ROLE OF BACTERIAL EFFECTORS SOPD AND SOPB IN PATHOGENICITY OF
SALMONELLA ENTERICA SEROVAR TYPHIMURIUM

by

Malina A. Bakowski

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for the degree of Doctor of Philosophy
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University of Toronto

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ABSTRACT

Role of bacterial effectors SopD and SopB in pathogenicity of *Salmonella enterica* serovar *Typhimurium*. A thesis submitted by Malina A. Bakowski in the year of 2009 for the degree of Doctor of Philosophy in the Graduate Department of Molecular Genetics, University of Toronto.

*Salmonella enterica* serovar *Typhimurium* is a facultative intracellular pathogen that has evolved to take advantage of the eukaryotic host cells it inhabits during infection. It uses bacterial effectors translocated into the host cell cytosol to manipulate host cell machinery and establish a replicative niche. In this thesis I study the function of two of these effectors, SopD and SopB, which have been shown to act cooperatively to induce phenotypes associated with gastroenteritis (fluid secretion and neutrophil influx into the intestinal lumen).

In addition to promoting gastroenteritis, SopD has also been implicated in systemic and persistent infection of mice. Although recently implicated in invasion, the precise function of SopD has remained elusive. Here I show that SopD affects membrane dynamics during *S. Typhimurium* invasion of epithelial cells. SopD promotes membrane sealing and macropinosome formation, events that may have important consequences for efficiency of bacterial cell entry *in vivo*. Furthermore, we demonstrate that SopD is recruited to the invasion site membranes through the phosphatase activity of SopB, suggesting a mechanism for their cooperative action during induction of gastroenteritis.

Unlike SopD, SopB has been a focus of intense research efforts and its role in invasion as a phosphoinositide phosphatase is well documented. However, we have observed that SopB also inhibits fusion of lysosomes with *Salmonella*-containing vacuoles (SCVs) following invasion. This ability depends on SopB-mediated reduction of negative membrane charge of the SCV during invasion by hydrolysis of the phosphoinositide PI(4,5)P₂. Membrane charge alterations driven by SopB result in removal of Rab GTPases from the SCV that depend on electrostatic
interactions for their targeting. Two of these Rabs, Rab23 and Rab35 were previously shown to promote phagosome-lysosome fusion. Therefore their removal from the SCV may promote SCV trafficking away from the degradative endocytic pathway of host cells. This represents a new mechanism by which an invasion associated effector controls SCV maturation.

Together, this work advances our knowledge of the interaction between *S. Typhimurium* and its host. This research also suggests a new mechanism by which pathogens other than *S. Typhimurium* could promote their intracellular survival.
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LIST OF ABBREVIATIONS

Akt/PKB: protein kinase B
APC: antigen presenting cell
BAR: Bin/Amphiphysin/Rvs
CAMP: cationic antimicrobial peptide
CFP: cyan fluorescent protein
DAG: diacylglycerol
DC: dendritic cell
Dot/Icm: defect in organelle trafficking/intracellular multiplication
DPI: diphenyleneiodonium
DQ-BSA: self-quenching BODIPY TR-X dye conjugate of bovine serum albumin
EE: early endosome
EEA1: early endosomal autoantigen 1
EGFR: epidermal growth factor receptor
ESCRT:
  FcγR: Fcγ receptor
FITC: fluorescein isothiocyanate
FYVE: Fab1/YOTB/Vac1/EEA1
GAP: GTPase-activating protein
GDI: guanine nucleotide dissociation inhibitor
GEF: guanine nucleotide exchange factor
GFP: green fluorescent protein
h: hour(s)
HGF-R: hepatocyte growth factor receptor
Hrs: hepatocyte growth factor-regulated tyrosine kinase substrate
Incs: inclusion membrane proteins
InlA/B: internalin A and internalin B
iNOS: inducible nitric oxide synthase
Inv: Invasin
Ins(1,4,5)P3: inositol-1,4,5-trisphosphate
JNK: Janus kinase
LAMP: lysosome-associated membrane protein
LBPA: lysobisphosphatidic acid
LE: late endosome
LMW-PTP: low-molecular weight protein tyrosine phosphatase
LPS: lipopolysaccharide
M6PR: mannose-6-phosphate receptor
mDia: mammalian Diaphanous-related formin
MeOH: methanol
MHC-I: class I major histocompatibility complex
MHC-II: class II major histocompatibility complex
Min: minute(s)
MLC: myosin light chain
MOI: multiplicity of infection
mRFP: monomeric red fluorescent protein
MTOC: microtubule organizing centre
MVB: multivesicular body
NADPH: nicotinamide adenine dinucleotide phosphate
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NHS-647: Alexa Fluor 647 carboxylic acid, succinimidyl ester
NO: nitric oxide
NSF: N-ethylmaleimide-sensitive factor
p.i.: post infection
PA: phosphatidic acid
PAK1: p21-activated kinase
PAMP: pathogen-associated molecular pattern
PBS: phosphate-buffered saline
PC: phosphatidylcholine
PE: phosphatidylethanolamine
PFA: paraformaldehyde
PH: phox homology domain
PH: plextrin homology domain
PI(3)P: phosphatidylinositol-3-phosphate
PI(3,4,5)P$_3$: phosphatidylinositol-3,4,5-trisphosphate
PI(3,4)P$_2$: phosphatidylinositol-3,4-bisphosphate
PI(3,5)P$_2$: phosphatidylinositol-3,5-bisphosphate
PI(4)P: phosphatidylinositol-4-phosphate
PI(4,5)P$_2$: phosphatidylinositol-4,5-bisphosphate
PI(5)P: phosphatidylinositol-5-phosphate
PI: phosphoinositide
PI3-K: phosphatidylinositol 3-kinase
PIP5-K: PI(4)P 5-kinase
PKC: protein kinase C
PLC: phospholipase C
PLD: protein lipase D
PM-GFP: GFP fusion targeted to the plasma membrane by myristoylation and palmitoylation
PMN: polymorphonuclear leukocyte
PS: phosphatidylserine
PX: phox
Rab: Ras gene from rat brain
Rac: Ras-related C3 botulinum toxin substrate
Rho: Ras homologous
RILP: Rab interacting lysosomal protein
RNS: reactive nitrogen species
ROCK: Rho kinase
ROS: reactive oxygen species
SCV: Salmonella-containing vacuole
SGEF: SH3-containing guanine nucleotide exchange factor
Sif: Salmonella-induced filament
SigD: Salmonella invasion gene D
SKIP: SifA and kinesin-interacting protein
SNARE: soluble NSF attachment protein receptor
SNX: sorting nexin
SopB: Salmonella outer protein B
SopD: Salmonella outer protein D
SPI: Salmonella pathogenicity
SPI: Salmonella Pathogenicity Island
SPI-1/2 T3SS: Salmonella pathogenicity island 1/2 encoded type III secretion system
T3SS: type III secretion system
TACO: tryptophan aspartate-containing coat protein
TLR: Toll-like receptor
VAP: vacuole-associated actin polymerizations
vATPase: vacuolar ATPase
VSVG: vesicular stomatitis virus glycoprotein
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CHAPTER 1
INTRODUCTION

PHAGOCYTOSIS OF PATHOGENS

The Process of Phagocytosis

Phagocytosis is a specialized form of endocytosis. Other forms of endocytosis include clathrin-dependent and -independent endocytosis, caveolae-dependent endocytosis, and macropinocytosis. While other forms of endocytosis (with the exception of macropinocytosis) take up particles that are $< \sim 0.5 \, \mu m$ in diameter, phagocytosis is the process by which cells internalize and destroy larger foreign objects, such as apoptotic cell bodies and microorganisms.

Once particles bind to phagocytic receptors, a phagocytic cup is formed on the surface of the phagocyte. The sealing of the phagocytic cup around the particle forms the phagosome, which through a series of fusion events with early endosomes, late endosomes and lysosomes matures into a degradative compartment, which is very acidic, oxidizing, and contains an assortment of hydrolases. In professional phagocytes (mainly polymorphonuclear neutrophils, monocytes and macrophages), phagosome maturation results in efficient degradation and clearance of foreign particles (Nagl et al., 2002). Depending on the phagocytic cargo, maturation can be accompanied by antigen presentation and activation of inflammatory signaling by the phagocyte.

Phagocytic Receptors

Phagocytosis is a complex, highly orchestrated process involving signal transduction events, and organized movement of membranes and the actin cytoskeleton. It begins with a clustering of specific phagocytic receptors upon contact with their specific ligands. Phagocytic cells encounter a variety of particles and correspondingly, express a variety of phagocytic receptors (Figure 1-1). Many phagocytic receptors can
directly recognize unique components of the surface of microorganisms, known as pathogen associated molecular patterns (PAMPs) (Heine et al., 2005). Receptors participating in this non-opsonin type of phagocytosis include mannose receptors that bind mannyl and fucosyl residues found on surfaces of bacteria and fungi (Ezekowitz et al., 1990, Schlesinger, 1993). Phagocytic scavenger receptors also participate in non-opsonin type of phagocytosis and are quite promiscuous, recognizing many ligands including lipopolysaccharides (LPSs) and lipoproteins (Underhill et al., 2002). Other phagocytic receptors recognize host-produced ligands, called opsonins, which can attach to microbe surfaces. For example complement-coated microorganisms are recognized by integrin-like complement receptors and IgG antibody-opsonized pathogens are recognized by Fcγ receptors (FcγRs) (Underhill et al., 2002).

Cells respond differently to different phagocytic cargo and different signaling cascades are elicited during phagocytosis depending on the specific receptor-ligand pairs. For example Src and Syk kinases are activated during FcγR-mediated phagocytosis (Scott et al., 2003) but integrin family and mannose receptor-mediated phagocytosis can occur independently of Syk (Allen et al., 1996). Rac and Cdc42 are required for actin and Arp2/3 recruitment during FcγR-mediated phagocytosis but during complement receptor 3-mediated phagocytosis Rho and Rho kinase (ROCK) are needed instead (Olazabal et al., 2002). During phagocytosis a variety of Toll-like receptors (TLRs) found on the plasma and intracellular membranes of eukaryotic cells recognize specific microbial PAMPs and initiate signaling cascades that lead to an appropriate innate immune response, such as cytokine production (Heine et al., 2005). TLR signaling has also been found to be an important determinant of phagosome fate (Blander et al., 2004). Activation of TLR signaling during phagocytosis by bacteria but not apoptotic cells affects phagosome formation as well as maturation (Blander, 2008).

In addition to receptor-ligand pairs dictating signaling cascades during phagocytosis the size of phagocytosed particles also has an impact on the signals required for this process. Phagocytosis of particles that are >3 µm in diameter requires phosphatidylinositol 3-kinase (PI3-K) but phagocytosis of smaller particles does not (Cox et al., 1999, Araki et al., 1996). Finally, receptor ligation is not required for all forms of
Figure 1-1: Receptors and signaling during phagocytosis of microbes. Many receptors can recognize microbes through direct binding or binding to opsonins attached to the microbial surface. Different types of scavenger receptors, carbohydrate-binding lectins (e.g., mannose receptor), and toll-like receptors (TLRs) bind directly to the surface components of the microbe. Fc receptors bind to immunoglobulin opsonized microbes, integrin-like complement receptors bind to microbes coated with complement proteins, and other integrins can also bind to microbes coated nonspecifically with various serum proteins, such as fibronectin. However, some bacteria can also bind specific integrins directly. Ligation of individual receptors or assortment of receptors induces intracellular signals, which can lead to phagocytosis and/or direct the fate of the internalized microbe and the phagocyte. For example, while TLRs do not directly promote phagocytosis, they cooperate in the detection of microbes. (Adapted from Underhill and Ozinsky, 2002).
particle uptake. In macropinocytosis, intracellular signals can also lead to membrane ruffling and particle internalization (Donepudi et al., 2008).

