseJ and SseJ CT were transiently transfected into HeLa cells with a plasmid expressing CA RhoA. SseJ CT colocalized with CA RhoA similar to full length SseJ in HeLa cells, and also appeared to induce tubulation of the membranous compartment where it was present (Figure 10). Thus, SseJ CT and full length SseJ localize to a similar membranous compartment in HeLa cells, and colocalize with RhoA.

Formation of the SseJ-RhoA complex is dependent upon pH

The SseJ-RhoA complex crystal structure provided by Dr. Jijie Chai revealed a histidine ($SseJ^{H391}$) present in the center of the SseJ-RhoA interface. Since histidine residues become protonated at around neutral pH we postulated that pH changes could regulate the formation of the SseJ-RhoA complex. Specifically, we hypothesized that decreasing the pH would disrupt the formation of the complex, since the neighboring RhoA residues were hydrophobic. We tested whether pH regulates the formation of the SseJ-RhoA complex using size exclusion chromatography with buffers of different pH. SseJ was mixed with a slight excess of RhoA preloaded with GTPγS, and separated at pH 6.5, pH 7.5 and pH 8.5. When the pH

of the buffer was pH 7.5 or pH 8.5, SseJ-RhoA formed a complex at a size corresponding to 1:1 complex of SseJ to RhoA (Figure 11) when compared to size exclusion standards. Contrary to our expectations, at pH 6.5 the elution mass of the SseJ-RhoA complex increased to approximately 2:2 of SseJ to RhoA (Figure 11). The shift in apparent mass at lower pH could reflect the oligomerization state of either SseJ or RhoA alone since this has not been tested. These data suggest that the histidine present in the SseJ-RhoA interface is not important for the complex to form, and may not be the only factor that influences complex formation in response to pH. No observable changes to SseJ activation by or binding to RhoA is observed when this histidine is mutated ($SseJ^{H391A}$) (not shown), supporting the idea that the histidine in the binding interface is not essential for complex formation.

Discussion

This chapter demonstrates that SseJ is activated by RhoA, RhoB, and RhoC, which share 85% identity. The carboxyl domain of SseJ that contains the catalytic residues is sufficient for binding RhoA. The binding of SseJ CT to RhoA is sufficient for activation of SseJ GCAT activity and for localization to membranes in HeLa cells, therefore the amino terminus of SseJ is dispensable for RhoA binding, SseJ activity, and localization to membranes. When the pH is lowered from pH 7.5 to 6.5, SseJ-RhoA forms a higher order complex, the significance of which, if any, is unknown.

SseJ coexpressed with RhoA, RhoB, and RhoC induces endosomal tubulation in HeLa cells, while coexpression with Cdc42, Rac1, or Rab7 did not (174). This suggests that SseJ enzymatic activity in cells is regulated at least in part by the three GTPases, RhoA, RhoB, or RhoC. Additional evidence from our laboratory suggests that SseJ does not modify

or influence RhoA GEF or GAP activity, but instead senses the activation state of RhoA to regulate enzymatic activity (47). Specifically, GTP-bound RhoA was found to have a higher affinity for SseJ than GDP-bound RhoA, and GTP-bound RhoA maximally stimulated SseJ enzymatic activity (47). Here we have shown that SseJ specifically binds RhoA, RhoB, RhoC, and Cdc42, but that only RhoA, RhoB, and RhoC can activate SseJ's enzymatic activity in vitro. When I purified ^{15}N-labeled Cdc42 for NMR studies, very few changes to the Cdc42 NMR spectrum were observed when SseJ was added, indicating that these proteins do not bind under the conditions examined, and that co-elution in our size exclusion experiments may have reflected impurities in the commercially acquired Cdc42.

The preference for a specific one of these Rho family GTPases (RhoA, RhoB, or RhoC) is not apparent in vitro, and this is corroborated with data in the next chapter that demonstrates that RhoA residues that are important for activation or binding are conserved between RhoA, RhoB, and RhoC. A number of eukaryotic effectors have been characterized for concurrent binding to RhoA, RhoB and RhoC, and it is difficult to establish which protein is the true binding partner of these effectors (229). Similarly, it is still unknown whether SseJ preferentially binds RhoA, RhoB, or RhoC in vivo. RhoB could be SseJ's preferred binding partner since it is an endosomal GTPase (1), and has been demonstrated to recruit the RhoA effector protein PKN1 to the endosomal compartment (155). Since the SseJ-RhoA complex is recruited to the endosomal compartment, and binding of SseJ to RhoA or RhoB occurs at the same surface, the importance of SseJ binding to RhoB for endosomal localization is not clear.

The fact that a eukaryotic protein regulates SseJ activity suggests that the tight and specific regulation of GCAT activity is important to virulence. All three bacterial GCATs

studied to date are regulated enzymatically by either an additional bacterial component or host cell component. Since GCATs can behave as lipases in the absence of cholesterol, unregulated activity in the bacterial cell could detrimentally affect the bacterial membrane and therefore survival. The expression of an active form of SseJ within *Salmonella* may have deleterious consequences on bacterial membrane stability, and therefore it may not be possible to isolate a constitutively active form of this protein because of the nature of its activity. Since SseJ is secreted into the mammalian cytosol it is unsurprising that it evolved to specifically sense a eukaryotic protein for activation. How SseJ evolved to specifically sense the activation state of RhoA over other Rho GTPases or other proteins remains unknown. RhoA is not normally an endosomally localized signaling molecule, therefore its recruitment to the endosomal compartment may have additional effects besides the activation of SseJ. It is plausible that RhoA recruitment to the endosomal compartment may result in the activation of endosomal effectors that are normally not spatially recognized by RhoA, and this may have additional effects on membrane regulation.

The significance of pH on the ability of the SseJ-RhoA complex to form either a dimer or a tetramer is unknown. The result that lowering the pH to 6.5 results in tetramer formation is unexpected. This result also suggests that at pH 7.5 there is most likely an equilibrium between the tetrameric and dimeric state of the SseJ-RhoA complex. Based upon the mutagenesis experiments described in the next chapter, we know that a specific RhoA surface is important for SseJ activation. However, a more extensive mutagenesis study on RhoA could reveal whether the tetrameric state of the complex may be important for enzymatic activation of SseJ, or for recruitment of these proteins to the endosome. The cytosolic pH of mammalian cells is around neutral pH (pH 7.4), therefore under

physiological conditions SseJ-RhoA likely forms a dimeric complex, although this may be in equilibrium with tetrameric complex.

An unpublished study in our laboratory demonstrated that $SseJ_{140-408}$ could not interact with RhoA. The co-crystal structure of SseJ-RhoA demonstrates that amino acids 121-139 form important contacts with RhoA, and the carboxyl domain of SseJ (SseJ CT; amino-acids 121-408) is sufficient for interaction with RhoA in vitro. RhoA can activate the SseJ CT in vitro to a level comparable to full-length SseJ, suggesting that the amino terminus is dispensable for SseJ activity. The amino terminus of T3SS effectors is known to be important for secretion, and was thought to be important for the specific localization of effectors in mammalian cells. When SseJ CT is co-expressed with RhoA into HeLa cells a similar localization is seen compared to full-length SseJ co-expressed with RhoA. However, to verify that the compartment is truly the endosomal compartment, staining of the compartment with LAMP-1 will need to be demonstrated. This data suggests that the amino-terminus is not necessary for SseJ localization to the endosomal compartment. The amino-terminus of SseJ may play a role in protein stability, since I have observed that purified SseJ CT aggregates more readily and is less stable in enzymatic assays compared to full-length protein. Additionally, SseJ enzymatic activity does not influence SseJ localization, since a catalytic mutant of SseJ still localizes to the endosomal compartment. Together, these data suggest that SseJ localization to the endosomal compartment is likely due to a protein-protein interaction with SseJ, or the SseJ-RhoA complex, and an endosomal protein. Identification of proteins that interact with SseJ-RhoA will be important for understanding how SseJ localizes to the endosomal compartment.

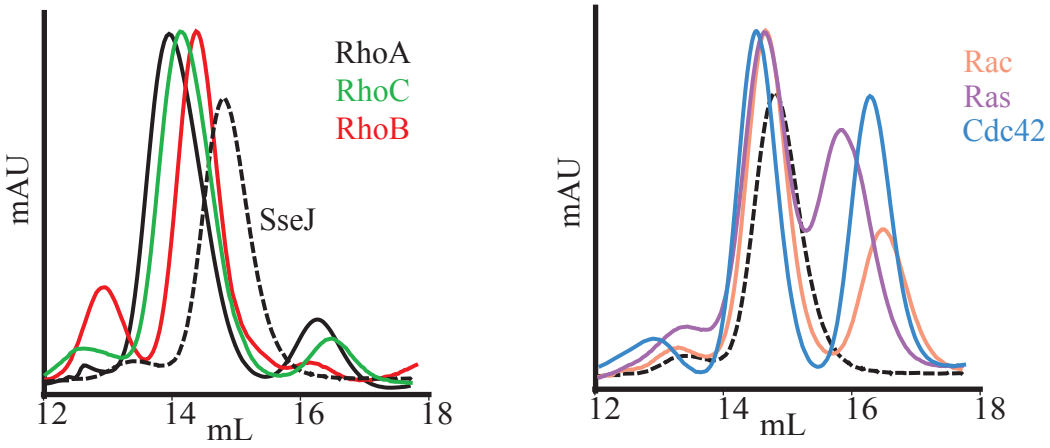

Figure 7. SseJ forms a complex with the small GTPases RhoA, RhoB, RhoC, and Cdc42, but not to Rac, or Ras. Size exclusion analysis of SseJ alone (dashed line), in the presence of RhoA (black), RhoC (green), RhoB (red) demonstrates SseJ can form a complex with RhoA, RhoB, and RhoC, and possibly Cdc42 (blue), but not Rac (orange) or Ras (purple). Excess GTPase was used in all experiments, and later peaks in each run reflect elution volume for each GTPase alone.

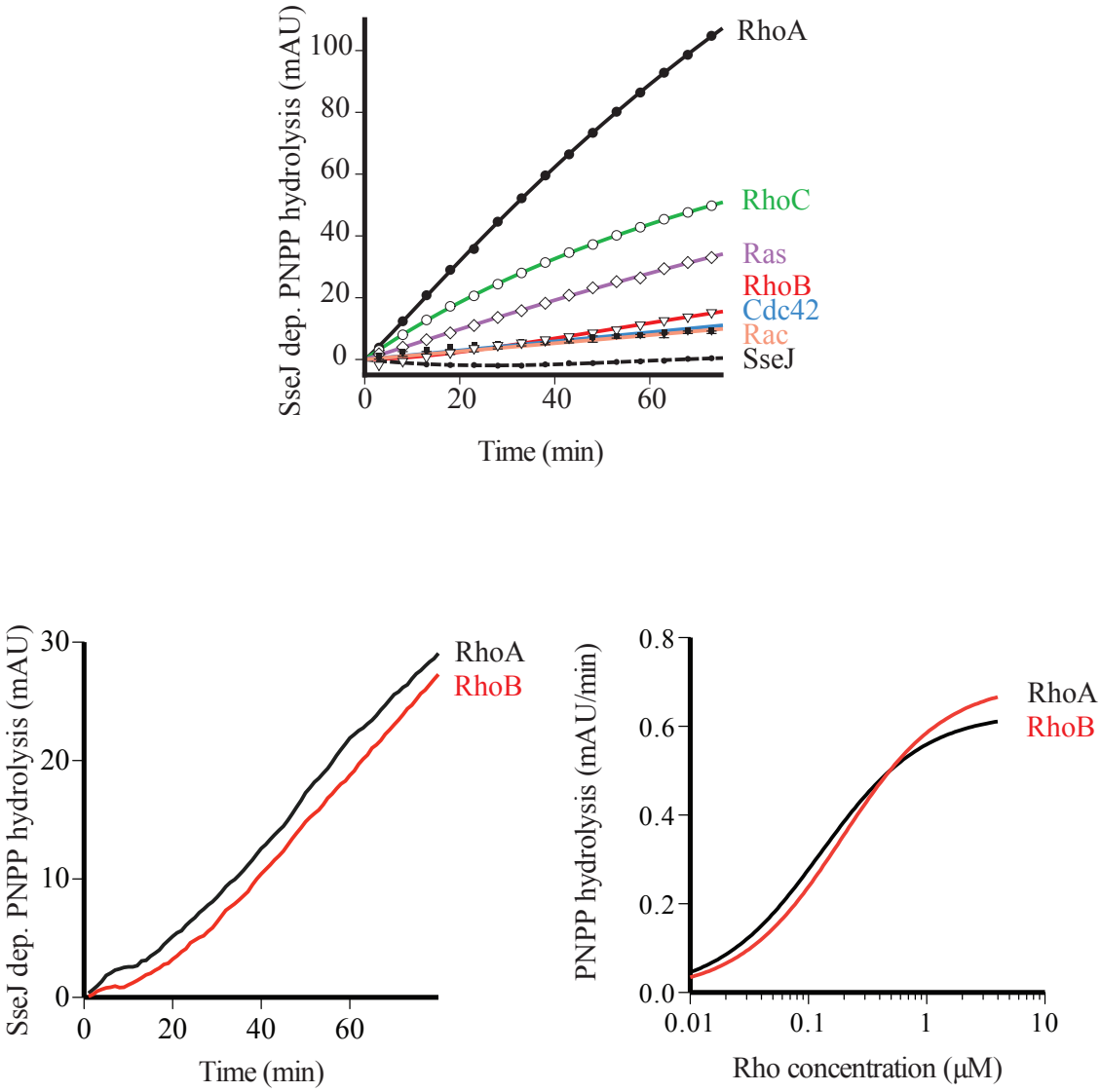

Figure 8. RhoA, RhoB, RhoC activate SseJ lipase activity. (Top) Representative experiment of SseJ (250 nM) dependent lipase activity is stimulated in the presence of 250 nM RhoA (black) and RhoC (green), but not RhoB (red), Cdc42 (blue), Rac (orange), or Ras (purple) (GTPases purchased from Cytoskeleton Inc.). Significant activation was determined by the ability of a GTPase to stimulate SseJ lipase activity in three experiments over SseJ alone. (Bottom) SseJ (250 nM) dependent lipase activity is stimulated equally in the presence of 250 nM RhoA (black) or RhoB (red) over time (left), or over different Rho concentrations (right) (GTPases purified in lab).