To date, a lot of information regarding phagocytosis is based on the well-studied FcγR-mediated phagocytosis and many proteins, lipids, and signaling cascades associated with this process have been characterized. However, substantial differences between this and other forms of phagocytosis can be identified and are bound to exist to accommodate the variety of internalized particles and contexts of internalization. Therefore, it is noteworthy that although a useful paradigm (and the main focus of the following section), information obtained from the study of FcγR-mediated phagocytosis is not always applicable to other forms of particle internalization. Interestingly, it is also becoming increasingly clear that diverse events and signaling that occur during particle engulfment can have significant impact on phagosome maturation.

**Particle Engulfment: Lipids and GTPases**

Many proteins involved in particle engulfment during phagocytosis have been identified. Among them are protein kinases and phosphatases, GTPases, lipid-modifying enzymes, adaptor complexes, actin-binding proteins and proteins regulating membrane fusion and fission (Groves et al., 2008, Rogers et al., 2008a, Swanson, 2008, Yeung et al., 2007). Briefly, in FcγR-mediated phagocytosis tyrosine residues of the receptors are phosphorylated upon receptor-ligand interaction and the tyrosine kinase Syk, adaptor proteins GRB2 and GAB2, and PI3-K type I are recruited to the cytoplasmic part of the receptor. This complex of cytosolic proteins generates a cluster of phosphorylated proteins and modified lipids, which further signal and recruit multiple other factors, resulting in actin polymerization and extension of membrane over the IgG-opsonized particle (Figure 1-2) (reviewed in (Swanson, 2008)). Phagocytosis of large particles also requires membrane to be delivered to the forming phagosome from intracellular stores such as recycling endosomes, late endosomes, lysosomes, and granules (Bajno et al., 2000, Braun et al., 2004, Niedergang et al., 2003, Rogers et al., 2008a, Touret et al., 2005). Overall, the engulfment of particles is a complex process requiring coordination of many factors. The order of this process is mainly bestowed by alterations in lipid
Figure 1-2: Localized lipid metabolism and lipid-protein interactions during FcγR-mediated phagocytosis. The ligation of FcγR results in receptor autophosphorylation and recruitment of the protein kinase Syk, adaptor proteins GRB2 and GAB2, and class I PI3K. Receptor ligation also leads to activation of PI4P5K (PIPK) and synthesis of PI(4,5)P₂. PI(4,5)P₂ plays an important role in phagocytosis by promoting actin remodeling. It binds to the actin capping protein profilin, removing it from the ends of actin filaments and it stimulates gelsolin to sever actin filaments (not shown). It also binds to and helps to activate WASP family proteins, which activate Arp2/3 complex-mediated actin assembly. As phagocytosis proceeds PI(4,5)P₂ is hydrolyzed by PLCγ to form IP3 and DAG, initially at the base of the phagocytic cup and then at the pseudopods. DAG can recruit proteins containing C1 domains to the phagocytic cup, such as PKCα/ε, which are essential for phagocytosis (Larsen et al., 2002). PI(4,5)P₂ is also converted by the class I PI3K to PI(3,4,5)P₃. The disappearance of PI(4,5)P₂ from the phagocytic cup coincides with the dissociation of actin from sealed phagosomes and appears to be necessary for completion of phagocytosis (Scott et al., 2005). PI(3,4,5)P₃ recruits proteins such as amphiphysin, dynamin (not shown), WAVE and myosin X to the phagocytic cup, thereby promoting the extension of pseudopods over the internalized particle. It may also help activate Rac1 during phagocytosis by recruiting Vav, a Rac1 GEF. Active Rac1 binds IRSp53, which mediates its interaction with WAVE, leading to Arp2/3 complex-mediated actin polymerization (Abou-Kheir et al., 2008). Cdc42 also becomes activated at the phagocytic cup and activates WASP, promoting actin polymerization. Finally, the glycerophospholipid PC is hydrolyzed by PLD to form PA, whose cone-like structure may promote membrane fission. During phagocytic cup formation additional membrane is delivered from intracellular stores to accommodate engulfment of large particles. Reviewed in (Yeung et al., 2006a). (Figure adapted from (Yeung et al., 2007, Yeung et al., 2006a).
composition of the phagocytic cup membrane and recruitment of small regulatory
GTPases.

1. Lipids

The membrane of the forming phagosome is derived from the plasma membrane but its lipid composition is rapidly remodeled by the concerted action of phospholipid kinases, phosphatases, hydrolases, as well as membrane fission and fusion with membranes from intracellular stores. Normally, the inner leaflet of the plasma membrane is largely composed of phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) (Yeung et al., 2007). Phosphoinositides (the phosphorylated products of PI) are present in much lower quantities than the other phospholipids, but have a significant role in many cellular processes, including phagocytosis (Takenawa et al., 2001a). Changes in distribution of phospholipids during phagocytosis can be observed in cells expressing fluorescent proteins fused to protein domains with various lipid-binding specificities (see Table 1-1) (Steinberg et al., 2008). This is a powerful approach as it allows direct, real time, visualization of localized and transient events and has been extensively used to determine the manner of lipid turnover during phagocytosis (Botelho et al., 2000, Vieira et al., 2001).

A schematic of lipid-protein interactions and a model time course of lipid metabolism during phagocytosis are presented in Figure 1-2 and Figure 1-3, respectively. Phosphatidylinositol-4,5-bisphosphate (PI(4,5)P$_2$) is predominantly found at the plasma membrane of resting eukaryotic cells and its production is further stimulated at the membrane of the forming phagocytic cup (Botelho et al., 2000). PI(4,5)P$_2$, like other phosphoinositides, can be bound by and affect the activity of many proteins (Takenawa et al., 2001a). In phagocytosis PI(4,5)P$_2$ activates a number of actin-regulatory proteins and increases the activity of WASP, which in turn stimulates actin polymerization via the Arp2/3 complex (Takenawa et al., 2001b). The increase in PI(4,5)P$_2$ levels is followed quickly by its disappearance from the cup base in part due to phosphorylation by PI3-K type I to form phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P$_3$] (Vieira et al., 2001).
Table 1-1: Protein binding domains of lipids.

<table>
<thead>
<tr>
<th>Lipid species</th>
<th>Protein-binding domain</th>
<th>Representative Protein(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI(3)P</td>
<td>• PX</td>
<td>• p40&lt;sup&gt;box&lt;/sup&gt;</td>
<td>(Kanai et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>• FYVE</td>
<td>• EEA1</td>
<td>(Dumas et al., 2001)</td>
</tr>
<tr>
<td>PI(3,4)P&lt;sub&gt;2&lt;/sub&gt;</td>
<td>• PH</td>
<td>• TAPP1, Akt*</td>
<td>(Dowler et al., 2000, Franke et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>• PX</td>
<td>• p47&lt;sup&gt;box&lt;/sup&gt;</td>
<td>(Kanai et al., 2001)</td>
</tr>
<tr>
<td>PI(3,5)P&lt;sub&gt;2&lt;/sub&gt;</td>
<td>-</td>
<td>• Atg18</td>
<td>(Dove et al., 2004)</td>
</tr>
<tr>
<td>PI(4,5)P&lt;sub&gt;2&lt;/sub&gt;</td>
<td>• PH</td>
<td>• PLD2, PLCδ</td>
<td>(Sciorrà et al., 2002, Lemmon et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>• FERM</td>
<td>• Ezrin</td>
<td>(Blin et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>• ANTH</td>
<td>• AP180</td>
<td>(Ford et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>• ENTH</td>
<td>• Epsin</td>
<td>(Ford et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>• Tubby</td>
<td>• Tubby</td>
<td>(Santagata et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>• AP2-α</td>
<td>• AP2-α</td>
<td>(Collins et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>• Polycationic motif</td>
<td>• WASP</td>
<td>(Lemmon, 2003)</td>
</tr>
<tr>
<td>PI(3,4,5)P&lt;sub&gt;3&lt;/sub&gt;</td>
<td>• PH</td>
<td>• Btk, myosin X, Akt*</td>
<td>(Cox et al., 2002, Baraldi et al., 1999, Stokoe et al., 1997)</td>
</tr>
<tr>
<td>DAG</td>
<td>• C1</td>
<td>• PKCɛ</td>
<td>(Cheeseman et al., 2006)</td>
</tr>
<tr>
<td>PS</td>
<td>• C2&lt;sup&gt;+&lt;/sup&gt;</td>
<td>• PKC</td>
<td>(Newton et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>• F2F8 type C or discoidin-type C2</td>
<td>• Lactadherin, Factor VIII</td>
<td>(Yeung et al., 2008)</td>
</tr>
<tr>
<td>PA</td>
<td>-</td>
<td>• Raf1</td>
<td>(Ktistakis et al., 2003)</td>
</tr>
<tr>
<td>Negative surface charge</td>
<td>• Polycationic motif with hydrophobic residues</td>
<td>• RGK GTPases</td>
<td>(Heo et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>• Polycationic motif with myristoylation</td>
<td>• MARCKS</td>
<td>(McLaughlin et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>• Polycationic motif with prenylation</td>
<td>• K-Ras4B</td>
<td>(Heo et al., 2006)</td>
</tr>
</tbody>
</table>

* Note: The PH domain of Akt can bind both PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub>. 
PI(3,4,5)P_3 recruits myosin X to the phagocytic cup and also promotes the recruitment, phosphorylation and activation of phospholipase C\_\gamma (PLC\_\gamma) that hydrolyses remaining PI(4,5)P_2 to inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol (DAG) (Cox et al., 2002, Falasca et al., 1998). IP3 stimulates release of calcium from the ER, which is needed for certain types of phagocytosis, depending on cell type and the type of Fc\_\gamma R that is mediating particle uptake (Garcia-Garcia et al., 2002). DAG remains in the membrane of the phagocytic cup and recruits and activates protein kinase C\_\alpha (PKC\_\alpha) or PKC\_\epsilon (Cheeseman et al., 2006, Larsen et al., 2000, Larsen et al., 2002). Phospholipase D1 (PLD1) and PLD2 are also needed for phagocytosis, and generate phosphatidic acid from PC (Corrotte et al., 2006, Ktistakis et al., 2003). Due to its cone-like shape, phosphatidic acid may promote membrane fission and phagosome sealing (Kooijman et al., 2003). Phospholipase A2 also participates in phagocytosis and generates arachidonic acid from PC and PE (Lennartz et al., 1997). Arachidonic acid has been suggested to play a role in membrane fusion as well as superoxide production, however discordant results make its role in phagosome formation uncertain at this time (reviewed in (Yeung et al., 2007)). In addition to these specific lipid modifications, the overall changes in lipid composition of membranes that translate into changes in total membrane charge have important regulatory consequences. For example, the disappearance of PI(4,5)P_2 alters the net surface charge of the cytoplasmic leaflet of phagocytic cup membranes leading to Rac1 and K-Ras dissociation from nascent phagosomes (Yeung et al., 2006b). Changes in membrane charge are increasingly being recognized for their contribution to cellular processes by targeting regulatory proteins, such as small GTPases that rely on electrostatic interactions for their localization to cellular membranes (Heo et al., 2006, Yeung et al., 2008, Yeung et al., 2007, Yeung et al., 2006b). The recent development of specific fluorescent protein probes will no doubt enable an in depth identification of changes occurring in membrane charge during phagocytosis and their immediate consequences.
Figure 1-3: Time course of lipid metabolism during phagosome formation. Changes in relative abundance of PI(4,5)P₂, PI(3,4,5)P₃, phosphatidic acid (PA), and diacylglycerol (DAG) are shown. During phagocytosis PI(4,5)P₂ is generated on the forming phagocytic cup but is also quickly hydrolyzed. Instead, DAG accumulates and persists on the phagosomal membrane up to a few minutes following sealing. PI(3,4,5)P₃ is formed on the phagocytic cup and lasts on the phagosome up to ~1 min after sealing. Evidence suggests that the 5-phosphatase SHIP mediates the disappearance of PI(3,4,5)P₃ from the phagosome. PA is also formed on the phagocytic cup and accumulates on the phagosome, remaining there until it is likely converted to other lipids, such as DAG. (Reviewed in and figure adapted from Yeung et al., 2007).
2. GTPases

During particle engulfment another important level of organization is provided by small GTPases, mostly of the Ras superfamily. The Ras superfamily consists of small monomeric GTPases that are subdivided into five main groups: Ras, Rho, Rab, Arf, and Ran (Wennerberg et al., 2005). These are switch like molecules that participate in and regulate many essential cellular functions and therefore their own activation is tightly regulated (Figure 1-4). These proteins associate with membranes by means of covalently associated acyl chains as well as non-covalent interactions with phospholipids or membrane proteins. They are activated by guanine nucleotide exchange factors (GEFs), which in turn are activated by upstream signals such as phosphorylation, binding to proteins, phosphoinositides or to phosphatidic acid. In their active state GTPases that participate in phagocytosis can bind and activate various effector enzymes that modify lipids or proteins that regulate actin polymerization, membrane trafficking, myosin contractility, and other activities. Once their function is no longer needed, GTPases are inhibited by GTPase-activating proteins (GAPs), which stimulate GTP hydrolysis, and by guanine nucleotide dissociation inhibitors (GDIs), which sequester GDP-bound proteins from membranes (Figure 1-4).

Some of the key GTPases that contribute to various forms of phagocytosis affect actin cytoskeleton dynamics at the site of particle engulfment. These include Rac1, Cdc42, RhoA, RhoG and Arf6 and their function and regulation is summarized in Figure 1-5 (Swanson, 2008). Other GTPases, such as Arf6 and Arf1 contribute to phagocytosis by affecting membrane dynamics. For example, in addition to activating PIP5K, thereby promoting formation of PI(4,5)P₂ and stimulating actin polymerization, (Honda et al., 1999) Arf6 was also found to mediate delivery of VAMP3-carrying vesicles from recycling endosomes to the forming phagosome (Braun et al., 2007, Niedergang et al., 2003). Arf1 contributed to membrane alterations by recruiting the endosome associated clathrin adaptor complex AP-1 (Braun et al., 2007, Niedergang et al., 2003). Yet another GTPase, dynamin II (which does not belong to the Ras superfamily), is needed for pseudopod extension around the target particle, either by controlling vesicle budding
Figure 1-4: Regulation of GTPase activity. GTPases act as molecular switches that cycle between an active (GTP-bound) and inactive (GDP-bound) conformation. In their active state they can interact with target protein effectors thereby affecting multiple cell functions and their activation is very tightly regulated. The guanine nucleotide exchange factors (GEFs) catalyze nucleotide exchange from GDP to GTP, thereby activating the GTPase. In contrast, GTPase-activating proteins stimulate GTP hydrolysis, which leads to inactivation of the GTPase. Also, guanine nucleotide exchange factors (GDIs) can keep GTPases in an inactive state by extracting them from membranes. (Adapted from Etienne-Manneville et al., 2002).
Figure 1-5: GTPases involved in regulating actin rearrangements during phagocytosis. The details and outcomes of activation of different GTPases during four different types of phagocytosis (phagocytosis of apoptotic cells, Fc receptor-mediated phagocytosis, EGFR-mediated macropinocytosis and complement receptor-mediated phagocytosis) are illustrated. The GTPase activating GEFs found to participate in phagocytosis are the RhoG-activating TRIO, and Rac1-activating Vav and the bipartite GEF ELMO/DOCK180. RhoG in its active state can interact with ELMO, bringing the ELMO/DOCK180 complex to the membrane surface where it activates Rac1. However, Rac1 activation can also occur independently of RhoG, through binding of the CrkII adaptor protein to ELMO or by the RacGEF Vav. Rac1 activation leads to actin polymerization and filament stabilization mainly through its activation of WAVE that activates the Arp2/3 complex. Rac1 also activates PIP5K and PAK1, a p21-activated kinase. PIP5K generates PI(4,5)P2 and PAK1 phosphorylates a number of proteins, such as LIM kinase and CTBP1/BARS. Phosphorylated LIM kinase inactivates cofilin, thereby increasing actin filament turnover and CTBP1/BARS mediates macropinocytic cup closure. Activated Cdc42 can also activate PAK and PIP5K, and it induces Arp2/3 complex-mediated actin polymerization through activation of WASP. Cdc42 (as well as RhoA, (Colucci-Guyon et al., 2005)) also activates mammalian Diaphanous-related formin (mDia), which leads to formin-dependent actin polymerization. Additionally, Arf6 can activate PIP5K, while RhoA activates ROCK that activates myosin that generates contractile forces necessary for particle engulfment and phagocytic cup closure. (Reviewed in and figure adapted from (Swanson, 2008)).
from endocytic compartments to the pseudopods (Gold et al., 1999, Groves et al., 2008) or by controlling actin dynamics (Otsuka et al., 2009). However, despite extensive study focused on the actions of GTPases during phagocytosis many details of their function and regulation are still unclear. For example, only a few GEFs that activate GTPases during phagocytosis have been identified (see Figure 1-5) and so far no GAPs or GDIs have been directly implicated in this process.

**Phagosome Maturation**

The nascent phagosome is a relatively harmless vacuole that matures into a degradative phagolysosome by sequential interactions with the endocytic machinery of the cell (Scott et al., 2003, Vieira et al., 2002). The phagosome interacts with endosomes and acquires their properties by completely fusing with each or, as proposed by the “kiss-and-run” model, by forming a transient hybrid organelle connected by pores that allow for selective exchange of endosomal membrane and luminal contents, followed by fission (Desjardins, 1995, Duclos et al., 2000, Luzio et al., 2007). Regardless of the mode of interaction, due to the association of the phagosome with early endosomes, late endosomes and eventually lysosomes, the lumen of the phagosome becomes progressively more acidic, oxidizing and rich in hydrolytic enzymes (Figure 1-5).

1. Early Endosomes and Phagosomes

The phagosome first interacts with the early endosomes, which are mildly acidic (pH 6.1) and lack hydrolytic activity (Mukherjee et al., 1997). The early, also called sorting, endosomes are where incoming endocytic cargo is segregated and directed for recycling or degradation (Scott et al., 2003). Cargo destined for recycling is restricted to tubules or small areas that bud off from the early endosome and traffic either to recycling endosomes or directly back to the plasma membrane. The remaining constituents of the early endosome are transformed into a multivesicular body (MVB) that fuses with late endosomes. The small GTPase Rab5 is a marker and critical regulator of early endosome maturation. Rab5 is involved in many early endosome functions including pinocytosis,
microtubule-dependent endosomal motility, early endosome-endocytic vesicle fusion and homotypic fusion of early endosomes (Simpson et al., 2005). Activated (GTP-bound) Rab5 recruits its specific effector proteins to the early endosome. One of Rab5 effectors is the PI3-K Vps34, which phosphorylates PI present in the early endosome membrane to form phosphatidylinositol-3-phosphate [PI(3)P] (Vicinanza et al., 2008). PI(3)P formation is a vital part of endosome maturation since Vps34 inhibition causes early endosome enlargement, mistargeting of lysosomal proteins and arrest of endosome maturation (Scott et al., 2003).

Like Rab5, PI(3)P features prominently in endosome maturation since it can target many different proteins to early endosomes by virtue of their FYVE (Fab1/YOTB/Vac1/EEA1) or phox (PX) domains, which specifically bind to the head group of this phospholipid (Birkeland et al., 2004). One of these proteins is the early endosomal autoantigen 1 (EEA1), which also interacts with Rab5. EEA1 tethers early endosomes with incoming endocytic vesicles and interacts with syntaxin-6 and syntaxin-13, soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs), which can catalyze membrane fusion. Other proteins implicated in endosome maturation that can interact with PI(3)P are the sorting nexins (SNXs) associated with recycling and early endosomes, hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) that interacts with the ESCRT complex to generate MVBs, and PIKfyve, a PI(3)P 5-kinase found on late endosomes that generates phosphatidylinositol-3,5-bisphosphate [PI(3,5)P$_2$] and is involved in maintenance of endosome morphology (Ikonomov et al., 2002, Carlton et al., 2005, Raiborg et al., 2003). The role of PI(3,5)P$_2$ and PIKfyve in endosome and phagosome maturation, as well as the interactions of PI(3)P and other PI(3)P-binding proteins remain to be determined. The details of how these proteins mediate endosome maturation are a subject of continued investigation.

The newly formed phagosomes rapidly acquire early endosome markers (Rab5, EEA1, PI(3)P, NSF, syntaxin-13), which are necessary for phagosome maturation (Scott et al., 2003). The transient acquisition of PI(3)P by the phagosome starts shortly after sealing and does not seem to play a significant part in particle internalization. However inhibition of PI(3)P production by wortmannin treatment or injection of Vps34-inhibitory antibodies prevents fusion of phagosomes with late endosomes/lysosomes. Eventually the
**Figure 1-5: Phagosome maturation.** Following engulfment the phagocytosed particle resides in the phagosome, which is initially relatively harmless. It has the features of early endosomes with an almost neutral luminal pH and associates with markers of early endosomes such as Rab5, the phosphoinositide PI(3)P and the Rab5 effector EEA1. Subsequently it acquires the characteristics of late endosomes, becoming more acidic because of the Rab7-dependent delivery of vATPase proton pumps to the phagosome membrane. At this time the phagosome also acquires the Rab7 effector RILP and other late endosome markers. Eventually the phagosome fuses with lysosomes to form the phagolysosome. The phagolysosome is enriched in hydrolases, such as cathepsin D, that become activated in the now acidic lumen of this compartment and effectively digest the phagocytosed particle.
phagosome loses early endosome markers and immediately acquires late endosomal markers.

2. Late Endosomes and Phagosomes

Late endosomes have a multivesicular structure, an acidic lumen (pH 5.5 – 6.0) and active proteases (Mukherjee et al., 1997). Markers of late endosomes include Rab7, syntaxin-7, mannose-6-phosphate receptors (M6PRs) and lysobisphosphatidic acid (LBPA) (Scott et al., 2003). They are also enriched in many lysosomal-associated membrane proteins (LAMPs). Vesicles from the Golgi that contain newly synthesized lysosomal components and MVBs fuse with late endosomes. In turn, late endosomes fuse transiently with lysosomes.