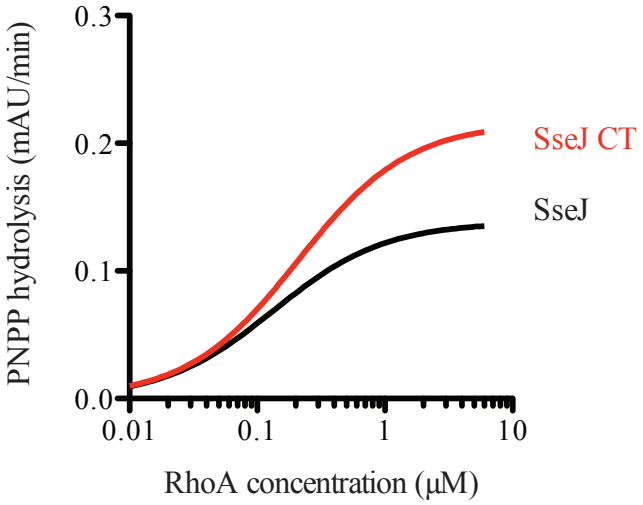

Figure 9. SseJ carboxyl domain has lipase activity in vitro. SseJ (250 nM) dependent lipase activity for either full length or carboxyl domain SseJ (SseJ CT; amino-acid 121-408) demonstrates both are activated by RhoA. Data was fit directly to the Michaelis-Menten equation using nonlinear regression using PRISM 5 (GraphPad), and are not statistically different from each other by t-test.

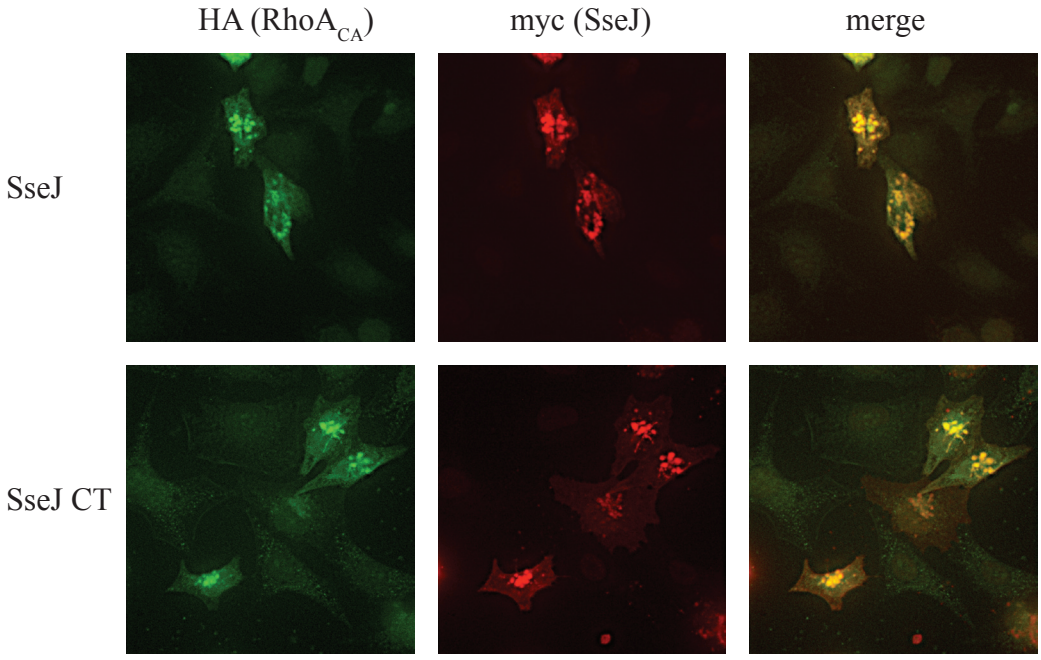

Figure 10. SseJ CT colocalizes with constitutively active (CA) RhoA in HeLa cells. HeLa cells were co-transfected with plasmids encoding myc-SseJ or myc-SseJ CT (amino acid 121-408), and HA-RhoA$_{CA}$ for 24 hours then stained with antibodies against HA (green), or myc (red). Images captured at 90X.

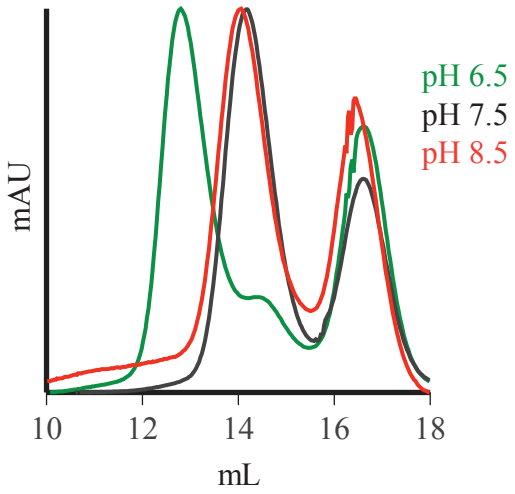

Figure 11. Stoichiometry of the SseJ-RhoA complex is influenced by pH. Size exclusion analysis of the SseJ-RhoA complex at different pH's demonstrate that SseJ-RhoA forms approximately a 1:1 complex at pH 7.5 and pH 8.5, but may form a higher order complex at pH 6.5.

CHAPTER 4. SSEJ BINDS TO THE SWITCH REGIONS OF RHOA

Results

SseJ competes with eukaryotic RhoA activated proteins, ROCK, PKN1, and Rhotekin for binding to RhoA

The observation that SseJ is activated upon binding RhoA-GTP demonstrated that SseJ is regulated similarly to eukaryotic effectors that interact with RhoA (46). We suspect that SseJ evolved to bind a similar RhoA surface as eukaryotic effectors, implying that SseJ may be able to compete effectively with eukaryotic effectors with adequate affinity for binding to RhoA. To test if SseJ activation by RhoA could occur in the presence of eukaryotic effectors, activation of SseJ lipase activity was measured using the chromogenic substrate PNPP and with increasing concentrations of three recombinant RhoA effector binding domains, Rhotekin-Hr1a, ROCK-RBD, or PKN1-Hr1a. These eukaryotic effectors competed with SseJ with similar kinetics, where at a 1:1 molar ratio of SseJ to eukaryotic effector, the rate of SseJ catalyzed PNPP hydrolysis in the presence of RhoA-GTP remained indistinguishable from when no effector is present (Figure 12). However, increasing concentrations up to 10 molar equivalents of eukaryotic effector relative to SseJ resulted in almost complete inability of RhoA to activate SseJ. In contrast the presence of 10 molar equivalents of a control protein, bovine gamma globulin, had no inhibitory effect on SseJ-RhoA-GTP deacylase activity (not shown).

I used native polyacrylamide gel electrophoresis to further explore the ability of SseJ to compete with the eukaryotic effectors Rhotekin-RBD (Figure 13 top) and ROCK-RBD (Figure 13 bottom), and observed that SseJ binds to RhoA in the presence of both effectors.

When SseJ, RhoA, and Rhotekin-RBD were mixed in a 1:1:1 ratio very little SseJ is unbound (Figure 13 top). With increased amount of Rhotekin there was no increase in free SseJ, suggesting that SseJ binds more tightly to RhoA than Rhotekin to RhoA. Interestingly, when SseJ and ROCK are mixed, ROCK shows altered migration in native gel compared to ROCK alone (Figure 13 bottom). This suggests that SseJ modifies ROCK in an unidentified manner. Similar to the competition between SseJ and Rhotekin, when SseJ, RhoA, and ROCK were mixed, there is little free SseJ, and instead increasing amounts of free ROCK were observed when it was added in excess. The binding affinity between RhoA and the binding domains of eukaryotic effectors have been measured for Rhotekin, PKN1, and ROCK, at 147 nM, 150 nM, and 130 nM respectively (21). These results suggest that either SseJ has a greater affinity than eukaryotic effectors for RhoA, and/or that SseJ recognizes a surface of RhoA-GTP that differs from that recognized by the eukaryotic RhoA binding proteins.

NMR studies reveal perturbation of distinct residues on the RhoA surface upon SseJ binding

A combination of heteronuclear 2D and 3D NMR was used to examine binding interactions between RhoA and SseJ. The resonances of backbone amides in NMR spectra are sensitive indicators of their environment, and interaction with another protein perturbs the resonances of residues located at the protein interface (246). Resonance assignments for RhoA-GDP and RhoA-GTPγS complexes, containing the first 181 residues and lacking the extreme C-terminal residues that are highly hydrophobic and contain a lipid modification site, have been previously published (48, 84). Multidimensional HNCA and HNCACB NMR spectra were collected for RhoA-GDP and RhoA-GTPγS complexes to verify backbone assignments under

new buffer conditions used for the current studies. 122 backbone amide resonances out of an expected 169 were assigned for RhoA-GTPγS while 150 backbone amide resonances were assigned for RhoA-GDP. Most of the missing assignments in RhoA-GTPγS correspond to residues in the nucleotide-binding and switch-regions of RhoA. The likely explanation is that the RhoA conformation induced by binding of GTP leads to weak self-association of RhoA-GTP mediated by the switch regions leading to excessive peak broadening and loss of resonances for residues involved in protein self-association.

Consistent with our previous observations that RhoA-GTPγS and SseJ form a stable complex, RhoA-GTPγS resonances observed in ^{1}H-^{15}N-TROSY spectra exhibit a uniform decrease in intensity with increasing concentrations of SseJ. The observed behavior is indicative of the formation of a large complex (> 50 kD) that is in slow exchange on the NMR time scale, making it difficult to study the binding surface between these proteins (Figure 14 bottom). However, in order to identify RhoA residues that directly interact with SseJ, ^{1}H-^{15}N-TROSY spectra were collected on the relatively weaker interaction between RhoA-GDP and SseJ, assuming that the interaction surface would be similar for both forms since SseJ can bind both RhoA-GTPγS and RhoA-GDP (47). Binding of RhoA-GDP to SseJ forms a complex that is in the fast to intermediate exchange regime on the NMR time scale (Figure 14 top). Under these conditions, RhoA resonances for residues at the binding interface selectively shift or broaden with increasing concentrations of SseJ.

As expected, a subset of RhoA-GDP resonances was observed to undergo chemical shift perturbations upon addition of SseJ. Resonances of residues of RhoA-GDP that were shifted or that underwent peak broadening were mapped onto the crystal structure of RhoA (Figure 15; PDB: 1A2B). The location of switch I and II and the insert helix of RhoA are

identified on the crystal structure (Figure 5). A large number of resonances of residues involved in RhoA nucleotide binding and the switch I and switch II region of RhoA were selectively broadened with increasing concentrations of SseJ. Residues in the insert helix of RhoA shifted upon binding to SseJ. Little to no changes to RhoA residues on the side opposite of the switch regions were observed. These observations indicated that SseJ binds to a surface of RhoA that includes the RhoA switch regions, similar to other eukaryotic effectors that recognize RhoA. Thus, SseJ likely competes with eukaryotic effector proteins for binding to the same surface on RhoA.

SseJ activation is mediated by the RhoA switch regions

Two classes of resonances were identified in the NMR titration experiments: resonances in switch I and switch II that broaden and disappear, and resonances in the insert helix that shift upon addition of SseJ. Typically resonances that broaden are indicative of a greater change to a residue's chemical environment than peak shifting, suggesting that the residues at the interface surface are more likely to be involved in binding, and residues in the insert helix that shifted are due to secondary effects of binding elsewhere on the protein. To identify the specific surface of RhoA involved in activating SseJ, alanine substitutions were introduced in the surface exposed residues of the two switch regions of RhoA. These RhoA residues have been mutated and studied previously (111, 132, 188). The RhoA insert helix was also replaced with the corresponding loop from Ras (RhoAΔRas) (245). These mutations were introduced into a constitutively active (CA) mutant of RhoA, His-RhoA^{G14V} (CA His-RhoA), and each mutant was affinity purified and tested for the ability to activate SseJ (Figure 16). Activity experiments were performed using 250 nM SseJ with increasing concentrations of

CA His-RhoA or each of the fourteen CA His-RhoA mutants. Lipase activity of SseJ similar to CA His-RhoA was observed for nine of the mutants generated, including CA His-RhoAΔRas (Figure 16 bottom). However, five of the CA His-RhoA mutants generated exhibited reduced ability to activate SseJ to varying degrees (Figure 16 top). I have tested additional RhoA residues for activation of SseJ in similar experiments, which were identified from previous crosslinking experiments as potentially being in the binding surface, and all the RhoA mutants tested for SseJ activation are summarized in Table 4.

One mutation within switch I (CA His-RhoA^{F39A}) of RhoA, demonstrated reduced activation of SseJ. At concentrations of 1000 nM, CA His-RhoA^{F39A} was able to activate SseJ to approximately 69% of the level observed for CA His-RhoA. Three mutations (R68A, L69A, and L72A) within switch II of RhoA significantly reduced the ability of RhoA to activate SseJ. The CA His-RhoA^{L69A} mutant exhibited a modest decrease (52%) in ability to activate SseJ compared to CA His-RhoA. In contrast, the CA His-RhoA^{R68A} and CA His-RhoA^{L72A} mutants were almost completely unable to activate SseJ enzymatic activity (2% and 17% respectively). A CA His-RhoA$^{F39A\ L72A}$ double mutant showed a more dramatic loss, activating SseJ to only 1% of the level of CA His-RhoA at comparable concentrations. Additionally RhoA^{F106}, which is a residue neighboring but not within the switch regions of RhoA, is important for activation of SseJ enzymatic activity since CA His-RhoA^{F106A} activated SseJ to only 6% of that compared to CA His-RhoA at 1000 nM.