The maturation of early phagosome into a late phagosome is characterized by acquisition of Rab7, its effector RILP, and LBPA (Harrison et al., 2003). Rab7 participates in late endosome to lysosome traffic: Rab7 down regulation stops traffic to lysosomes while its up regulation causes the formation of enlarged late endosomes/lysosomes. The Rab7 effector RILP was found to promote movement of late endosomes and/or lysosomes on microtubules by a dynein/dynactin-mediated process (Harrison et al., 2003). It also mediated displacement of phagosomes from cell periphery to the perinuclear region, which is required for their fusion with lysosomes (Vieira et al., 2002).

3. Lysosomes and Phagolysosomes

Lysosomes are more acidic than late endosomes (pH 4.5 – 5.5) and contain mature proteases (Mukherjee et al., 1997). However, due to many features shared with late endosomes, no known protein or lipid markers can absolutely identify the lysosome. Instead, fluid-phase marker pulse-chase techniques have been used extensively as the best available method to label lysosomes (Scott et al., 2003). In this approach cells are incubated in media that contains a fluid-phase marker (eg. a fluorescent dextran) and take
it up by constitutive pinocytosis. Following this initial pulse, the cells are washed and incubated in media lacking the fluid-phase marker. Within one to two hours the vesicles containing the marker mature, and it becomes concentrated in late endosomal/lysosomal compartments. Unfortunately, there is no guarantee of exclusive labeling of lysosomes using this method. A more stringent approach uses a fluid-phase marker called DQ-BSA that only becomes fluorescent upon proteolytic cleavage in degradative compartments of the cell, the lysosomes (Reis et al., 1998). Therefore using DQ-BSA as the fluid-phase marker allows for selective labeling of lysosomes.

The late phagosome eventually fuses with lysosomes thereby maturing into a phagolysosome. This is accompanied by reduction of late phagosome markers on the phagosome and its enrichment in lysosomal components such as LAMP1, mature proteases, and additional V-type ATPases (proton pumps) that acidify the phagosome lumen (Scott et al., 2003). Rab7 and RILP are necessary but not sufficient for fusion of phagosomes with lysosomes since PI3-K inhibition does not prevent acquisition of Rab7 and RILP by the phagosome but effectively arrests phagosome maturation (Scott et al., 2003). There are many outstanding questions in the field regarding phagosome maturation into a phagolysosome. Two quantitative proteomic studies indicated that there are likely more distinct fusion steps during phagosome maturation that probably occur with subpopulations of the main classes of endosomes (Gotthardt et al., 2006, Rogers et al., 2007).

Once fully mature, the phagolysosome with its highly acidic lumen and many active hydrolases is microbicidal and capable of degrading many substrates. Additionally, eukaryotic cells evolved other mechanisms that ensure efficient killing of ingested microbes and their delivery to this compartment.

**Additional Microbicidal Phagosome Factors**

Factors in addition to the acidic environment of and active hydrolases in the phagolysosome lumen contribute to the microbicidal nature of the phagosome. Generators of toxic reactive oxygen and nitrogen species (ROS and RNS) and cationic
antimicrobial peptides (CAMPs) are also delivered to the phagosome. Additionally, phagosomes contribute to adaptive immunity by facilitating antigen presentation.

1. ROS and RNS

Deliberate production of reactive oxygen species and oxidative killing of microbes is catalyzed by the Nox-family NADPH oxidases integrated into cell membranes, which catalyze the conversion of O$_2$ into superoxide anions (reviewed in (Robinson, 2008, Grandvaux et al., 2007)). In humans seven differentially expressed and regulated members of the Nox-family have been identified, five Nox proteins (Nox1-5) and two dual oxidases (Duox1 and Duox2). Nox2, originally known as gp91$^\text{phox}$, is predominantly expressed in phagocytes. The phagocyte oxidase becomes activated during phagocytosis to produce superoxide, a precursor of microbicidal reactive oxygen species (ROS). ROS production is a crucial part of immunity and patients with chronic granulomatous disease (CGD) who lack superoxide-producing activity in their phagocytes suffer from recurring and life-threatening infections (Segal et al., 2009).

Because reactive oxygen species are toxic to host and microbe alike, the activity of NADPH oxidase is precisely regulated (Robinson, 2008). In phagocytes, Nox2 forms a mutually stabilizing complex in the membrane with p22phox, and provides a docking site for soluble regulatory proteins. Activation of Nox2 requires the GTPase Rac plus the Nox organizer p47phox and the Nox activator p67phox, all found in the cytoplasm of resting cells. During phagocytosis the p47phox-p67phox complex and Rac are recruited to the phagosome membrane where they assemble with Nox2 and p22phox to form the active oxidase (Grandvaux et al., 2007). Additionally, in dendritic cells (DCs), recruitment of Nox2 is Rab27a-dependent (Jancic et al., 2007).

Nitric oxide (NO) is another microbicidal free radical generated in the phagosome. NO is generated by three different isoforms of nitric oxide synthase (NOS) with the help of multiple cofactors (Persichini et al., 2006). Type 2, or inducible, nitric oxide synthase (iNOS) uses L-arginine to produce NO in phagocytes in response to infection. The induction of iNOS occurs at a transcriptional level and can be initiated by
certain inflammatory cytokines or bacterial LPS, leading to activation of NFκB transcription factor and NO production at the phagosome (Persichini et al., 2006).

2. CAMPs

Another antimicrobial feature of the phagosome is the presence of CAMPs, which facilitate pathogen killing by binding to microorganisms and disrupting their essential processes or structural components. They are an ancient part of the immune system and are able to kill many different microorganisms including bacteria, fungi, and enveloped viruses (Brown et al., 2006a). They are found inside cells, in neutrophils and macrophages, and also extracellularly, in mucosal surfaces. Many CAMPs have been identified with many different structures but generally they are amphipathic, with discreet regions of cationic and hydrophobic residues. Commonly their killing activity is mediated by charge interactions and insertion into membranes causing increased membrane permeability of the targeted microorganism (Brown et al., 2006a).

3. Antigen presentation

Finally, antigen presentation is a less direct, but still relevant, microbial killing mechanism facilitated by phagocytosis. Internalization of microbes into phagosomes causes proteolytic processing of microbial antigens. This produces antigenic peptides that are loaded onto and presented by class I major histocompatibility complex (MHC-I) and class II (MHC-II) molecules to T cells (Ramachandra et al., 2009). The presentation of peptides derived from the phagosome and not the cytosol by MHC-I is called MHC-I cross processing or cross presentation. Although all nucleated cells can process and present cytosolic antigens on MHC-I, only phagocytic antigen presenting cells can take up and process antigens for MHC-I cross presentation, allowing them to promote a specific immune response to pathogens by MHC-I-restricted CD8+ T cells in addition to MHC-II-restricted CD4+ T cells (Ramachandra et al., 2009).
Prevailing over Phagocytosis: Pathogen Strategies

Pathogens have become adept at manipulating and interacting intimately with the hosts they invade. To prevent their destruction via phagosome-mediated degradation bacterial pathogens have acquired the abilities to avoid the phagosome altogether by

1. rapidly killing the phagocytosing cells (eg. *Salmonella* spp., *Shigella flexneri*, *Yersinia* spp.),
2. preventing their recognition by phagocytic receptors (eg. *Neisseria* spp., *Staphylococcus aureus*, *Streptococcus pyogenes*),
3. inhibiting the process of phagocytosis (eg. Enteropathogenic *E. coli*, *Yersinia* spp., *Pseudomonas aeruginosa*), and/or
4. disrupting the phagosome membrane and escaping into the cytosol (eg. *Shigella flexneri*, *Listeria monocytogenes*).

Other bacterial pathogens have instead learned to finely control phagocytosis itself by

1. preventing phagosome maturation, and
2. invading cells that are normally non-phagocytic and therefore less adept at dealing with bacterial invaders.

What we are learning about bacterial control of phagocytosis showcases the fine interaction between pathogen and host. The two aspects of bacterial control of the phagocytic process (preventing phagosome maturation and invading non-phagocytic cells) are described below.

1. Preventing Phagosome Maturation

Many bacterial pathogens choose to enter cells in a phagosome or vacuole, subsequently remodeling this compartment into one suitable for survival and further replication (Figure 1-7). With the exception of the obligate intracellular bacterium *Coxiella burnetii*, which lives inside a phagolysosome-like compartment (Voth *et al.*, 2007) (Figure 1-7G), other vacuole-adapted pathogens avoid fusion with lysosomes. To prevent lysosome fusion, the pathogen-containing phagosome can segregate from the endocytic route (as in the case of *Chlamydia*, *Legionella*, and *Brucella*), or become
Figure 1-7: Disruption of phagosome maturation by bacterial pathogens.
Intracellular pathogens are phagocytosed or enter non-phagocytic cells by triggered phagocytosis or a zipper mechanism of invasion. Once inside the host cell, some bacteria rapidly lyse the phagosome and escape into the cytosol (A). Others remain in the phagosome but remodel it to their advantage. *Chlamydiae* segregate from the endocytic route and form a unique inclusion vacuole by interacting with Golgi-derived vesicles (B). *Legionella* segregates from the endocytic route at the early endosome (EE) stage and remodels its phagosome to acquire properties of the ER (C). *Mycobacterium* arrests phagosome maturation at the early endosome stage (D). *Brucella* segregates at the late endosome (LE) stage and like *Legionella*, its phagosome acquires characteristics of the ER (E). *Salmonella* selectively interacts with early and late endosomes, but blocks fusion with lysosomes (Lys) (F). Unlike the other intracellular pathogens, *Coxiella* survives inside a phagosome that undergoes the full maturation process, including fusion with lysosomes (G). (Adapted from (Alonso et al., 2004)).
arrested at a specific phagosome maturation step prior to delivery of lysosomes (Salmonella, discussed later, and Mycobacterium tuberculosis) (Figure 1-7D,F). Certainly, a lot has been learned about pathogen control of the phagosome environment, but manipulation of phagosome maturation by bacteria is often subtle and transient. Therefore significant gaps in our knowledge on this topic are still evident. New information is bound to be instructive on the process of phagocytosis in general.

_Chlamydia_ spp.

Chlamydiae are obligate intracellular bacteria that replicate in a non-acidic vacuole, the inclusion, segregated away from late endosomes and lysosomes (Valdivia, 2008) (Figure 1-7B). After internalization into epithelial cells the inclusion does not associate with early or late endosomal markers, including EEA1, Rab5, Rab7, Rab9 nor LAMP1. It does however acquire recycling endosome markers Rab4 and Rab11, as well as Rab1, involved in ER-Golgi trafficking (Rzomp et al., 2003). Some species specificity in inclusion biogenesis exists, as _C. trachomatis_ inclusions associate with Rab6 while _C. pneumoniae_ inclusions associate with Rab10. If the bacteria are internalized by DCs or macrophages the inclusion also becomes associated with LAMP1, perhaps due to rapid phagosome maturation of these professional antigen-presenting cells. Once in the cell, the inclusion migrates in a dynein- and bacterial protein-dependent manner to the microtubule-organizing centre (MTOC), where it remains close to the Golgi apparatus, acquiring cholesterol and sphingolipids via vesicles from the Golgi (Figure 1-7[2]). Inclusions have also been suggested to interact with MVBs and perhaps autophagosomes to obtain more nutrients and lipids. _Chlamydia_ inclusion membrane proteins (IncCs) are thought to be secreted into the host cell cytosol by a type III secretion system and to participate in inclusion biogenesis and its segregation from lysosomes (Valdivia, 2008). However, Chlamydiae are genetically intractable, and despite ongoing progress in this field precisely how the inclusion avoids fusion with endocytic compartments is not known.
Legionella pneumophila

Following Legionella invasion, smooth vesicles, mitochondria and the ER sequentially surround the Legionella-containing phagosome (Isberg et al., 2009). This compartment never acquires late endosomal/lysosomal markers such as Rab7 and lysosomal-membrane glycoproteins. Instead, ER-derived proteins associate with the Legionella phagosome, including the SNARE protein Sec22b, Rab1 (Derre et al., 2004, Kagan et al., 2004) and eventually soluble ER-derived proteins such as glucose-6-phosphatase and protein disulfide isomerase (Robinson et al., 2006) (Figure 1-7[3]). The formation of the replicative vacuole depends on Legionella’s dot/Icm (defect in organelle trafficking/intracellular multiplication) type IV secretion system that delivers Legionella proteins across host-cell membranes (Marra et al., 1992, Berger et al., 1994, Nagai et al., 2002). More than 100 dot/Icm substrates have been identified but the function of only a few is known (Isberg et al., 2009). The Legionella RalF protein is delivered by the dot/Icm system and recruits and activates the small GTPase Arf1, implicated in ER to Golgi traffic, to the phagosome (Nagai et al., 2002). Similarly, recruitment of Rab1 to the phagosome depends on another dot/Icm substrate, SidM/DrrA. Interestingly, SidM activates Rab1 by both acting as a GDI dissociation factor and promoting nucleotide exchange (Ingmundson et al., 2007, Machner et al., 2007). Other Legionella proteins also target Rab1. LidA binds to Rab1 (and other Rabs) (Machner et al., 2006) and LepB is a Rab1 GAP (Ingmundson et al., 2007). However, the specific targeting of Rab1 by Legionella effectors is puzzling as mutants lacking proteins that affect Rab1 function have only modest defects in formation of their replicative phagosomes (Neumeister et al., 2002). The aspects of pathogenesis that Rab1 manipulation by Legionella effectors contributes to are likely at present waiting to be uncovered.

Brucella spp.

Brucella are intracellular pathogens that preferentially infect the macrophages of their hosts, but their interactions with the host cell are subtle and therefore poorly understood. They require smooth LPS to enter macrophages via lipid rafts and this mode of entry has been linked to their intracellular survival (Naroeni et al., 2002, Porte et al., 2003). As a result of this specific invasion mechanism, the Brucella-containing
phagosomes do not fuse with lysosomes and instead are able to interact with the ER (Celli et al., 2003) (Figure 1-7[5]). Additionally, the smooth LPS itself can impair MHC-II-dependent antigen presentation presumably enabling Brucella to dampen the adaptive immune response (Forestier et al., 2000, Forestier et al., 1999). Upon invasion of epithelial cells (and similarly in macrophages) Brucella initially resides in early endosomal compartments, positive for Rab5 and EEA1. Next, they traffic to an ER-like compartment via the route of autophagosomes, acquiring LAMP1, Sec61b (an autophagosome marker) and ER markers sec1b, calnexin and ribophorin (Gorvel et al., 2002) (Figure 1-7[5]). Like Legionella, Brucella express a type IV secretion system and it is anticipated that secreted virulence factors that affect their interaction with host cells will be identified.

Mycobacterium tuberculosis

Mycobacterium tuberculosis causes a developmental arrest of the phagosome in which it resides (Figure 1-7[4]). The phagosome retains early endosome characteristics, such as the presence of Rab5 (but not its effector, EEA1), and excludes the late-endosomal Rab7 from its membrane. M. tuberculosis phagosomes exclude the vacuolar ATPase and do not acidify. Lipoarabinomannan, a glycosylated phosphatidylinositol released from the M. tuberculosis cell envelope may be partly responsible for this maturation arrest as it inhibits the activity of the class III PI3-K Vps34 thus blocking accumulation of PI(3)P on the phagosome (Fratti et al., 2003). Bacterial proteins also contribute to lysosome fusion avoidance. A bacterial phosphatase secreted into host-cell cytosol called SapM degrades PI(3)P (Vergne et al., 2005) and protein phosphatases PtpA and PtpB may interfere with host trafficking by affecting vacuolar sorting proteins (Bach et al., 2008). PknG is a secreted bacterial protein kinase highly homologous to eukaryotic serine/threonine kinases, whose activity is required to prevent phagosome-lysosome fusion (Walburger et al., 2004). Additionally, a few host-cell proteins were identified that contribute to the block of M. tuberculosis phagosome maturation. These include Rab14 (Kyei et al., 2006) and Rab22a (Roberts et al., 2006), which were recruited to M. tuberculosis phagosomes, and coronin 1 (initially named TACO). Rab14 was found to promote fusion of early endosomes with phagosomes, allowing these to
maintain the character of early compartments (Kyei et al., 2006), while Rab22a blocked acquisition of Rab7 normally associated with maturation of endosomes into late endosomes (Roberts et al., 2006). Coronin 1 regulates calcium-dependent signaling processes, and is found on the phagosomes containing viable but not dead bacteria. It causes a cytosolic influx of calcium that activates the calcium-dependent phosphatase calcineurin, required for blocking phagosome-lysosome fusion during infection of cells with *M. tuberculosis*. Precisely how Rab14, Rab22a, and coronin 1 are recruited to bacteria-containing phagosomes and how calcineurin prevents lysosome fusion with these compartments is unknown. Lastly, *M. tuberculosis* modulates the phagosome environment to its advantage by inhibiting iNOS recruitment through lowering the retention on phagosomes of the scaffolding protein EB50 that binds to iNOS and links it to the actin cytoskeleton (Davis et al., 2007).

2. Invading Non-Phagocytic Cells

Pathogenic bacteria will often invade less bactericidal, normally non-phagocytic cells (such as epithelial and endothelial cells) to avoid encountering professional phagocytes and other circulating immune factors or to traverse epithelial layers, gaining access to preferred sites of infection within their host. Two general strategies for pathogen entry of non-phagocytic cells have been recognized, the zipper and the trigger mechanism.

Zipper Mechanism of Invasion

The zipper mechanism of entry is mediated by the interaction of a bacterial adhesin to a host cell surface receptor normally involved in cell-cell adhesion or modulation of cytoskeleton dynamics. Such receptors are often integrated into downstream signaling cascades that lead to actin and membrane rearrangements.

In the case of *Listeria monocytogenes* the adhesins are internalin A (InlA) and B (InlB) (Alonso et al., 2004). InlA is covalently linked to the bacterial peptidoglycan and contains several leucine-rich repeats (LRRs). Its host receptor is E-cadherin, normally
involved in intercellular adherence and formation of adherence junctions. Upon binding of InlA to E-cadherin, actin remodeling occurs, promoted by α- and β-catenins that normally link E-cadherin to cytoskeleton fibers present in adherence junctions. InlB is linked to the bacterial cell wall by its interaction with lipotechoic acids. InlB also contains LRRs and interacts with at least three cell surface proteins, gC1qR (a receptor for a component of the complement cascade), the tyrosine kinase receptor Met (also known as the hepatocyte growth factor receptor, HGF-R), and glycosaminoglycans (GAGs). Binding of InlB to Met causes activation of PI3-K, generation of PI(3,4,5)P₃, activation of Rho GTPases, and actin rearrangements (Cossart et al., 2003).

*Yersinia pseudotuberculosis* and *Y. entercolitica* express three adhesins called invasin, YadA, and Ail (Alonso et al., 2004). Ail mediates entry into epithelial cells but it is not known how. However, both invasin and YadA bind a subset of β1-integrin receptor family of proteins, presumably to gain entry into M cells and be transcytosed across the intestinal epithelium. Invasion by both *Yersinia* species depends on tyrosine and serine-threonine kinases, activation of PI3-K, Rac1 and the Arp2/3 complex. Other host molecules that become activated upon bacterial adhesion are NFκB, MAPK p38, and JNK (Alonso et al., 2004).

**Trigger Mechanism of Invasion**

Bacterial proteins (the effectors) that are translocated into the host-cell cytosol mediate the trigger mechanism of invasion, utilized by both *Shigella flexneri* and *Salmonella enterica* (discussed at length later) to enter cells. Here, ligation of host-cell receptors to stimulate bacterial uptake is dispensable. Instead, multiple effectors delivered into the cell target signaling cascades regulating cytoskeleton and membrane dynamics. The trigger mechanism of invasion is akin to macropinocytosis in terms of both its morphology as well as ability to proceed in the absence of ligation of host-cell receptors.

During invasion of cells by *S. flexneri* the initial contact between bacteria and host cells is mediated by host cell receptors CD44 and α5β1 integrin in lipid rafts (Ogawa et al., 2008). Binding of these receptors by the bacteria induces early actin cytoskeleton
rearrangements but efficient internalization also requires the translocation of effectors via the Mxi-Spa T3SS, which stimulate massive membrane ruffling and macropinocytosis. This process of internalization requires the activation of Rac1 and Cdc42 and subsequent recruitment of Arp2/3. Five bacterial proteins are implicated in induction of actin rearrangements so far (Ogawa et al., 2008). First, IpaC, which is part of the S. flexneri translocon, stimulates Rac1 and Cdc42 activation by an unknown mechanism. The effector IpgB1 mimics the activated form of RhoG and thereby elicits Rac1 activation and membrane ruffling through the ELMO-Dock180 pathway. The bacterial VirA cysteine protease destabilizes microtubules, which leads to further activation of Rac1. The IpgB2 effector can also mimic an active GTPase, in this case the active RhoA, and its expression in eukaryotic cells leads to formation of stress fibers and membrane ruffling (Alto et al., 2006). IpgD acts differently, as it is a phosphoinositide-4-phosphatase that hydrolyzes PI(4,5)P₂ to phosphatidylinositol-5-phosphate [PI(5)P]. Disappearance of PI(4,5)P₂ causes a dissociation of the actin cytoskeleton from the plasma membrane, facilitating bacterial entry. Finally, IpaA binds vinculin and enhances its interaction with actin filaments, leading to localized actin depolymerization, perhaps promoting closure of the phagocytic cup around the bacteria.

The triggered internalization by Salmonella is similar to that of S. flexneri. For example, both pathogens initiate invasion at cholesterol rich lipid rafts and both hydrolyze PI(4,5)P₂ during invasion. They also both target GTPases of the Rho family to manipulate the actin cytoskeleton. However, differences between these processes exist. Instead of mimicking active GTPases like S. flexneri IpgB1 and IpgB2, the Salmonella effectors SopE and SopE2 act as GEFs in order to activate Rac1 and Cdc42 (Schlumberger et al., 2005). Also, the assortment of activated GTPases, although achieving the same end of internalization is not identical as Salmonella does not seem to activate RhoA during invasion (Criss et al., 2003). Is this a case of applying different solutions to the same problem, or do such differences have functional consequences? Unlike Salmonella, S. flexneri is a cytosol-adapted pathogen, escaping the phagosome shortly after its formation. Therefore S. flexneri might find it unnecessary to regulate factors associated with phagosome maturation. Additionally, S. flexneri enters epithelial cells almost exclusively via the basolateral side, while Salmonella is able to also enter
them apically. The two poles of the cell have a very different composition and signaling
events that promote entry on one side may not be relevant on the other. However, at this
time we do not have information on the full extent of effector functions secreted by either
pathogen and additional bacterial proteins that affect triggered phagocytosis may still be
identified. Therefore the functional consequences of these differences remain to be
determined, but are bound to be useful in dissecting signaling pathways acting during
macropinocytosis and triggered bacterial invasion alike.
**SALMONELLA ENTERICA**

*Salmonella enterica* serovar Typhimurium Infection

*Salmonella enterica* infections are a serious health problem in developing countries and contamination with these bacteria is a constant concern of the food industry. *Salmonella enterica* serovar Typhimurium (S. Typhimurium) is a facultative intracellular pathogen. It causes gastroenteritis in humans and cattle, and a systemic infection in mice, which is used as a model of human typhoid fever (caused by *Salmonella enterica* serovar Typhi). Central to S. Typhimurium infection is its ability to invade and colonize a variety of cell types within its host, including non-phagocytic intestinal epithelial cells and phagocytic cells, such as macrophages (Leung *et al.*, 1991, Tsolis *et al.*, 1999). Following invasion, the bacteria inhabit a membrane-bound compartment called the *Salmonella*-containing vacuole (SCV) where they establish a niche conducive to their replication (Meresse *et al.*, 1999).