SseJ binds the RhoA switch regions

To determine whether the RhoA residues involved in activating SseJ were also those involved in directly binding SseJ, each mutant that showed a decrease in ability to activate

SseJ was tested for the ability to bind GST-SseJ. Purified GST-SseJ was mixed with CA His-RhoA or each of the five mutants CA His-RhoA and glutathione beads (Figure 17). CA His-RhoA pulled down with GST-SseJ, but not with GST control. Each of the five mutants that exhibited reduced ability to stimulate SseJ activity was unable to interact with SseJ in these pull down assays. Thus, all of the mutations identified that altered the ability of RhoA to activate SseJ also decreased the ability of RhoA to bind to GST-SseJ indicating that the binding and activation surfaces of RhoA for SseJ are the same. The RhoA residues that are involved in SseJ binding are highlighted in orange, while those residues not important for activation in the same study are highlighted in teal (Figure 18).

SseJ, ROCK, and PKN1 have partially overlapping contact surfaces on RhoA

The mutational analysis showed that residues in switch II of RhoA are important for activation of SseJ. These findings were consistent with our results showing that SseJ could effectively compete with eukaryotic effectors for binding to RhoA. A combination of evidence from co-crystal structures of eukaryotic effector binding domains with RhoA and mutational studies have demonstrated the specific residues important in mediating the interaction between these effectors and RhoA (64, 111, 147). Interestingly, two invariant leucines, $RhoA^{Leu69}$ and $RhoA^{Leu72}$, which are implicated in most small GTPase contacts with eukaryotic effectors or eukaryotic regulators are also involved in the SseJ interaction with RhoA (63). When residues that are involved in PKN1 (Figure 19 right) or ROCK (Figure 19 left) binding to RhoA are compared to the residues within RhoA that are involved in binding SseJ it is apparent that binding of each effector or SseJ to RhoA must be mutually exclusive since similar residues within RhoA are involved. PKN1, ROCK, and SseJ specifically sense

the nucleotide bound state of RhoA, RhoB, and RhoC and are not activated by other small GTPases such as Cdc42 or Rac1 (46, 63, 111). PKN1 and ROCK both bind to $RhoA^{Glu40}$ within switch I, and this is the only residue used by these eukaryotic effectors that differs between RhoA and other small GTPases. $RhoA^{Glu40}$ is not critical to SseJ's interaction with RhoA; the only residue used by SseJ that varies between RhoA and other small GTPases is outside of the switch region in $RhoA^{Phe106}$, suggesting the possibility that this residue is used by SseJ to distinguish RhoA from other small GTPases. However, SseJ is not unique in recognizing this residue, since $RhoA^{Phe106}$ in addition to $RhoA^{Glu40}$, appears to be used by another RhoA eukaryotic effector, mDia1 to distinguish RhoA from other small GTPases (132, 188, 245). Therefore we conclude that SseJ uses a similar surface to bind to RhoA as eukaryotic proteins and likely competes effectively by a relatively increased affinity for this surface.

Discussion

This chapter has demonstrated the structural basis by which SseJ is activated by the nucleotide bound state of RhoA. SseJ has properties similar to eukaryotic small GTPase activated proteins that bind to the conformational sensitive switch regions. Activation of SseJ by RhoA is mutually exclusive to binding eukaryotic effectors, and SseJ can compete effectively by potentially having a greater affinity for activated RhoA. The affinity of SseJ CT to RhoA was measured to be 45 nM assessed by isothermal titration calorimetry by Dr. Jijie Chai's group, and this affinity is at least one order of magnitude greater than the affinities of RhoA for the binding domains of eukaryotic effector proteins, although these were measured by a different technique (21). NMR experiments coupled with mutational

studies examining the effect of SseJ binding to RhoA demonstrate that SseJ perturbs a large number of residues on the RhoA surface, but that only a subset of these residues are on the activation surface.

Eukaryotic RhoA binding proteins that preferentially sense either the GTP- or GDP-bound state of RhoA specifically recognize the switch regions of RhoA. This chapter demonstrated through RhoA protein NMR and mutational analysis of RhoA that SseJ also specifically recognizes the switch regions of RhoA. Amino acid substitutions of two residues whose conformations are nucleotide-dependent, $RhoA^{Phe39}$ within switch I or $RhoA^{Arg68}$ within switch II, dramatically decrease the ability of RhoA to interact with and activate SseJ. Consistent with SseJ having a similar binding surface on RhoA as eukaryotic effectors, SseJ activation by RhoA can be competed by three eukaryotic effectors, PKN1, ROCK, and Rhotekin, implying mutually exclusive binding. Many eukaryotic effectors including PKN1, ROCK, and Rhotekin utilize coil-coiled motifs to interact with a few residues within the RhoA switch regions (63), whereas SseJ does not have a recognizable Rho binding domain, and interacts with a large surface of RhoA including the switch regions. This predicted convergent evolution of SseJ to recognize a similar surface of RhoA as eukaryotic effectors adds to the paradigm that bacterial effectors evolved very different mechanisms to mimic the behavior of eukaryotic proteins (79).

Regulation of SseJ activity by RhoA suggests that the lipase or GCAT activity of SseJ when unregulated may be detrimental to the bacteria. The GCATs studied to date have the ability to deacylate any phospholipid with a glycerol backbone, including bacteria lipids. If SseJ was active inside bacteria, it could potentially deacylate bacterial phospholipids which might substantially compromise the bacterial membrane content. Additionally, the

importance of SseJ regulation by the eukaryotic signaling protein RhoA, suggests that spatial and temporal regulation of SseJ within mammalian cells is important. Specifically, if SseJ was proteolytically processed like other bacterial GCATs, the ability to regulate SseJ activity would be dependent on degradation. Instead, SseJ regulation by the activation state of RhoA allows tight regulation of SseJ GCAT activity within cells on the endocytic membrane. It would be interesting to determine if SseJ can use bacterial phospholipids in addition to mammalian phospholipids as substrates. Another important question is whether SseJ activity within *Salmonella* may have detrimental effects on bacterial survival and growth in culture or in infection. Although we have so far been unable to make a SseJ constitutively active mutant, this question could still be tested by expressing SseJ and RhoA in bacterial cells and examining effects on bacterial membrane permeability and bacterial survival.

The previous chapter demonstrated that SseJ specifically binds RhoABC, but not to Cdc42 or Rac1 (46), suggesting that specific residues important for interaction must differ between RhoA and Cdc42 and Rac1. We identified a single amino-acid, Phe106, that is variable between RhoA and Cdc42 or Rac1 that is involved in the RhoA interaction with SseJ, which is located outside of the switch regions of RhoA but is critical for RhoA binding and activation of SseJ. This residue, Phe106, is not utilized by the two effectors crystalized with RhoA, PKN1 or ROCK (64, 147), but is required by mDia1 which has been crystalized with RhoC and mDia1 has been demonstrated to bind RhoA as well (188). These results indicate that SseJ uses conserved residues to differentiate the nucleotide bound state of RhoA as eukaryotic effectors and likely distinguishes RhoABC from other Rho GTPases in a similar manner to at least one eukaryotic effector. It is tempting to speculate that the different binding surface and greater affinity allows SseJ to compete effectively with

eukaryotic effectors in vitro, though in vivo the relatively large excess of RhoA may make this possibility irrelevant.

Table 4. The effects of RhoA mutations on their ability to activate SseJ. No difference is denoted as ND.

RhoA mutation	Ability to activate SseJ compared to WT
R5A	ND
R5K	ND
R5Q	ND
Q29E	ND
V38A	ND
F39A	69%
R68A	2%
D65A	ND
Y66A	ND
L69A	52%
P71A	ND
L72A	17%
P75A	ND
D76A	ND
T100V	ND
H105A	ND
F106A	6%
ΔRas	ND
F39A L72A	1%
R129T	ND
R129T G152Q	ND
P141T	ND
P141T G152Q	ND
D146A	ND
N149V	ND
G152Q	ND
G152Q F154Y G155D	ND
G152Q G155D	ND
F154Y G155D	ND
G155D	ND
Δ182-193	ND

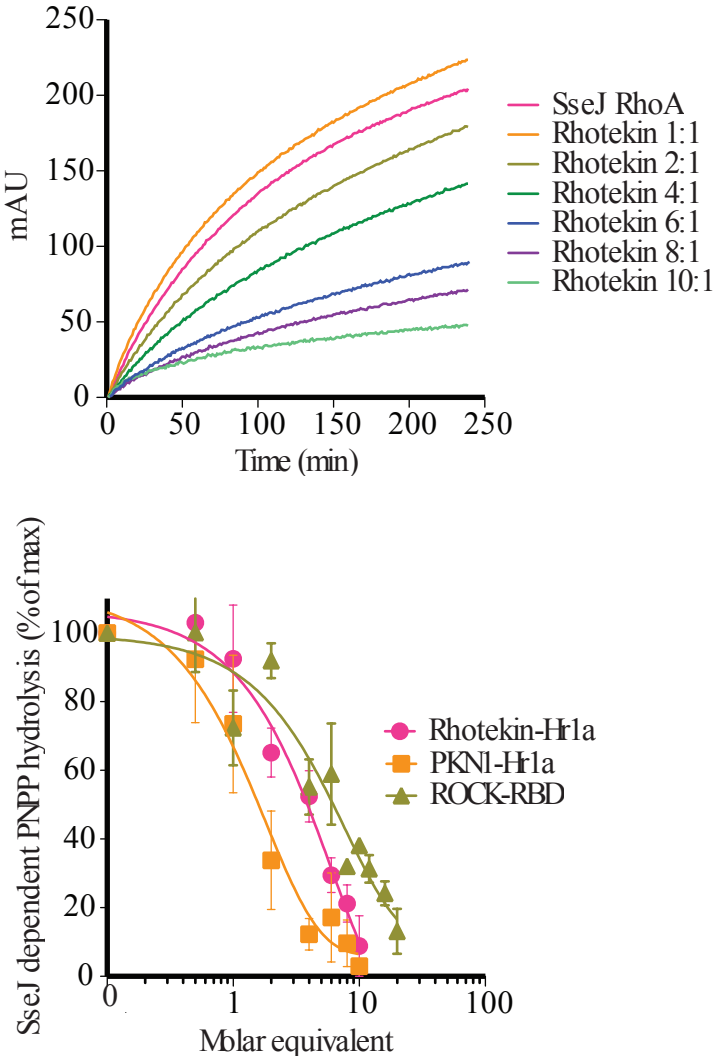

Figure 12. SseJ competes with eukaryotic RhoA activated proteins, ROCK, PKN1, and Rhotekin for activation by RhoA. SseJ dependent lipase activation by RhoA was assayed by hydrolysis of PNPP using 250 nM SseJ and RhoA-GTPγS, with (Top) increasing equivalents of the RhoA binding domain of Rhotekin, and (Bottom) increasing equivalents of the RhoA binding domains of Rhotekin, PKN1, and ROCK. Average of three experiments with standard errors plotted demonstrate that SseJ can compete with all three eukaryotic effectors equally.

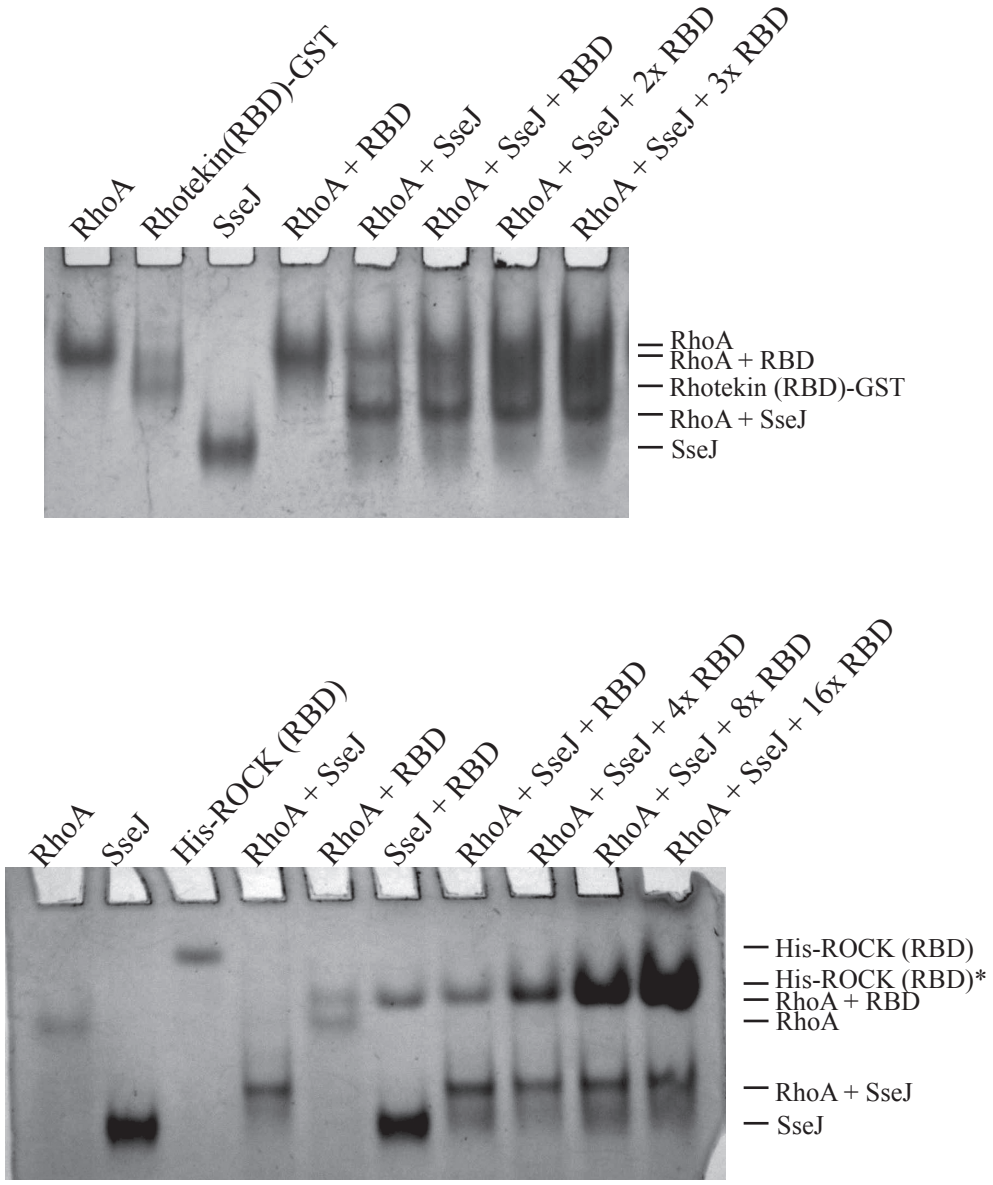

Figure 13. SseJ competes with eukaryotic RhoA activated proteins, Rhotekin and ROCK for binding to RhoA. (Top) The interaction and ability of SseJ to compete with Rhotekin for binding to RhoA was determined by native polyacrylamide gel electrophoresis and Coomassie staining. (Bottom) The interaction and ability of SseJ to compete with ROCK for binding to RhoA was determined by native gel and Coomassie staining. ROCK-RBD was altered by SseJ alone and is shown as (His-ROCK (RBD)*).

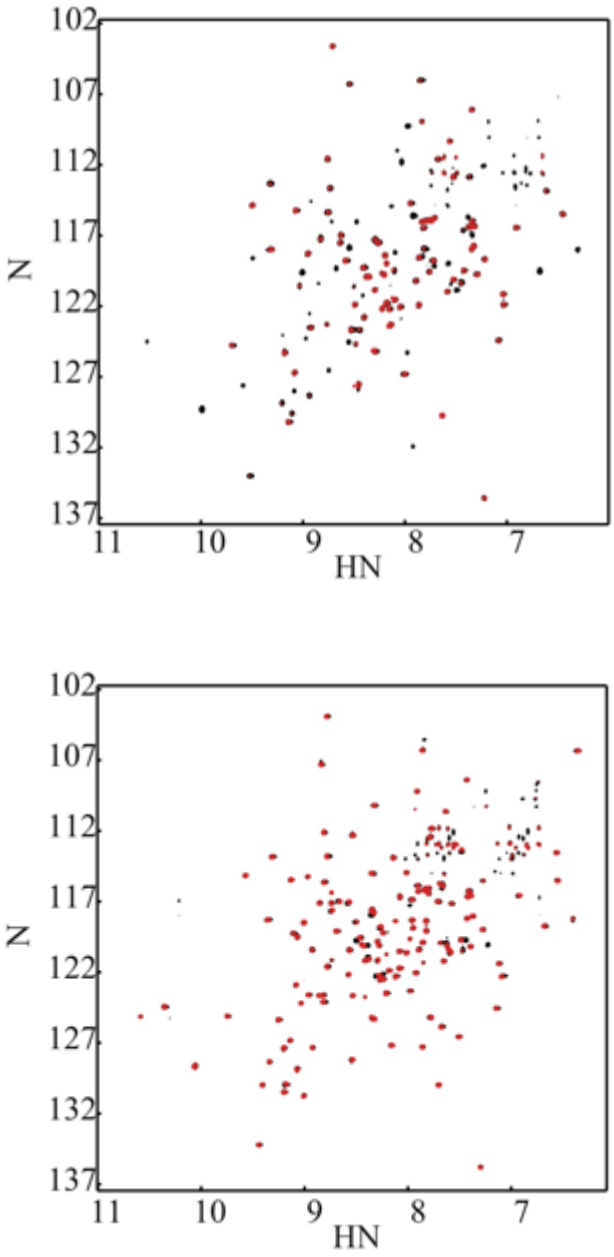

Figure 14. SseJ perturbs resonances in the NMR spectra of $RhoA_{1-181}$ bound to GDP and GTPγS. Overlay of ^{1}H ^{15}N-RhoA of (Top) 300 μM ^{1}H ^{15}N-RhoA-GTPγS in the absence (black) and presence (red) of 75 μM SseJ. (Bottom) 300 μM ^{1}H ^{15}N-RhoA-GDP in the absence (black) and presence (red) of 75 μM SseJ.

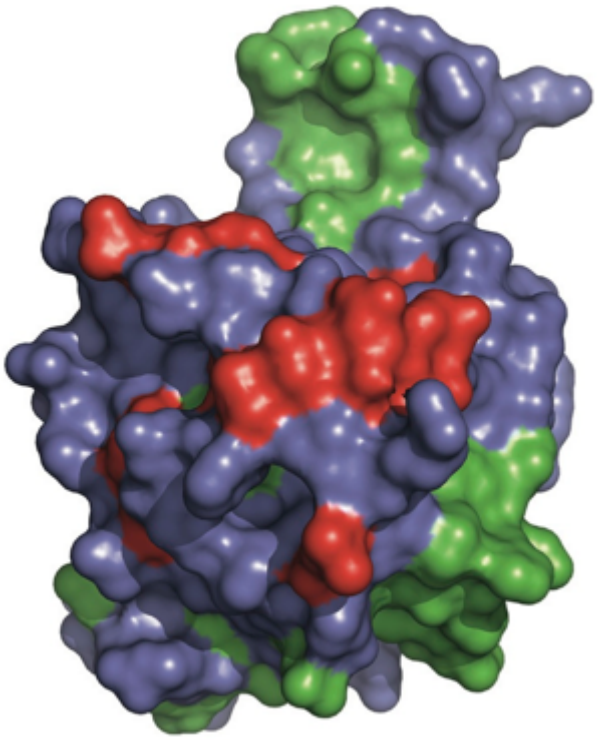

Figure 15. RhoA NMR reveals residues perturbed upon binding SseJ. SseJ changes chemical environments of a subset of RhoA-GDP resonances, the resonances of ^{1}H ^{15}N-labeled RhoA-GDP residues that broaden (red) or shift (green) upon addition of SseJ are mapped onto the crystal structure of RhoA-GTPγS (PDB: 1A2B).

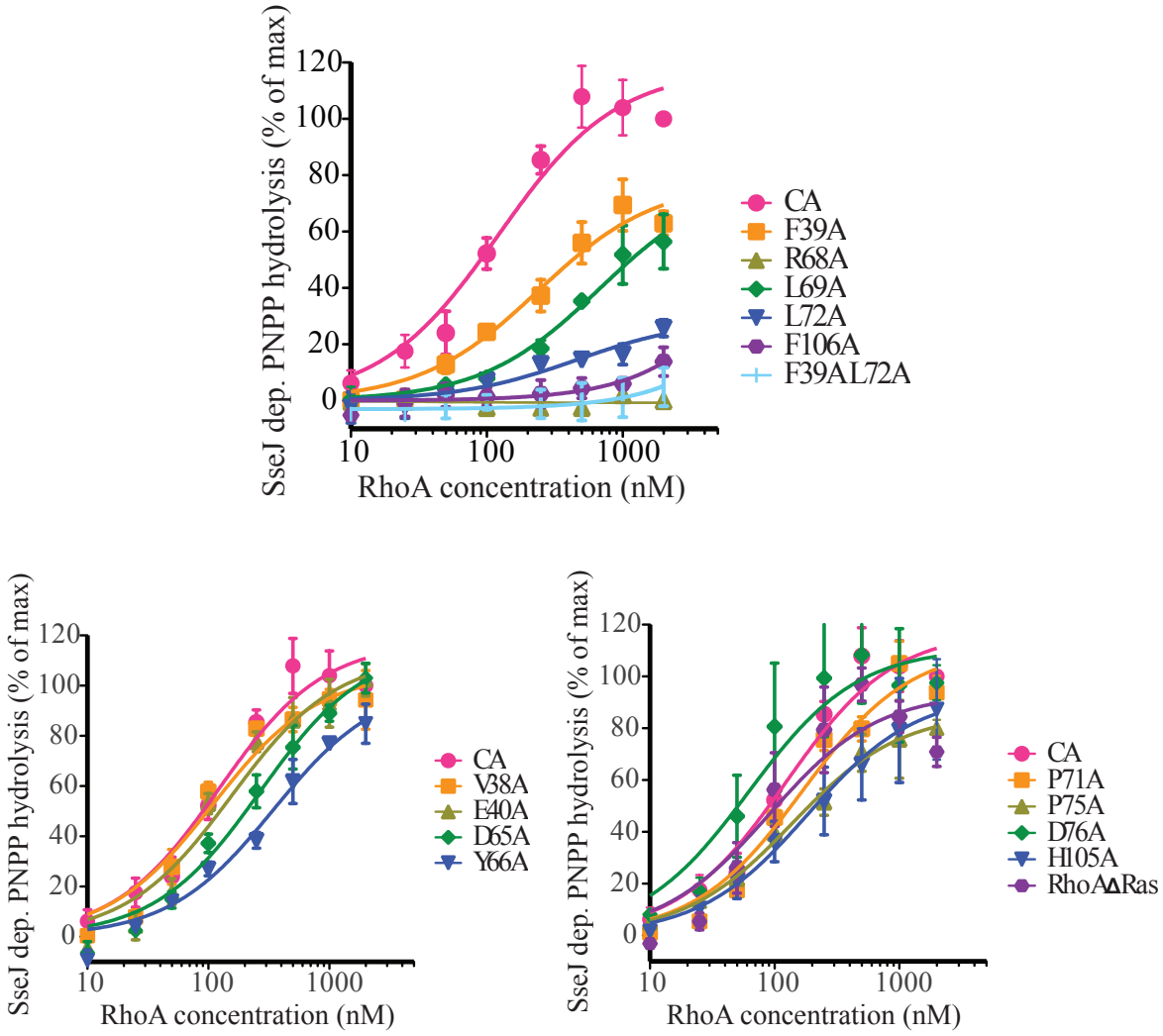

Figure 16. SseJ activation is mediated by the RhoA switch regions. (Top) Lipase assays in the presence of 250 nM SseJ and increasing concentrations of CA His-RhoA, CA His-RhoA^{F39A}, CA His-RhoA^{R68A}, CA His-RhoA^{L69A}, CA His-RhoA^{L72A}, CA His-RhoA^{F106A}, or CA His-RhoA$^{F39A\ L72A}$ demonstrate that these residues are important for activation of SseJ. (Bottom) Lipase assays in the presence of 250 nM SseJ and increasing concentrations of CA His-RhoA, CA (left) His-RhoA^{V38A}, CA His-RhoA^{E40A}, CA His-RhoA^{D65A}, CA His-RhoA^{Y66A}, and (right) CA His-RhoA^{P71A}, CA His-RhoA^{D76A}, CA His-RhoA^{H105A}, or CA His-RhoAΔRas demonstrate that these residues are not important for activation of SseJ.

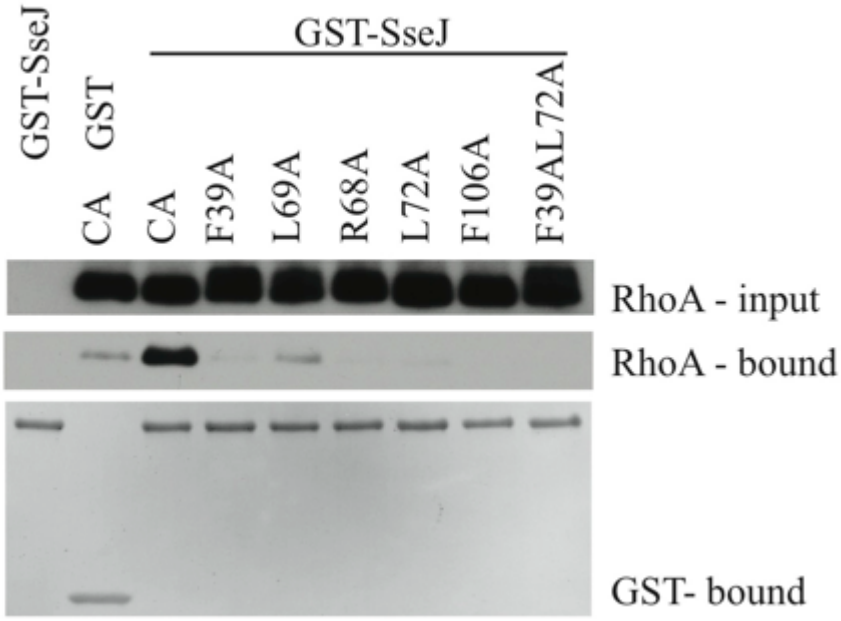

Figure 17. SseJ binds the RhoA switch regions. Purified GST-SseJ and CA His-RhoA, CA His-RhoA^{F39A}, CA His-RhoA^{R68A}, CA His-RhoA^{L69A}, CA His-RhoA^{L72A}, CA His-RhoA^{F106A}, or CA His-RhoA$^{F39A\ L72A}$ were mixed with glutathione beads and each input and bound sample was blotted with nickel-HRP for the CA His-RhoA and mutants. The bound samples were also evaluated for GST-SseJ by staining of the membrane with Ponceau S.

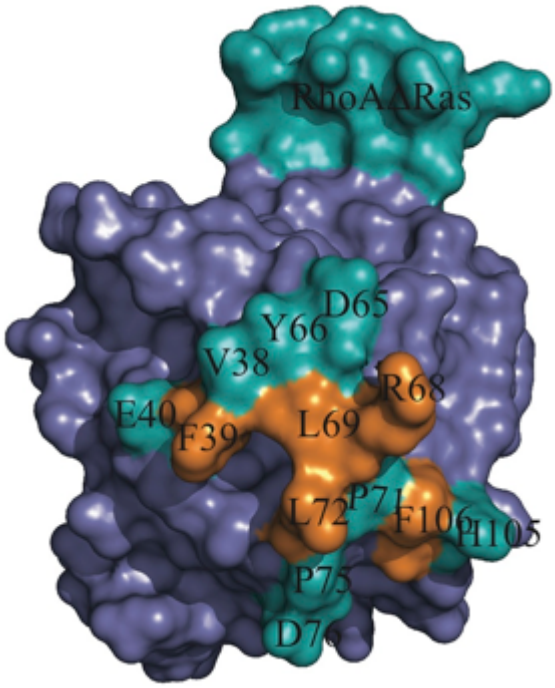

Figure 18. SseJ activation and binding are mediated by the RhoA switch regions. Residues that are important for RhoA activation of and binding to SseJ are highlighted on the crystal structure of RhoA-GTPγS in orange (PDB: 1A2B). Residues evaluated but not involved in activation of SseJ are highlighted in teal.