**S. Typhimurium Type III Secretion Systems and Effectors**

To invade as well as control the fate of the SCV the bacteria employ protein effectors that they inject into the host-cell cytosol using one of the two type three secretion systems (T3SSs) at their disposal. Once translocated into the host cell, the effectors are able to direct various host-cell processes to the advantage of the bacteria. However, despite identification of many *Salmonella* effectors and recognition of their significant role in virulence, relatively little is known about their specific functions, host-cell targets and mechanisms of action (see Table 1-2). An in depth introduction to the SopD and SopB/SigD (herein called SopB) effectors (the focus of this thesis) is provided in subsequent sections (pp 49 – 56).

The two *Salmonella* T3SSs are encoded on separate genomic regions, the *Salmonella* Pathogenicity Islands (SPIs), SPI-1 or SPI-2 (Hansen-Wester *et al.*, 2001). These T3SSs (and their associated effectors) are differentially regulated, with SPI-1-encoded T3SS (SPI-1 T3SS) expressed early, together with the unique assortment of
effectors it translocates. The SPI-1 T3SS translocated effectors are critical for bacteria mediated invasion, early SCV biogenesis, and the intestinal phase of infection (Lostroh et al., 2001, Santos et al., 2001b, Wallis et al., 2000). The SPI-2 encoded T3SS (SPI-2 T3SS) is expressed a few hours following invasion and is responsible for translocating effectors that further direct SCV maturation, intracellular bacterial survival, and the systemic phase of infection (Hensel, 2000, Waterman et al., 2003). Notably, a growing understanding of the two T3SSs and the interplay between effectors translocated by each is revealing that their roles are not that strictly segregated. While SPI-2 T3SS has been shown to affect the intestinal phase of infection, the SPI-1 T3SS translocated effectors can also play roles in late stages of S. Typhimurium’s intracellular life style and SCV maturation (Brawn et al., 2007, Coburn et al., 2005, Hapfelmeier et al., 2005, Steele-Mortimer et al., 2002, Wasylnka et al., 2008).

**S. Typhimurium Invasion of Epithelial Cells**

Invasion of non-phagocytic cells by S. Typhimurium is characterized by massive ruffling of the plasma membrane akin to macropinocytosis that drives bacterial uptake and is accompanied by formation of large endocytic vacuoles (macropinosomes). At least four S. Typhimurium effectors (SipA, SopB, SopE, and SopE2) translocated through the host-cell plasma membrane by the SPI-1 T3SS initiate this process by affecting the host-cell cortical actin cytoskeleton (Patel et al., 2005) (Figure 1-8). The initiation of actin driven membrane ruffling is followed by fission of bacteria-containing vacuoles and empty vacuoles from the plasma membrane. Prior work has demonstrated a role for the SopB effector in SCV fission and macropinosome formation (Hernandez et al., 2004, Terebiznik et al., 2002).
<table>
<thead>
<tr>
<th>Effector</th>
<th>Identified biochemical activity</th>
<th>Host target(s)/binding partner(s)</th>
<th>Virulence function and/or phenotype</th>
<th>Reference(s)</th>
</tr>
</thead>
</table>
| AvrA (also by SPI-2 T3SS) | Cysteine protease and acetyltransferase | IκBα, MKK4/7 | • prevents NF-κB activation by deubiquitinating  
• IκBα − dampens immune response  
• stabilizes tight junctions  
• represses JNK signaling pathway and apoptosis  
• prevents NF-κB activation by deubiquitinating  
• stabilizes tight junctions  
• represses JNK signaling pathway and apoptosis  
• stabilizes tight junctions  
• represses JNK signaling pathway and apoptosis  
• stabilizes tight junctions  
• represses JNK signaling pathway and apoptosis  | (Ye et al., 2007, Liao et al., 2008, Jones et al., 2008, Collier-Hyams et al., 2002, Geddes et al., 2005) |
| PipD             | Cysteine protease homolog          | ?                                  | • required for long-term systemic infection in mice                                                 | (Lawley et al., 2006, Wood et al., 1998)                                      |
| SipA/SspA        | ?                                | F-actin                           | • induces enteritis  
• induces chemokine expression by phosphorylation of JUN and p38MAPK  
• disrupts tight junctions  
• promotes juxtanuclear SCV positioning  
• invasion (promotes actin assembly, prevents depolymerization)  
• induces enteritis  
• induces chemokine expression by phosphorylation of JUN and p38MAPK  
• disrupts tight junctions  
• promotes juxtanuclear SCV positioning  
• invasion (promotes actin assembly, prevents depolymerization)  
• induces enteritis  
• induces chemokine expression by phosphorylation of JUN and p38MAPK  
• disrupts tight junctions  
• promotes juxtanuclear SCV positioning  
• invasion (promotes actin assembly, prevents depolymerization)  
• induces enteritis  
• induces chemokine expression by phosphorylation of JUN and p38MAPK  
• disrupts tight junctions  
• promotes juxtanuclear SCV positioning  
• invasion (promotes actin assembly, prevents depolymerization)  | (Boyle et al., 2006, Brawn et al., 2007, Figueiredo et al., 2009, McGhie et al., 2004, Raffatellu et al., 2005, Zhang et al., 2002, Zhou et al., 1999a, Zhou et al., 1999b) |
| SipB/SspB        | ?                                | Caspase-1, cholesterol            | • required for fluid secretion in bovine ligated ileal loops  
• activates caspase-1  
• disrupts mitochondria  
• induces apoptosis  
• is a T3SS translocator that binds cholesterol  
• required for long-term systemic infection in mice  
• required for fluid secretion in bovine ligated ileal loops  
• activates caspase-1  
• disrupts mitochondria  
• induces apoptosis  
• is a T3SS translocator that binds cholesterol  
• required for long-term systemic infection in mice  | (Hayward et al., 2005, Hernandez et al., 2003, Hersh et al., 1999, Lawley et al., 2006, Santos et al., 2001a, van der Velden et al., 2003, Wood et al., 1996) |
| SipC/SspC        | Actin binding                     | F-actin                           | • T3SS translocator  
• cytoskeleton rearrangements  
• actin nucleation, bundling  
• T3SS translocator  
• cytoskeleton rearrangements  
• actin nucleation, bundling  | (Hayward et al., 1999, Fu et al., 1998, Chang et al., 2005, Chang et al., 2007) |
| SlrP (and SPI-2 T3SS) | LRR protein                       | ?                                  | • interferes with antigen presentation in DCs  
• interferes with antigen presentation in DCs  | (Halici et al., 2008) |
| SopA             | HECT E3 ubiquitin ligase           | HsRMA1                            | • induces enteritis  
• promotes invasion  
• promotes bacterial escape from SCV in HeLa cells  
• induces enteritis  
• promotes invasion  
• promotes bacterial escape from SCV in HeLa cells  | (Raffatellu et al., 2005, Zhang et al., 2005, Zhang et al., 2006) |
| SopB/SigD        | Inositol polyphosphate phosphatase | PI(4,5)P2, Cdc42                   | • induces enteritis  
• promotes invasion (membrane fission and macropinosome formation, actin rearrangements)  
• SCV membrane dynamics/SCV trafficking  
• Sif formation  
• SCV positioning  
• Anti-apoptotic via Akt activation  
• Disrupts tight junctions  
• induces enteritis  
• promotes invasion (membrane fission and macropinosome formation, actin rearrangements)  
• SCV membrane dynamics/SCV trafficking  
• Sif formation  
• SCV positioning  
• Anti-apoptotic via Akt activation  
• Disrupts tight junctions  | (Boyle et al., 2006, Bujny et al., 2008, Dukes et al., 2006, Hernandez et al., 2004, Knodler et al., 2005a, Mallo et al., 2008, Mason et al., 2007, Patel et al., 2006, Rogers et al., 2008b, Terebiznik et al., 2002, Wasylnka et al., 2008, Zhang et al., 2002) |
Table 1-2: *S. Typhimurium* effectors (cont’d)