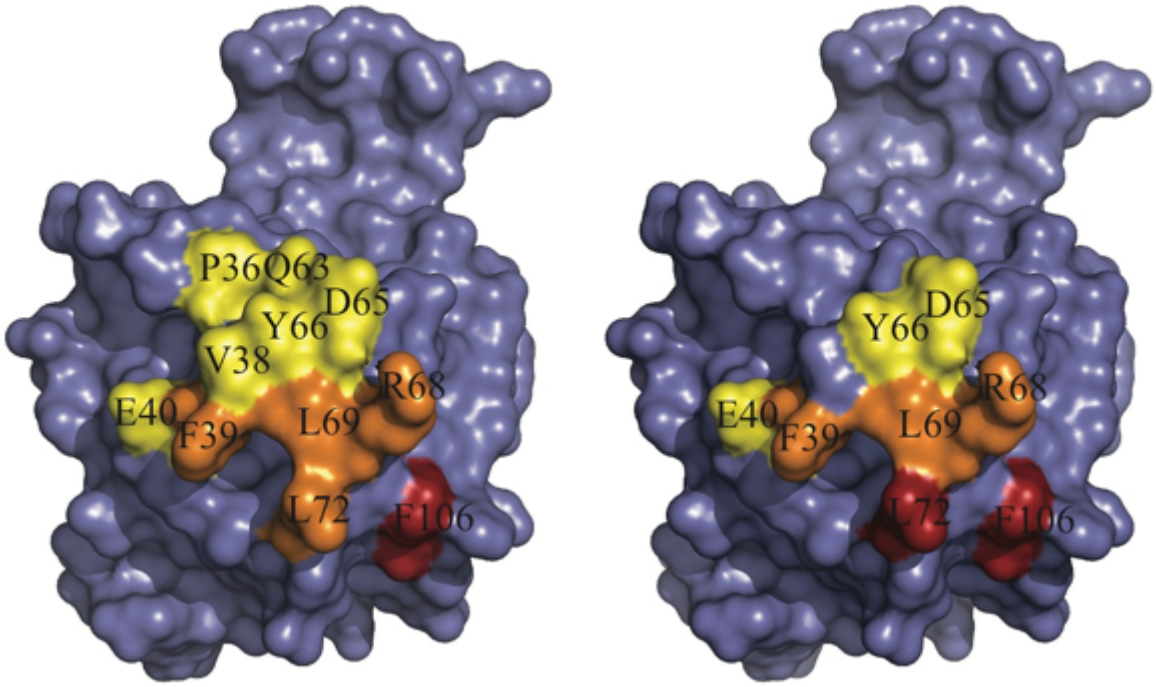

Figure 19. SseJ, ROCK, and PKN1 have partially overlapping contact surfaces on RhoA. Residues involved in RhoA binding to ROCK (left) or PKN1 (right) are highlighted in yellow, residues involved in SseJ binding are highlighted in red, and residues involved in binding both ROCK and SseJ (left) or PKN1 and SseJ (right) are highlighted in orange on the RhoA-GTPγS crystal structure (PDB: 1A2B).

CHAPTER 5. SSEJ ACTIVATION BY RHOA IS REQUIRED FOR VIRULENCE

Results

SseJ localization to endosomal compartments is dependent upon binding RhoA

Six *sseJ* mutant genes predicted to synthesize proteins with altered RhoA binding surfaces were generated based upon information regarding the SseJ-RhoA binding surface provided by Dr. Jijie Chai. SseJ alone localizes to and alters LAMP-1-positive membranous compartments and recruits RhoA to this location upon transfection (174, 191). These *sseJ* mutants were screened for the ability to localize to and alter LAMP-1 positive endosomal compartments by expression of these genes in HeLa cells. HeLa cells were transiently transfected with epitope-tagged SseJ and CA RhoA, and SseJ mutants monitored for colocalization and recruitment of CA RhoA and for their ability to cleave PEDA1. Five of the SseJ mutants tested ($SseJ^{Q172L}$, $SseJ^{R177A}$, $SseJ^{D368L}$, $SseJ^{I373D}$, $SseJ^{Q387L}$) demonstrated localization to membranous compartments and co-localization with CA RhoA (Figure 20). $SseJ^{F121D}$ did not recruit CA RhoA and was diffuse within cells (Figure 20). $SseJ^{R177A}$, which recruited RhoA, and $SseJ^{F121D}$ that was diffuse in cells were purified and tested for binding to RhoA and activation by RhoA. At 250 nM there was no activation of deacylase activity for either $SseJ^{R177A}$ or $SseJ^{F121D}$ in the presence of RhoA-GTPγS. Pull-down experiments using purified GST-RhoA and His-tagged $SseJ^{R177A}$ or $SseJ^{F121D}$ indicate that $SseJ^{R177A}$ binds to RhoA, whereas $SseJ^{F121D}$ does not. This result suggests SseJ must bind to RhoA in order to localize to membranes in addition to the requirement of RhoA for enzymatic activity.

SseJ mutants localized to the endosomal compartment, as HeLa cells transfected with epitope-tagged SseJ colocalized with LAMP-1, except the $SseJ^{F121D}$ mutant that

demonstrated a diffuse localization pattern (Figure 21). Each of the five SseJ mutants that localized to membranous compartments with CA RhoA also co-localized with LAMP-1, but visually did not appear to modify the LAMP-1-positive compartment like wild-type SseJ. The SseJ catalytic mutant ($SseJ^{3x}$), localized to LAMP-1 similarly to $SseJ^{R177A}$. These data were consistent with the requirement of SseJ activity for modification of the endosomal compartment (46, 191). Each mutant was examined for stability in transfection by Western blot of myc-tagged SseJ, the one SseJ mutant ($SseJ^{F182D}$) that was unstable was not included in any further analyses (Figure 22 top). Each of the six SseJ mutants was purified and their CD spectra were compared to WT and the catalytic mutant (3x) to demonstrate no changes to the global secondary structure due to these point mutations (Figure 22 bottom). The mutations generated were all within loops and on the surface of the SseJ structure, and were unlikely to have had an effect on protein folding. Additional mutations in SseJ (N128A, K133A, F174A, E186A, H192A, Q370A, L371A, H391A) were also tested and have no effect on binding of RhoA, or activation by RhoA. These data demonstrate that SseJ colocalization with LAMP-1 is dependent on the ability of SseJ to bind RhoA within cells.

SseJ binding to RhoA is important for phospholipase A1 activity in HeLa cells

Incubation of HeLa cells transfected with SseJ with PEDA1 results in the formation of fluorescent cholesterol dependent upon SseJ catalytic activity (46). The six SseJ mutants were transiently transfected into HeLa cells and PEDA1 was added for two hours to visualize SseJ activity by fluorescence microscopy. Cells transfected with SseJ were able to cleave PEDA1 measured as a statistically significantly increase in fluorescence above levels observed for untransfected cells as previously demonstrated (Figure 23) (47). In contrast, no

increase in fluorescence was detected for the *sseJ* mutants tested, indicating that they must alter SseJ activation by RhoA (Figure 23). This section demonstrates that alteration of the SseJ binding interface with RhoA can alter SseJ enzymatic activity.

SseJ activation by RhoA is important for *Salmonella* survival in mice

To verify that these proteins are expressed and translocated by *S.* Typhimurium, HeLa cells were infected with a Δ*sseJ* mutant expressing SseJ^{F121D}-HA and SseJ^{R177A}-HA as previously examined for SseJ-HA (173). SseJ^{F121D}-HA was diffusely localized within HeLa cells when delivered by *S.* Typhimurium, whereas SseJ^{R177A}-HA was localized to LAMP-1 positive Sifs (Figure 24).

To determine whether SseJ binding and activation by RhoA is required for *S.* Typhimurium virulence in mice we tested the virulence phenotype by competitive index of strains chromosomally expressing SseJ-HA, the catalytic mutant (SseJ^{3x}-HA), SseJ^{F121D}-HA which cannot bind RhoA, and SseJ^{R177A}-HA which can bind RhoA but has no detectable activity in cells. To verify that the plasmid-based antibiotic resistance markers did not influence the competitive index ratios, the chromosomally expressed wild-type SseJ-HA strain containing either a Kan^{R} (pWSK129) or Amp^{R} (pWSK29) marker were competed and found to compete equally (0.90 ± 0.14) (Figure 25). SseJ^{3x}-HA, a catalytic inactive mutant, expressed on the chromosome competed against wild-type (SseJ-HA) exhibits a competitive index defect of 0.38 ± 0.18, which is comparable to previous competition results from Δ*sseJ* strains expressing SseJ^{3x} on a plasmid competed against wild-type (0.44 ± 0.06) (Figure 25) (173). When wild-type (SseJ-HA) was competed against the strain expressing either SseJ^{F121D}-HA or SseJ^{R177A}-HA phenotypes of 0.42 ± 0.19, and 0.36 ± 0.15 were observed

(Figure 25). These competitive indices are not significantly different from the wild-type (SseJ-HA) competed against a strain expressing catalytically inactive SseJ^{3X}-HA. These results demonstrate that SseJ activation by RhoA is required for systemic virulence in mice, and that specific interactions at the SseJ binding surface with RhoA is necessary but not sufficient for full virulence.

Discussion

This chapter examined SseJ residues in the SseJ-RhoA interface originally identified by the crystal structure of the SseJ-RhoA complex. We used SseJ mutants in the binding interface with RhoA to reveal that a specific interaction between SseJ and RhoA is required for enzymatic activation of SseJ GCAT activity and systemic virulence for mice. Furthermore, simple targeting of RhoA to the endosome away from its normal functions, does not by itself complement the virulence defect observed in infection of susceptible mice. Instead, it appears that only RhoA activation of SseJ GCAT activity is required for SseJ contribution to systemic virulence.

SseJ localization to the endosomal compartment was previously demonstrated to be independent of enzymatic activity and was presumed to be independent of binding RhoA, since expression of SseJ within host cells results in recruitment of RhoA to the endosomal compartment. A mutation in the SseJ binding interface, SseJ^{F121D}, has allowed us to establish that SseJ binding to RhoA is required for SseJ localization to the endosomal compartment, since SseJ^{F121D} is diffusely localized upon transient expression within HeLa cells. This suggests that SseJ localization to the endosome is dependent on at least the nature of and possibly the formation of the SseJ-RhoA complex. Upon reflection, there is likely enough

endogenous RhoA present in HeLa cells to allow transfected SseJ to localize at the endosome. SseJ does not influence the ability of RhoA to cycle between the GDP and GTP bound states (47), so how SseJ remains on the endosome despite its relatively weaker interaction with GDP-bound RhoA remains an important question. Perhaps SseJ binding to RhoA-GDP in the cytosol, followed by the subsequent activation of RhoA by an endogenous GEF, and interaction with an endosomal protein moves this complex to the endosomal compartment. Once at the endosomal compartment the unusual localization of RhoA may be sufficient to prevent the normal cycling of GTP/GDP since endogenous GEFs and GAPs may not be present at this compartment. It is unknown how SseJ-RhoA localizes to the endosomal compartment, but identification of this mechanism is important to understand the complexity of SseJ regulation.

Since RhoA is not normally localized to the endosomal compartment, we speculate that an unidentified host factor is involved in recruitment of the complex to the endosomal vacuole. This factor is unlikely to be RhoB since RhoB is at best equivalent to RhoA in binding and activation of SseJ, and since RhoA is recruited to the endosome upon binding SseJ. Identification of the additional proteins that SseJ interacts will be important to address how SseJ localizes to the endosome. Our discovery suggests that SseJ activity in addition to being temporally regulated by the signaling state of RhoA is also spatially regulated, further indicating how very specific regulation of SseJ enzymatic activity is important for virulence.

If a SseJ mutant that is active in the absence of binding RhoA can be isolated, it would be useful to further our understanding of how the regulation of SseJ enzymatic activity is important to virulence. Unfortunately, the complex crystal structure of SseJ-RhoA did not provide insight into how RhoA activates SseJ. A structure of SseJ in the absence of RhoA,

and comparison to the activated form might reveal how activation of SseJ GCAT activity mechanistically occurs. In the complex crystal structure $SseJ^{R177}$ forms an intramolecular hydrogen bond with $SseJ^{E186}$, and since $SseJ^{R177A}$ still binds RhoA but cannot be activated by RhoA, perhaps this interaction is critical for SseJ activation. This possibility was especially intriguing given the close proximity of the active site residues to $SseJ^{R177}$. Additional mutational analysis including $SseJ^{E186A}$ had no effect on binding or activation of SseJ. We attempted to address how SseJ is activated using protein NMR. Our rational was to compare an SseJ CT NMR spectrum with same protein in the presence of RhoA to identify residues that change conformation and therefore might be involved in activation. We purified ^{15}N-labeled SseJ CT and assessed the 2D NMR spectrum. Unfortunately, the SseJ CT spectrum was not well dispersed and consequently we could not use it to identify the residues involved in activation. Therefore, insight into the mechanism by which SseJ is activated by RhoA remains elusive.

A number of lipase structures in the substrate-free and bound states reveals that activation occurs by the opening of a lid, which initially covers the active site residues and prevents substrate binding (25, 35, 57, 220, 230). The lid is opened at a lipid-water interface and this phenomenon is known as interfacial activation. SseJ enzymatic activity occurs in solution, suggesting that it does not undergo the interfacial activation process. In the SseJ-RhoA crystal structure, there is a hydrophobic pocket that may cover up SseJ catalytic residues when SseJ is not bound to RhoA, and this may represent a lid-like structure that is seen for many lipases. Instead of lid opening at a lipid-water interface, SseJ's lid may have evolved to open upon binding RhoA.

We demonstrate a similar virulence defect in mice for *S.* Typhimurium expressing catalytically inactive SseJ^{3x}, SseJ^{F121D}, or SseJ^{R177A}. These results show that specific SseJ binding to RhoA is necessary for virulence. However, binding is not sufficient for virulence, indicating that recruitment alone of RhoA by SseJ is not a virulence mechanism. These data were somewhat expected since RhoA is in excess of SseJ during *Salmonella* infection. Instead, sensing of the RhoA activation state by SseJ to regulate its GCAT activity at a specific time appears important for the endosomal maturation of the SCV, which is important for virulence.

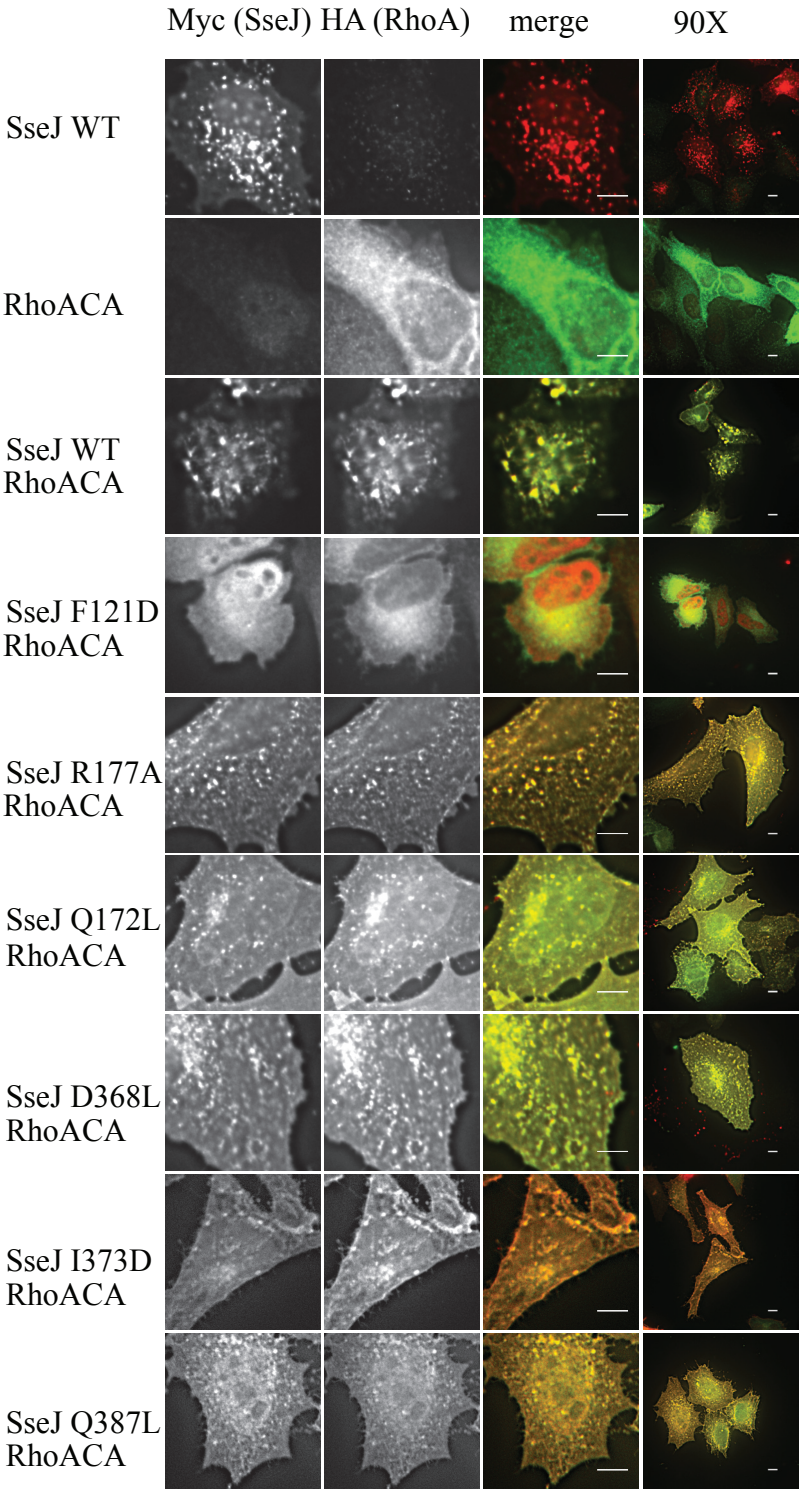

Figure 20. SseJ point mutant localization with CA RhoA. HeLa cells transfected for 24 hours with plasmids encoding myc-SseJ, myc-SseJ^{F121D}, myc-SseJ^{Q172L}, myc-SseJ^{R177A}, myc-SseJ^{D386L}, myc-SseJ^{I373D}, or myc-SseJ^{Q387L} and CA HA-RhoA were stained with anti-myc (red) and anti-HA (green) antibodies. Scale bar, 5 μM.

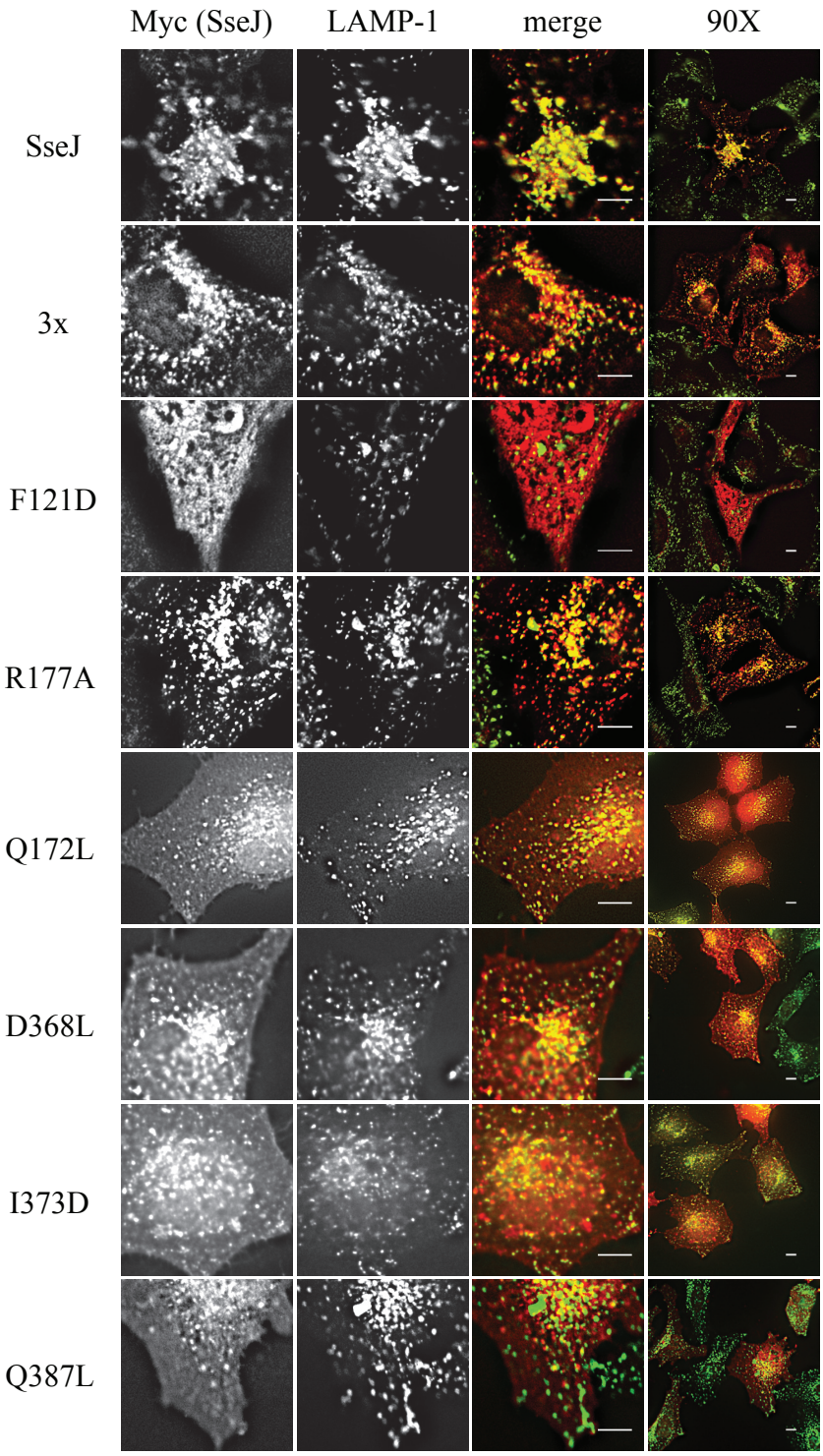

Figure 21. SseJ point mutants that bind RhoA localize to LAMP-1. HeLa cells transfected for 24 hours with plasmids encoding myc-SseJ, myc-SseJ^{3x}, myc-SseJ^{F121D}, myc-SseJ^{Q172L}, myc-SseJ^{R177A}, myc-SseJ^{D386L}, myc-SseJ^{I373D}, or myc-SseJ^{Q387L} and CA HA-RhoA were stained with anti-myc (red) and anti-LAMP-1 (green) antibodies. Scale bar, 5 μM.

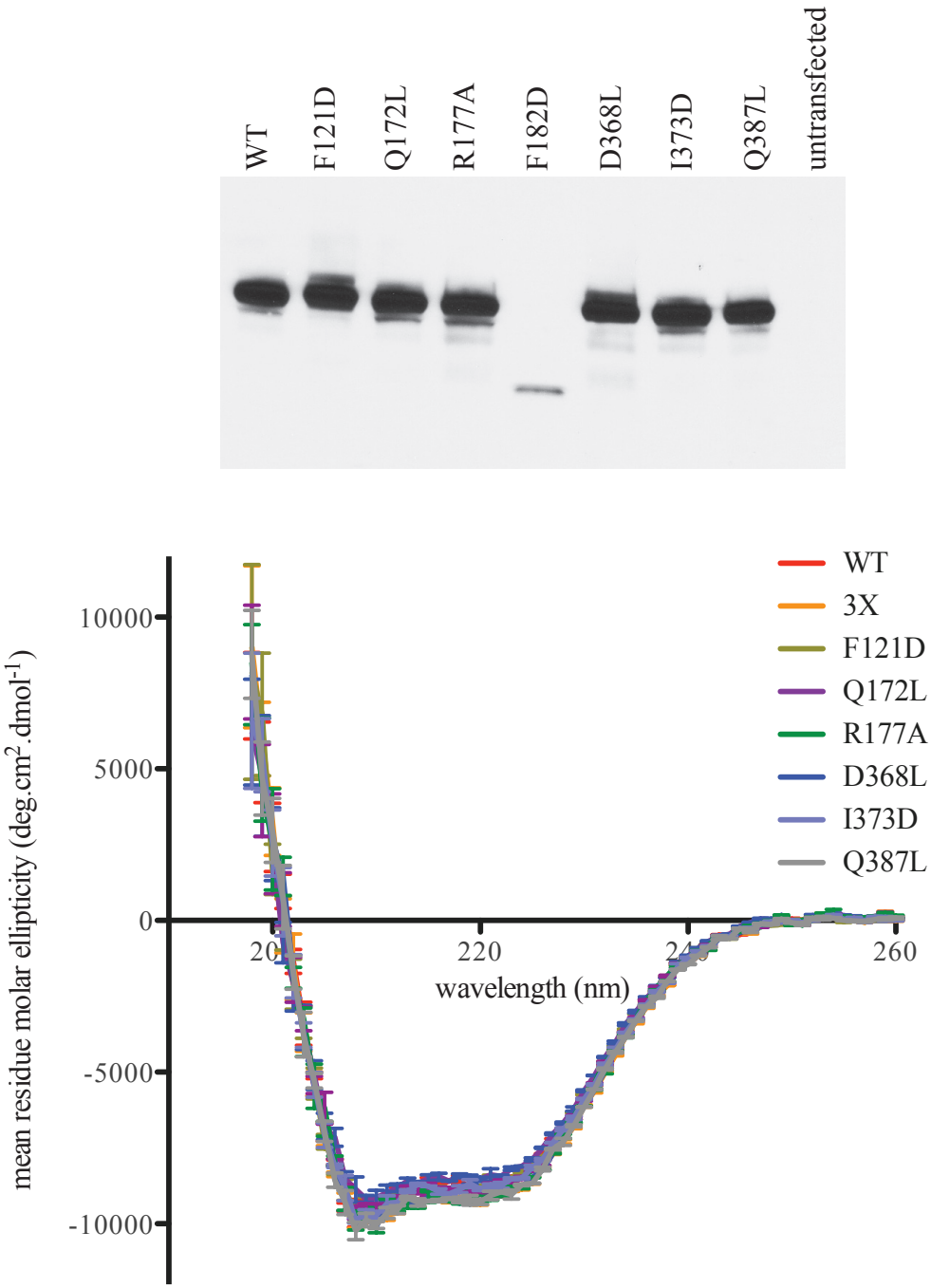

Figure 22. SseJ point mutants are stable in transfection and properly folded. (Top) HeLa cells were transfected for 24 hours with plasmids encoding myc-SseJ, myc-SseJ^{F121D}, myc-SseJ^{Q172L}, myc-SseJ^{R177A}, myc-SseJ^{F182D}, myc-SseJ^{D386L}, myc-SseJ^{I373D}, or myc-SseJ^{Q387L} and were blotted using anti-myc antibodies to detect SseJ constructs. SseJ^{F182D} was unstable and not included in other analyses. (Bottom) SseJ point mutants were purified and folding compared to WT SseJ by CD spectroscopy. Mean residue molar ellipticity and errors measured at 25°C are plotted for each protein.