<table>
<thead>
<tr>
<th>Effector</th>
<th>Identified biochemical activity</th>
<th>Host target(s)/binding partner(s)</th>
<th>Virulence function and/or phenotype</th>
<th>Reference(s)</th>
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<tbody>
<tr>
<td><strong>SPI-1 T3SS translocated (cont’d)</strong></td>
<td></td>
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<tr>
<td>SopE and SopE2</td>
<td>GEF</td>
<td>Cdc42, Rac1</td>
<td>• actin polymerization • promote invasion • induce proinflammatory cytokines (activate MAPK pathways) • disrupt tight junctions • SopE2 is required for long-term systemic infection in mice</td>
<td>(Boyle <em>et al.</em>, 2006, Hobbie <em>et al.</em>, 1997, Lawley <em>et al.</em>, 2006, Patel <em>et al.</em>, 2006, Zhang <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td>SptP (also by SPI-2 T3SS)</td>
<td>GAP and tyrosine phosphatase</td>
<td>Cdc42, Rac/vimen- tin?, Raf</td>
<td>• actin depolymerization • down-modulates Erk activation • reduces proinflammatory cytokines (TNF-α, IL-8) • induces iNOS expression</td>
<td>(Lin <em>et al.</em>, 2003, Murli <em>et al.</em>, 2001, Cherayil <em>et al.</em>, 2000, Fu <em>et al.</em>, 1999)</td>
</tr>
<tr>
<td>SseI/SrfH/Gtg B</td>
<td>?</td>
<td>Filamin, TRIP6</td>
<td>• Actin rearrangements • Promotes phagocyte motility and accelerates systemic spread of infection • required for long-term systemic infection in mice</td>
<td>(Lawley <em>et al.</em>, 2006, Miao <em>et al.</em>, 2003, Worley <em>et al.</em>, 2006)</td>
</tr>
<tr>
<td>SspH1 (also SPI2)</td>
<td>LRR protein</td>
<td>?</td>
<td>• inhibits NF-xB-dependent gene expression</td>
<td>(Miao <em>et al.</em>, 1999, Haraga <em>et al.</em>, 2003)</td>
</tr>
<tr>
<td>SteA (also by SPI-2 T3SS)</td>
<td>?</td>
<td>?</td>
<td>• localizes to the TGN • required for long-term systemic infection in mice</td>
<td>(Geddes <em>et al.</em>, 2005, Lawley <em>et al.</em>, 2006)</td>
</tr>
<tr>
<td>SteB (also by SPI-2 T3SS)</td>
<td>?</td>
<td>?</td>
<td></td>
<td>(Geddes <em>et al.</em>, 2005)</td>
</tr>
<tr>
<td><strong>SPI-2 T3SS translocated</strong></td>
<td></td>
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<tr>
<td>GogB</td>
<td>Similarity to LRR proteins</td>
<td></td>
<td></td>
<td>(Coombes <em>et al.</em>, 2005)</td>
</tr>
<tr>
<td>Effector</td>
<td>Identified biochemical activity</td>
<td>Host target(s)/binding partner(s)</td>
<td>Virulence function and/or phenotype</td>
<td>Reference(s)</td>
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<td>---------------</td>
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<td>--------------</td>
</tr>
<tr>
<td>PipB2</td>
<td>?</td>
<td>Kinesin-1</td>
<td>• Sif formation&lt;br&gt;• SCV centrifugal displacement at late stages of infection and cell-to-cell spread&lt;br&gt;• interferes with antigen presentation in DCs</td>
<td>(Halici et al., 2008, Henry et al., 2006b, Knodler et al., 2005b, Szeto et al., 2009)</td>
</tr>
<tr>
<td>SifA</td>
<td>GEF for RhoA family GTPases?</td>
<td>SKIP, Rab7, RhoA family GTPases</td>
<td>• Sif formation&lt;br&gt;• SCV stability&lt;br&gt;• maintains SCV in juxtanuclear location&lt;br&gt;• interferes with MHC class II antigen presentation&lt;br&gt;• needed for virulence in mice&lt;br&gt;• redirects secretory vesicles to SCV</td>
<td>(Beuzon et al., 2000, Boucrot et al., 2005, Brumell et al., 2002, Halici et al., 2008, Harrison et al., 2004, Kuhle et al., 2006, Mitchell et al., 2004, Ohlson et al., 2008, Stein et al., 1996)</td>
</tr>
<tr>
<td>SifB</td>
<td>?</td>
<td>?</td>
<td>• localizes to SCV and Sifs</td>
<td>(Alto et al., 2006, Freeman et al., 2003)</td>
</tr>
<tr>
<td>SopD2</td>
<td></td>
<td></td>
<td>• Sif formation&lt;br&gt;• interferes with antigen presentation in DCs&lt;br&gt;• virulence in mice&lt;br&gt;• required for long-term systemic infection in mice</td>
<td>(Brumell et al., 2003, Halici et al., 2008, Jiang et al., 2004, Lawley et al., 2006)</td>
</tr>
<tr>
<td>SpiC/SsaB</td>
<td>?</td>
<td>Hook3, TassC</td>
<td>• involved in ordered secretion of translocators and SPI-2 effectors, hence phenotypes listed below may be due to its role in SPI-2 T3SS effector delivery&lt;br&gt;• promotes vacuole-associated actin polymerizations (VAP) formation&lt;br&gt;• Sif formation&lt;br&gt;• inhibits fusion of LE/lysosomes with SCV&lt;br&gt;• required for long-term systemic infection in mice</td>
<td>(Freeman et al., 2002, Lawley et al., 2006, Uchiya et al., 1999, Yu et al., 2004, Yu et al., 2002, Shotland et al., 2003)</td>
</tr>
<tr>
<td>SpvC (also by SPI-1 in vitro)</td>
<td>Phosphothreonine lyase</td>
<td>MAPKs (phospho-Erk, p38, JNK)</td>
<td>• down regulates cytokine release by irreversibly inactivating MAPKs</td>
<td>(Mazurkiewicz et al., 2008, Zhu et al., 2007, Li et al., 2007)</td>
</tr>
<tr>
<td>SpvB (independent of either T3SS but cytotoxicity depends on SPI-2 T3SS)</td>
<td>Actin ribosyltransferase</td>
<td>actin</td>
<td>• blocks actin polymerization by ADP-ribosylating actin&lt;br&gt;• inhibits VAP formation&lt;br&gt;• causes delayed macrophage cytotoxicity&lt;br&gt;• activates caspase-3</td>
<td>(Browne et al., 2008, Browne et al., 2002, Gotth et al., 2003, Miao et al., 2003, Otto et al., 2000, Tezcan-Merdol et al., 2001)</td>
</tr>
<tr>
<td>SpvD</td>
<td>?</td>
<td>?</td>
<td>• contributes to virulence in mice</td>
<td>(Gulig et al., 1992, Matsui et al., 2001)</td>
</tr>
<tr>
<td>Effector</td>
<td>Identified biochemical activity</td>
<td>Host target(s)/binding partner(s)</td>
<td>Virulence function and/or phenotype</td>
<td>Reference(s)</td>
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<tr>
<td>SPI-2 T3SS translocated (cont’d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| SseF | ? | dynein | • Sif formation  
• maintains SCV in juxtanuclear location  
• interferes with antigen presentation in DCs  
• microtubule bundling  
• redirects secretory vesicles to SCV | (Abrahams et al., 2006, Deiwicks et al., 2006, Guy et al., 2000, Halici et al., 2008, Kuhle et al., 2006, Kuhle et al., 2002, Ramsden et al., 2007b) |
| SseJ/SifC | Deacylase and acyl-transferase, cholesterol esterification | RhoA, cholesterol | • negative regulation of Sifs  
• SCV membrane dynamics  
• esterifies cholesterol  
• promotes lipid droplet formation  
• required for long-term systemic infection in mice | (Freeman et al., 2003, Lawley et al., 2006, Nawabi et al., 2008, Ohlson et al., 2005, Ohlson et al., 2008, Ruiz-Albert et al., 2002) |
| SseK (also SPI1) | ? | ? | • secreted 9 h p.i. | (Kujat Choy et al., 2004) |
| SseK2 | ? | ? | • required for long-term systemic infection in mice  
• secreted 21 h p.i. | (Kujat Choy et al., 2004, Lawley et al., 2006) |
| SseL | deubiquitinase | Ubiquitin, IκBα | • enhances delayed macrophage killing  
• impairs IκBα ubiquitination and degradation thereby suppressing NF-κB activation and dampening the immune response | (Rytkonen et al., 2007, Coombes et al., 2007, Le Negrate et al., 2008) |
| SspH2 | LRR protein | Filamin, profilin | • decreases rate of actin polymerization in vitro  
• interferes with antigen presentation in DCs | (Halici et al., 2008, Miao et al., 2003, Miao et al., 1999) |
| SteC | Kinase (with similarity to Raf-1) | ? | • VAP formation  
• specific colonization factor for the chick infection model | (Geddes et al., 2005, Morgan et al., 2004, Poh et al., 2008) |
**Figure 1-8: S. Typhimurium invasion of epithelial cells.** To induce its own uptake into non-phagocytic cells, *S. Typhimurium* uses a SPI-1 encoded T3SS to inject a number of effectors into the cell that promote actin and membrane remodeling that manifests as massive ruffles at the plasma membrane and culminates in bacterial uptake by a mechanism resembling macropinocytosis. First, *S. Typhimurium* secretes through the T3SS the SipB and SipC proteins that form a translocon pore in the plasma membrane of the cell. The translocon allows for the delivery of other bacterial effectors into the host cell cytosol. Other than being an integral part of the translocon, SipC also nucleates and bundles actin filaments, while a translocated *S. Typhimurium* effector SipA helps to stabilize the formed filaments. *S. Typhimurium* also translocates the SopE and SopE2 effectors that act as GEFs and catalyze nucleotide exchange on Rac1 and Cdc42 to activate these GTPases (while SopE can act as a GEF for both Rac1 and Cdc42, SopE2 seems to be selective for Cdc42). Early experiments suggested that active Cdc42 can interact with N-WASP leading to Arp2/3 activation and actin polymerization during bacterial invasion, but this idea has been called into question. Rather, it is believed that Cdc42 activation contributes mostly to induction of proinflammatory signaling while Rac1, by interacting with the multimeric WAVE2 complex via the PIR121 subunit, activates the Arp2/3 complex during invasion and promotes polymerization of branched actin filaments leading to ruffle formation. The SopB effector also contributes to host cell GTPase activation, but it does so indirectly. SopB is a phosphoinositide phosphatase that hydrolyzes PI(4,5)P2 and presumably via these phosphoinositide alterations activates SGEF, a RhoG GEF. The activation of RhoG contributes to membrane ruffling through a currently undetermined mechanism. Additionally SopB-mediated PI(4,5)P2 hydrolysis at the invasion site promotes the fission of nascent SCVs from the plasma membrane presumably by helping to detach polymerized actin at the base of invasion ruffles and stimulates antiapoptotic signaling via the activation of Akt (not shown). Finally, to restore cell surface morphology following bacterial internalization, the SptP effector acts as a GAP and deactivates the host cell GTPases involved in the bacterial invasion process, terminating actin polymerization events and membrane ruffling.
The *Salmonella*-Containing Vacuole

1. SCV trafficking

Maturation of the SCV has been extensively studied in many cell types (Steele-Mortimer *et al.*, 1999, Garcia-del Portillo *et al.*, 1993b, Garcia-del Portillo *et al.*, 1993a, Meresse *et al.*, 1999, Garcia-del Portillo *et al.*, 1995, Brumell *et al.*, 2001, Rathman *et al.*, 1997, Garvis *et al.*, 2001). This work established that *S*. Typhimurium actively modifies the SCV by manipulating host-cell factors to develop it into a unique intracellular compartment permissive for bacterial growth, distinct from a classical phagosome. Many host-cell markers that associate with this compartment were identified (recently reviewed (Steele-Mortimer, 2008)). For example, initially the SCV associates transiently with markers of early endosomes such as EEA1, the transferrin receptor, and Rab5 (Steele-Mortimer *et al.*, 1999), followed by acquisition of Rab7, lysosomal glycoproteins such as LAMP1, and the vATPase (Garcia-del Portillo *et al.*, 1993a), while excluding the M6PR which delivers lysosomal hydrolases to the endosomal system (Garcia-del Portillo *et al.*, 1995) (Figure 1-9).

2. SCV and Rab small GTPases

Members of the Rab family of small GTPases seem to be crucial players involved in controlling SCV maturation. In their GTP-bound (active) state they associate with membranes and interact with their individual (eukaryotic) effectors mediating events such as membrane fusion, fission, and tethering in a specific and highly organized fashion (Grosshans *et al.*, 2006). As *S*. Typhimurium actively remodels the SCV, it came as little surprise that the pattern of recruitment/retention of individual Rabs on the SCV is distinct from that of a model phagosome containing the ΔinvA/inv strain of *S*. Typhimurium, (which lacks a SPI-1 T3SS and is degraded in lysosomes). A “Rab screen”, comparing WT *S*. Typhimurium SCVs to this model phagosome revealed that their Rab profiles differ in several aspects (Smith *et al.*, 2007) (Figure 1-10).
Figure 1-9: SCV biogenesis. Interactions of the SCV with endosomal markers (left), compared to markers acquired by the model phagosome strain of *Salmonella*, the ΔinvA/inv strain (right).
Figure 1-10: Localization of Rab GTPases on WT or ΔinvA/inv SCVs. WT and ΔinvA/inv model phagosomes were scored for their association with 48 Rab GTPases at indicated times following internalization. The degree of shading represents the percent of vacuoles colocalizing with the Rab GTPase according to the scale shown (0 to 75%). *, P < 0.05, comparing differences between WT and ΔinvA/inv vacuoles at each time point. Figure taken from Smith et al., 2007.
First, several Rabs are acquired and retained by the SCV while absent from the model phagosome. Rab5A/B/C are present on the SCV at high levels early during invasion, while the model phagosome never acquires these Rabs to the same extent at any point following uptake. Interestingly, although *S.* Typhimurium limits the interaction of the SCV with late endosomes (LEs)/lysosomes, it does acquire Rab7 (normally associated with LEs) much earlier than the model phagosome. Importantly, the acquisition of LAMP1 and fluid endosomal content by the SCV is Rab7 dependent (Meresse *et al.*, 1999), yet *S.* Typhimurium seem to recruit Rab7 and Lamp1 but exclude other LE markers (Brumell *et al.*, 2001). It is possible that a specific Rab7+/LAMP1+ endosome, lacking LE markers such as the M6PR, is interacting with the SCVs.