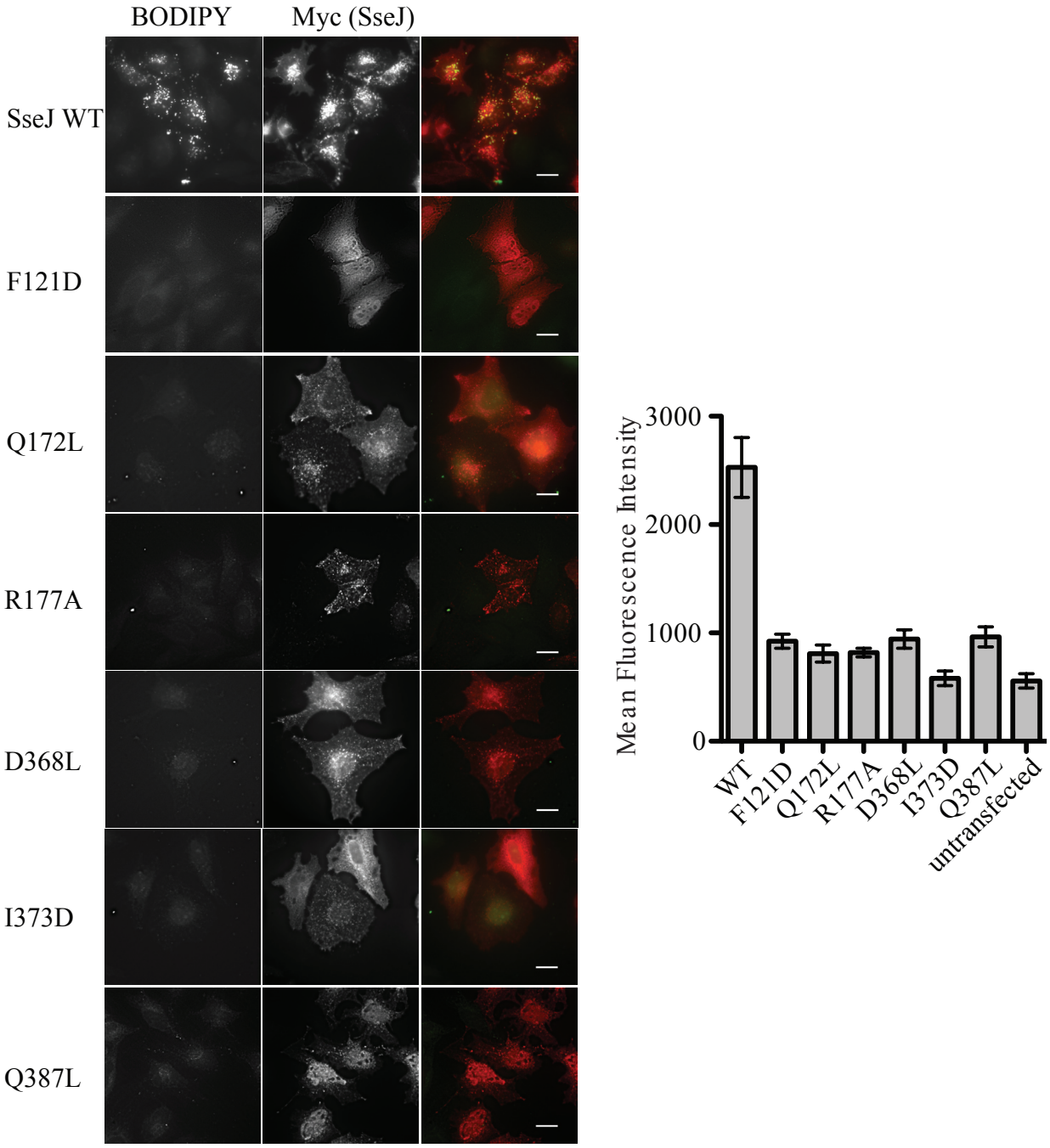

Figure 23. SseJ activation by RhoA is essential for phospholipase activity within HeLa cells. (Left) HeLa cells were transfected with a plasmid encoding myc-SseJ, myc-SseJ^{F121D}, myc-SseJ^{Q172L}, myc-SseJ^{R177A}, myc-SseJ^{D368L}, myc-SseJ^{I373D} or myc-SseJ^{Q387L} for 22 hours and then incubated for 2 hours with 3.5 μM PEDA1 (green) and stained with anti-myc (red) antibodies. Scale bar, 20 μM. (Right) Mean intensity of BODIPY fluorescence was measured using the NIS-Elements image analysis software for 10 fields. Statistical analysis (one way ANOVA followed by Dunnett's multiple comparison) demonstrate that transfection of wild-type but not each of the SseJ mutants tested resulted in a significant increase in cellular BODIPY fluorescence compared to untransfected cells ($p < 0.0001$).

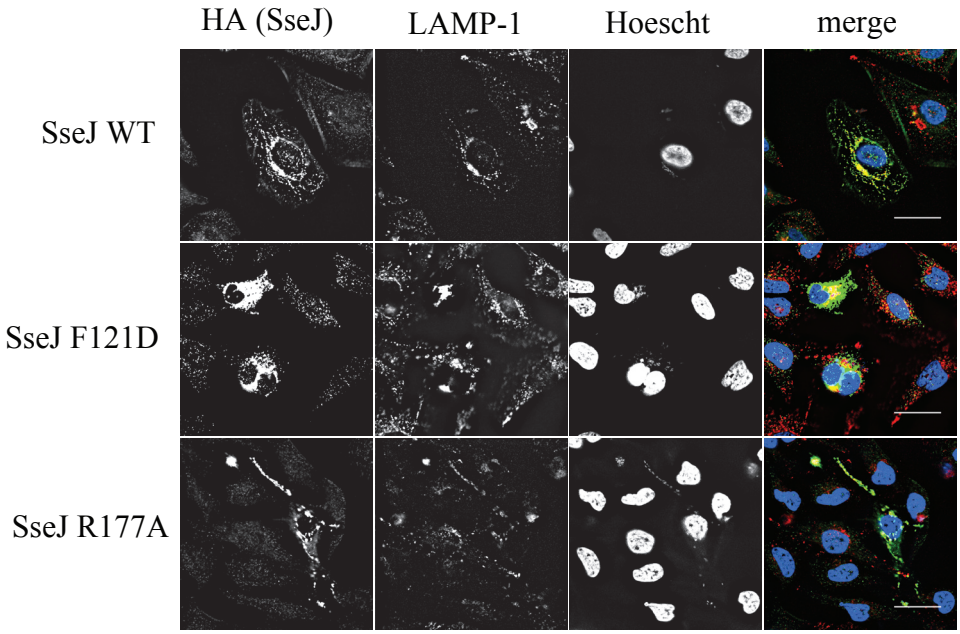

Figure 24. SseJ point mutants are translocated into HeLa cells during infection. HeLa cells were infected for 15 hours with an *sseJ* mutant strain of *S.* Typhimurium expressing p*sseJ*-HA or p*sseJ*^{F121D}-HA, or p*sseJ*^{R177A}-HA and were immunostained with antibodies against SseJ-HA (green), LAMP-1 (red), Hoechst 33342 nuclear stain (blue). Scale bar, 20 μM.

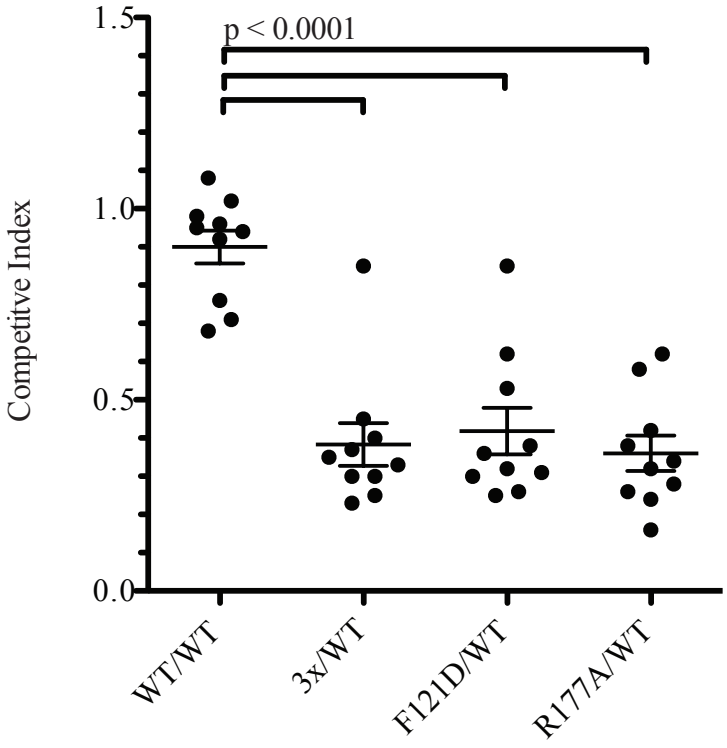

Figure 25. SseJ activation by RhoA is required for virulence. Female BALB/c mice were inoculated intraperitoneally with a 1:1 mixture of $5x10^4$ *S.* Typhimurium expressing (strain 1) wild-type SseJ with pWSK129 (Kan) competed against (strain 2) wild-type SseJ, the catalytic mutant $SseJ^{3x}$, $SseJ^{F121D}$ mutant, or $SseJ^{R177A}$ mutant with pWSK29 (Amp^{50}). The competitive index (CI) was calculated by dividing the ratio of the strain 2 to the strain 1 in the output by the ratio of strain 2 to strain 1 in the input. The graph demonstrates the CI within the spleen of each individual mouse (10 per competition) and denotes the mean and standard deviation per group. Statistical analysis by one way ANOVA followed by Dunnett's multiple comparison demonstrates that the CI value for the wild-type against wild-type competition is significantly different from the values derived for the competition between wild-type against the catalytic mutant $SseJ^{3x}$, $SseJ^{F121D}$ mutant, or $SseJ^{R177A}$ mutant.

CHAPTER 6. CONCLUSIONS AND FUTURE PERSPECTIVES

Conclusions

The goal of this study was to determine structurally how SseJ interacts with RhoA. This work demonstrated that SseJ specifically interacts with the small GTPases RhoA, RhoB, and RhoC at the switch regions. This recognition of the switch regions is similar to the region that eukaryotic Rho binding proteins recognize, and in line with this, SseJ can compete with eukaryotic effectors for binding to RhoA. This study identified the specific RhoA residues important for SseJ activation, and highlights that the majority of these residues are similar to ones used by eukaryotic effectors. SseJ utilizes its catalytic carboxyl domain to bind RhoA, and this domain is sufficient for the localization of SseJ to the endosome in cells. Although the crystal structure of the SseJ-RhoA complex has been solved, the mechanism by which SseJ becomes activated by RhoA is still not clear. Eukaryotic effectors bind RhoA with an autoinhibitory domain, that when not bound to RhoA blocks effector function, in contrast it appears that SseJ does not use a similar mechanism for activation.

This work demonstrated that SseJ must bind RhoA in order to localize to the endocytic compartment since an SseJ mutant that could not bind RhoA was distributed diffusely in HeLa cells. This suggests that there is an endosomal protein that recognizes the SseJ-RhoA complex and localizes it to the endosomal compartment. This study showed that activation of SseJ enzymatic activity is important to virulence, and the recruitment of RhoA away from endogenous functions is not important to virulence, since the SseJ mutant that cannot be activated by RhoA, or the SseJ mutant that does not bind RhoA, have virulence defects in mice similar to when the catalytic activity of SseJ is abolished. This study has

used structural and biochemical studies to understand SseJ interaction with RhoA, but there remain many unresolved questions. This chapter will address some of these questions, with the overarching goal of understanding how SseJ enzymatic activity contributes to virulence.

How is SseJ activated by RhoA?

The mechanism of SseJ activation by RhoA is not currently known. The co-crystal structure of SseJ-RhoA provided us with a snapshot of SseJ in what is most likely its active conformation, although additional changes to the structure may occur upon binding of either phospholipid or cholesterol substrates. Unlike most eukaryotic effectors of RhoA, SseJ does not use a coiled-coil motif Rho binding domain. Instead, short alpha-helices and loops of SseJ interact with RhoA over the RhoA switch regions. Interestingly, the catalytic residues of SseJ are deep within a pocket that is lined with hydrophobic residues. It is tempting to speculate that when SseJ is not bound to RhoA, the catalytic residues are unable to bind substrates because the hydrophobic pocket is blocked. If this is the case, mutations of these hydrophobic residues surrounding the catalytic site that abolish the hydrophobic interactions might generate a constitutively active SseJ. A number of SseJ mutations deleting the neighboring loops that interact with RhoA had been tested, but none generated a constitutively active SseJ. The hydrophobic pocket could alternatively be used by SseJ to bind the acyl chain substrate, and may not be important for activation. A structure of SseJ-RhoA bound to a lipid, or a structure of SseJ alone may allow us to dissect how these hydrophobic residues are important, and may give us insight into the mechanism that SseJ activation by RhoA occurs.

What mammalian cell factor localizes SseJ-RhoA to the endosomal compartment?

Intriguingly, data shown in chapter 5 demonstrated that SseJ must be able to bind RhoA in order for SseJ to localize to the endosomal compartment. This suggests that at minimum, SseJ must transiently interact with RhoA in the cytoplasm to localize to the endosome. Additionally, it suggests that another mammalian factor is responsible for recognizing the SseJ-RhoA complex and for trafficking these proteins to the endosome. This factor is most likely a mammalian membrane protein that is normally targeted to the endosomal compartment. We are currently attempting to identify this protein by yeast two-hybrid screen with the SseJ-RhoA complex against a mammalian and yeast library. Since the protein is most likely a membrane protein, an alternative method to identify this factor may be necessary, and could include mass spectrometry identification of immunoprecipitated proteins from mammalian membranes. Identification of the factor that localizes SseJ-RhoA to the endosome is important to contribute to the understanding of how effector proteins are localized to specific mammalian compartments, since this is not a well-studied phenomenon for any effector protein.

What are the intracellular substrates of SseJ?

SseJ is a GCAT enzyme that specifically converts cholesterol to cholesterol ester using an acyl chain donated from a phospholipid both in vitro and in cells (47, 145, 170). *Salmonella* infection results in the recruitment of at least 30% of total cellular cholesterol to the SCV (42). Our unpublished observations demonstrate that the recruitment of cholesterol is dependent on SseJ enzymatic activity. The presence of cholesterol in membranes affects cellular processes and can decrease membrane fluidity and reduce the permeability of polar

molecules (201). Additionally, cholesterol plays a key role in lipid raft function and assembly. Lipid rafts function to separate and concentrate membrane proteins in specific regions, and they are important for sorting lipids and proteins to different membranes, where they ultimately play important roles in signal transduction pathways (200). The accumulation of a raft lipid like cholesterol can lead to accumulation of other raft lipids in endosomes/lysosomes. The endosomal compartment is typically low in raft lipids like cholesterol, and the accumulation of raft lipids leads to the formation of abnormal endosomes that disturb membrane transport (143, 196, 199). It is plausible that *Salmonella* recruitment of cholesterol to the endosomal compartment, which is dependent upon SseJ activity, affects membrane and protein trafficking.

Previous studies have demonstrated that the accumulation of cholesterol ester and subsequent formation of lipid droplets in cells upon *Salmonella* infection is partially dependent on SseJ (170). These data suggest that cholesterol is a major substrate of SseJ, and that esterified cholesterol is the major product of SseJ enzymatic activity. Cholesterol esters are less water-soluble than cholesterol and tend to intercalate into lipid droplets rather than stay in membranes. Lipid droplets consist of a core of neutral lipids such as cholesterol esters, and are surrounded by a layer of phospholipids and associated proteins (149). Lipid droplet production can recruit and therefore alter the availability of different proteins involved in membrane trafficking, such as a number of Rab GTPases (26, 78, 144). A consequence of *Salmonella* increasing the production of cholesterol esters and lipid droplets may be the sequestration of proteins away from their normal functions in membrane regulation. The removal of cholesterol ester from the SCV may destabilize the membrane, and this may explain in part why SseJ destabilizes the SCV in the absence of SifA.

Altogether, recruitment and esterification of cholesterol appears to be important to *Salmonella*, since an *sseJ* mutant that is unable to esterify cholesterol is defective in virulence (173).

An additional substrate of SseJ is the phospholipid acyl chain donor. It is not known whether there is a specific phospholipid donor preferred by SseJ, either by virtue of a tighter interaction, or because of the phospholipid's availability in infection at the endosomal compartment. Most likely, similar to other GCATs, SseJ will show no preference for a particular glycerophospholipid in vitro. Phosphatidylserine is a negatively charged phospholipid that is normally found in the plasma membrane and in endosomes/lysosomes, and is important for the targeting of proteins with cationic charged regions such as Rac1 (71). Upon *Salmonella* infection, the levels of PS on the SCV early in infection are reduced dependent on SopB activity, and this reduction in PS affects the targeting of host cell proteins to the SCV (12). SseJ co-expressed in HeLa cells with a marker for PS demonstrates SseJ localization to PS, and SseJ modification of PS distribution inside cells (Figure 26). Additionally, at 18 hours post-infection *Salmonella* inside the SCV are surrounded by a fluorescent analog of phosphatidylserine that is exogenously added to HeLa cells, suggesting PS is available and could be used as a phospholipid substrate for SseJ (Figure 27). It will be interesting to determine whether SseJ binds to PS, and regulates this phospholipid's localization in infection.

One study assessed the lipid ratio in AC29 cells (CHO cell line lacking host acyl-coenzyme A:cholesterol transferase [ACAT]) infected with *Salmonella* expressing wild-type or Δ*sseJ*. These data did not show a significant decrease in any of the three major phospholipids, phosphatidylcholine (PC), phosphatidylserine (PS), or

phosphatidylethanolamine (PE), while showing a 20-fold increase in cholesterol ester (170). This information suggests that SseJ will have no preference for a particular phospholipid in cells, and that the cholesterol esterification activity and not the deacylase activity of SseJ is most important for virulence. When we assessed the ability of SseJ to use either PS (DOPS) or PC (DOPC) as a substrate in vitro, we found that SseJ could use both phospholipids (Figure 28). It is currently not known whether SseJ preferentially deacylates phospholipids carrying acyl groups with specific chain lengths or saturation states. Testing the specificity of SseJ for specific acyl groups in vitro would provide additional insight into the preferred phospholipid of SseJ in cells.

SseJ does not hydrolyze lysophospholipids (145), which suggests that SseJ converts phospholipids to lysophospholipids, and that lysophospholipids potentially accumulate on the SCV as a result of SseJ phospholipase activity. The presence of lysophospholipids in a single bilayer leaflet can increase membrane curvature simply because of the uneven distribution of cylindrical and cone shaped phospholipids compared to the inverted cone shaped lysophospholipids (124). Also, mammalian phospholipases have been implicated in brefeldin A (BFA)-stimulated endosomal and Golgi tubulation events (30, 54). These data imply that SseJ's phospholipase activity may be important for modifying the endosomal curvature and potentially for the formation of Sifs upon *Salmonella* infection. Examining SseJ's ability to modify the curvature of membranes in vitro may be one way to address to how alteration of phospholipid content affects membrane curvature. These experiments are technically challenging and my attempts to use an in vitro system to assay vesicle formation, membrane fission, and membrane tubulation has been so far unsuccessful (180).

What are the effects of SseJ modification of the endosomal compartment protein content?

Salmonella recruit cholesterol to the SCV in a manner dependent on the SPI-2 T3SS, and this alteration recruits the glycosylphosphatidyinositol (GPI)-anchored receptor CD55 to the SCV (42). GPI-anchored proteins are selectively retained in cholesterol-rich endocytic compartments (43), therefore it is likely that additional GPI-anchored proteins are recruited to the SCV upon infection. We examined the ability of SseJ to modify the localization of the microtubule motor kinesin in cells since this protein is regulated by a number of *Salmonella* effectors during infection. A subtle change in the ability of kinesin to extend to the cell periphery is seen when co-transfected with wild type but not catalytically inactive SseJ (Figure 29). These data suggest that SseJ enzymatic activity may alter the localization of kinesin. Interestingly, the overexpression of SseJ in mammalian cells antagonizes the ability of coexpressed SifA to interact with cholesterol-enriched vesicles and also to LAMP-1 positive endosomes (170). Although this effect is not seen in the context of infection, it does provide some insight into how SseJ enzymatic activity may function in cooperation with SifA. Overall, a large number of proteins may be altered as a result of SseJ enzymatic activity, and identifying each of them may not be possible until there are greater technological advances in comparing relative membrane protein contents within specific compartments.

Does SseJ interact with SifA through RhoA?

A summary of the evidence for an interaction of SseJ with SifA has been reviewed in the introduction. In addition to the intracellular phenotypic evidence for a link between these

SPI2 effectors there is biochemical evidence to suggest SseJ and SifA are linked through RhoA. Despite much speculative evidence of SseJ and SifA cooperation in infection, very little is actually known about the mechanism or proteins involved in their interaction. Assessment of proteins that possibly bind SifA, SseJ, or various complexes by yeast two-hybrid or coimmunoprecipitation could help identify the other participating proteins. It is still a possibility that SseJ requires RhoA activation by SifA for its enzymatic activity, and that a large protein complex is necessary for membrane modifications and tubulation to occur.

Currently, SseJ is one of the most well studied SPI-2 effector protein. Despite this there remain many unresolved questions regarding why it is important for *Salmonella* virulence. Understanding each of the SPI-2 effectors in isolation may provide a basic understanding of how *Salmonella* interacts with host cells and modifies cellular membrane dynamics. Ultimately, to fully understand the intricate interaction of *Salmonella* with mammalian cells it may be necessary to study the molecular mechanisms of the effectors collectively.

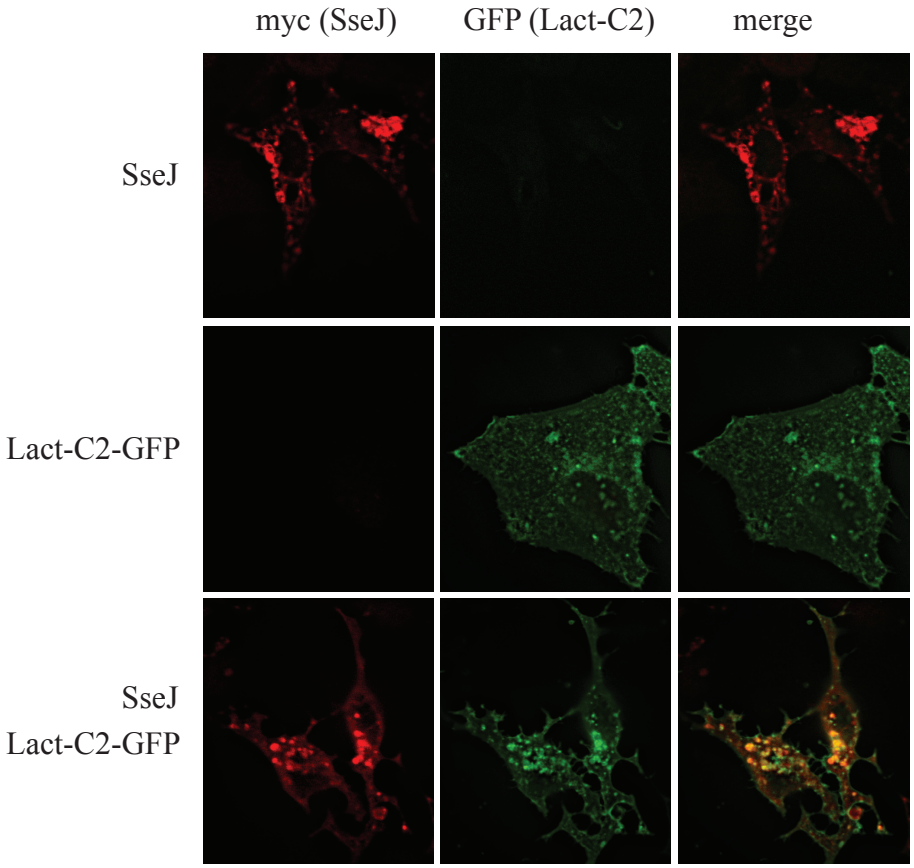

Figure 26. SseJ colocalizes with phosphatidylserine and alters phosphatidylserine localization in HeLa cells. SseJ and Lact-C2-GFP were transfected into HeLa cells for 24 hours and stained for SseJ (red). Lact-C2-GFP (green) contains the C2 domain of lactadherin, which tightly and specifically binds the phospholipid, phosphatidylserine.

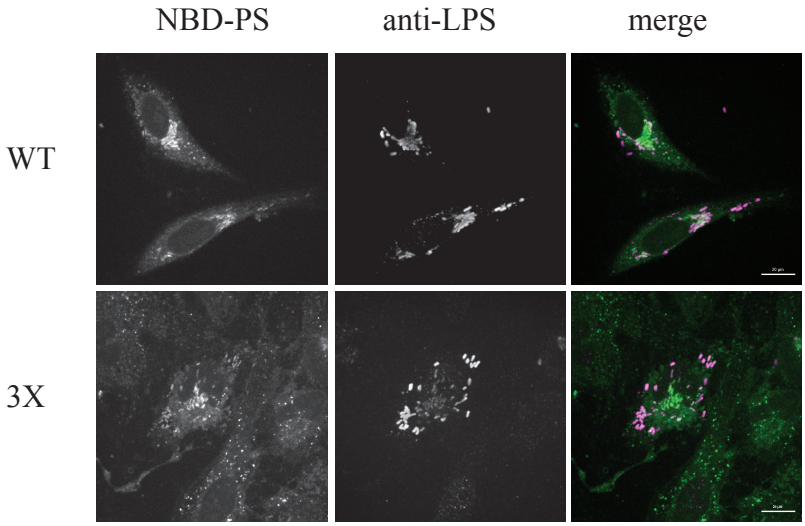

Figure 27. Fluorescently labeled phosphatidylserine surrounds *S.* Typhimurium at late stages of infection independent of SseJ enzymatic activity. HeLa cells were infected for 17.5 hours with *S.* Typhimurium expressing wild-type (WT) or catalytic mutant (3X) SseJ, 5 μM 18:1 NBD-PS (green) was added for 30 minutes and bacteria were visualized with anti-LPS antibodies (purple). Scale bar, 20 μM.

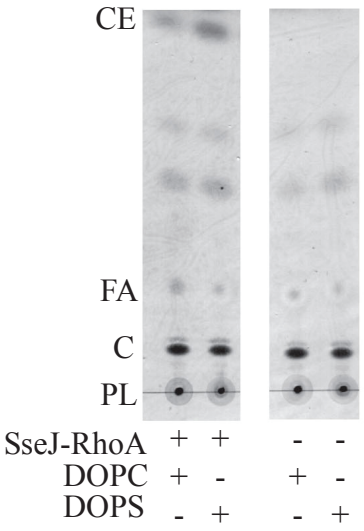

Figure 28. SseJ can use both phosphatidylcholine and phosphatidylserine as an acyl chain donor. Liposomes containing a 60:10:30 ratio of cholesterol: oleic acid: DOPC/DOPS were incubated for 30 minutes with SseJ-RhoA or no proteins and separated on TLC and stained with amido black.

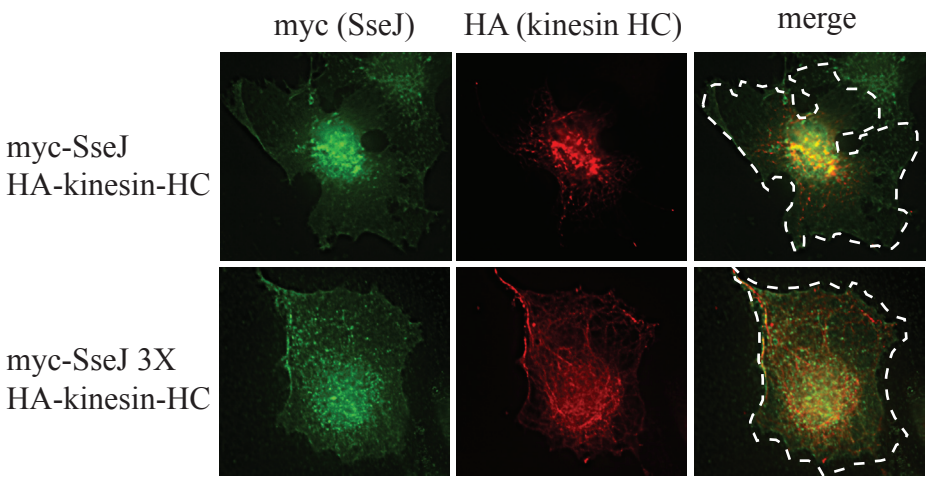

Figure 29. SseJ modifies the localization of kinesin in HeLa cells. HeLa cells were cotransfected with myc-SseJ and HA-kinesin-HC for 24 hours and stained for SseJ (green) or kinesin (red).

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VITAE

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