However, Bujny *et al.* have proposed an alternative explanation for a lack of M6PR on the SCV. They found that sorting nexin 1 (SNX1) is recruited to WT SCVs by the action of a *S.* Typhimurium effector, SopB, and seems to remove M6PR positive membrane from this compartment (Bujny *et al.*, 2008). This implies that *S.* Typhimurium may not avoid fusion of the SCV with undesirable endocytic compartments but, alternatively, can control recycling pathways in the host cell to remove unwanted factors. Accordingly, while the model phagosome never strongly associates with Rab11A/B (implicated in endocytic recycling), the SCV does so extensively. In fact, Rab11A was shown to play a significant role in recycling of CD44 (hyaluronic acid receptor involved in adhesion) from the SCV and syntaxin 13 was implicated in a distinct recycling pathway responsible for recycling of MHC class I. Both of these recycling events proved to be important for the acquisition of LAMP1 by the SCV (Smith *et al.*, 2005). Therefore, endocytic recycling is important for SCV maturation and bacteria specifically recruit recycling factors to the SCV to remodel this compartment.

A subset of Rabs was associated with the model phagosome but excluded from the SCV. This included Rab8B, Rab13, Rab23, Rab35, and to a lesser extent Rab9 and Rab32. Intriguingly, Rab23 and Rab35 were shown to promote phagosome-lysosome fusion and the selective exclusion of these Rabs from the SCV may serve to block or delay fusion of lysosomes with the SCV (Smith *et al.*, 2007). How *S.* Typhimurium manages to control the association of specific subsets of the Rab GTPases and other factors remains to be discovered. However, Rab8B, Rab13, Rab23, and Rab35 share two
common features in their carboxy-termini that help them anchor to their target membranes in host cells: prenylation and a polybasic domain (Heo et al., 2006). As these features target them to negatively charged membranes (Heo et al., 2006), they may also provide a convenient mechanism for S. Typhimurium to exploit and bring about specific yet widespread Rab dissociation from the SCV.

3. Advanced SCV maturation

Several hours after invasion, S. Typhimurium expresses the SPI-2 T3SS to deliver effectors across the SCV. This second set of effectors directs positioning of the SCV in the perinuclear region of host cells in close proximity to the Golgi apparatus, induces the formation of elongated filaments termed Salmonella-induced filaments (Sifs) and plays a role in bacterial replication. During this maturation process, the bacteria modulate the cellular machinery by interacting with the host cell cytoskeleton, by manipulating the molecular motor proteins involved in movement along the actin or microtubule filaments and by blocking SCV fusion with lysosomes.

4. SCV and the cytoskeleton

The uptake of S. Typhimurium induces remodeling of the actin cytoskeleton at the plasma membrane. Additionally immunofluorescence experiments following polymerization of actin at later time points during S. Typhimurium invasion showed that at 4h p.i F-actin starts assembling in the vicinity of the SCV, defined as vacuole-associated actin polymerizations (VAP) (Meresse et al., 2001). By 8h p.i the actin cytoskeleton forms a nest around bacteria clusters and persists up to 16-24h. This second burst of actin polymerization is SPI-2 dependent and is important for the bacteria since actin depolymerisation agents such as latrunculin B inhibit bacterial replication. The SPI-2 effector SspH2 has been identified by a two-hybrid screen to interact with filamin and profilin, two actin-binding proteins (Miao et al., 2003) (Figure 1-11A). Profilin is a small molecule that interacts directly with monomeric G-actin and enhances actin
Figure 1-11: Modulation of host cell machinery during bacterial growth. Several hours after invasion, SCVs localize to a perinuclear region associated with the Golgi network. There the bacteria establish a ‘nest’ and modify host cell machinery using a variety of secreted virulence factors. **A**, Alterations of the host cytoskeleton. **B**, Bacterial factors that regulate actin and microtubule motors to control SCV positioning. **C**, Modulation of the endocytic pathway.
polymerization. The amino-terminal domain of SspH2 binds to filamin and its carboxy-
terminal domain to profilin. SspH2 co-localizes with actin filaments and in vitro
decreases the rate of actin polymerization. However an SspH2 deletion mutant does not
affect the formation of VAP in HeLa cells suggesting that other SPI-2 effectors may play
a role in the modulation of the host cell actin cytoskeleton.

The virulence factor SpvB has been identified as a mono ADP-ribosylating
enzyme that targets G-actin and interferes with actin polymerization (Miao et al., 2003,
Tezcan-Merdol et al., 2005, Tezcan-Merdol et al., 2001). Site-directed mutagenesis
demonstrates that the ADP-ribosylating activity of SpvB is essential for Salmonella
virulence in mice (Lesnick et al., 2001) and a SpvB deletion mutant strain of S.
Typhimurium inhibits VAP formation and actin polymerization (Miao et al., 2003).

Previous analysis reported that Sifs are formed along a scaffold of microtubules
(Brumell et al., 2002) and that the disruption of the microtubule network leads to an
inhibition of Sif formation (Garcia-del Portillo et al., 1993b). Two S. Typhimurium
effectors, SseG and SseF, have been identified as microtubule-associated proteins
required for Sif formation (Deiwick et al., 2006, Kuhle et al., 2004). These two effectors
play a role in the positioning of the SCV at the MTOC during S. Typhimurium invasion
(for review see (Ramsden et al., 2007a)). SseF and SseG are translocated to the
cytoplasm and are localized to Sifs. Both effectors colocalize with microtubules and
induce strong microtubule bundling in the host cell (Kuhle et al., 2004). However the
molecular targets of these two proteins as well as the precise mechanism by which
SseF/G modulate the microtubule network remains unknown.

5. SCV and molecular motors

In eukaryotic cells the actin cytoskeleton and the microtubule network provide
“tracks” for the transport of vesicles or cargo within the cell. Molecular motors such as
dynein and kinesin traffic along the microtubules in a movement directed towards the
minus end or the plus end of the tubules, respectively. Inhibition of the activity of either
motor prevents Sif formation and impairs S. Typhimurium replication (Guignot et al.,
As soon as 30 and 60 min p.i, *S. Typhimurium* recruits the small GTPase Rab7 to the SCV (Brumell *et al.*, 2001, Harrison *et al.*, 2004, Marsman *et al.*, 2004, Meresse *et al.*, 1999, Smith *et al.*, 2007) which interacts with the Rab7-interacting lysosomal protein (RILP) (Figure 3B). RILP recruits dynein and allows the SCV to move towards the perinuclear region of the cell at early stages of invasion (Guignot *et al.*, 2004, Harrison *et al.*, 2004, Marsman *et al.*, 2004). At later stages of the infection (>6 hours) Rab7 is associated with Sifs and is required for their formation contrary to RILP, which is absent from Sifs (Harrison *et al.*, 2004). The interaction between Rab7 and RILP has been shown to be blocked by the *S. Typhimurium* effector SifA leading to an inhibition of dynein recruitment to the SCV (Harrison *et al.*, 2004) (Figure 1-11B). SifA also interacts with the host protein SKIP (SifA and kinesin-interacting protein), which binds to kinesin (Boucrot *et al.*, 2005) (Figure 1-11B). Functional analysis of the role of SKIP revealed that its depletion, like the absence of SifA, leads to an accumulation of kinesin on the SCV, showing that SKIP uncouples kinesin from the vacuole. Furthermore, it has been shown that the SPI-1 effector SipA, which persists several hours after bacterial entry, cooperates with SifA to down-regulate kinesin recruitment and to ensure perinuclear positioning of the SCV (Brawn *et al.*, 2007). Although SifA modulates the recruitment of kinesin to the SCV through its interaction with SKIP, a second SPI-2 effector, PipB2, influences the SCV-kinesin interaction. Indeed, PipB2 triggers the recruitment of kinesin-1 to the SCV, acting antagonistically from the SifA-SKIP complex (Brawn *et al.*, 2007, Henry *et al.*, 2006b). The recruitment of opposing microtubule motors appears to be very dynamic and controlled by different *S. Typhimurium* effectors.

Recently myosins, which interact with the actin cytoskeleton, have been implicated in SCV dynamics (Wasylko *et al.*, 2008). The actin-based motor myosin II was found to be recruited to the SCV and played a role in the positioning of the SCV during infection. Inhibition of myosin II, like inhibition of actin polymerization or mutation of the SPI-1 effector SopB, prevents the positioning of the SCV in the perinuclear region. Activation of myosin II is regulated by phosphorylation of the myosin light chain (MLC) by multiple kinases including ROCK. The authors showed that activation of myosin II by overexpressing constitutively active mutants of Rho GTPases, RhoA, Cdc42, RhoG or ROCK, complements the positioning of ΔsopB *S. Typhimurium*.
Moreover SopB was sufficient to induce myosin II phosphorylation, suggesting that SopB modulates SCV positioning through the activation of Rho GTPases and myosin II.

6. SCV and the endocytic pathway

During maturation the SCV interacts with late endosomes, acidifies gradually and acquires some LE/lysosomal markers (discussed above). However, studies of interactions of the late endosomal system with SCVs in macrophages and epithelial cell lines have yielded conflicting results. While the majority of research supports the concept that the SCV is segregated from LE/lysosomes (Hashim et al., 2000, Ishibashi et al., 1990, Buchmeier et al., 1991, Carrol et al., 1979, Rathman et al., 1997), others disagree (Oh et al., 1996). Most recently, based on data acquired by live cell imaging using Dextran as a general marker of the lysosomal compartment, Drecktrah et al. found that the SCV extensively interacts with the endosomal system and associates significantly with lysosomes in HeLa cells as soon as 30 min p.i. and up to several hours after invasion in a vATPase, microtubule and Rab7 dependent manner (Drecktrah et al., 2007). The authors of this study proposed that delay rather than avoidance of lysosome fusion is the key step in establishment of a replicative niche by *S. Typhimurium*. However, the difference between late endosomes and lysosomes remains hard to define as they share many markers and a common morphology, with the true distinction derived from their nature as non-degradative and degradative compartments, respectively.

The SPI-2 effector SpiC contributes to the inhibition of the cellular trafficking by *S. Typhimurium* (Uchiya et al., 1999) (Figure 1-11C). By examining the proteolysis of an internalized ligand the authors showed that in macrophages *S. Typhimurium* interferes with the normal cellular trafficking to the lysosomes in a SpiC dependent manner. SpiC interacts with the host protein Hook3 which is involved in positioning of the Golgi complex by linking Golgi membranes to microtubules (Shotland et al., 2003, Walenta et al., 2001). Overexpression of SpiC induces the disruption of the Golgi apparatus and an abnormal distribution of lysosomes similar to the effect of overexpression of a dominant-negative construct of Hook3. SpiC, via its interaction with Hook3, may alter the lysosomal compartment and thereby block SCV-lysosome fusion.
7. SCV and the secretory pathway

If the maturation of the SCV implies interaction with the endocytic route it has also been shown that the vacuole receives cargo from the Golgi apparatus (Kuhle et al., 2006) (Figure 1-11C). Following the transport of the vesicular stomatitis virus glycoprotein (VSVG) from the Golgi to the plasma membrane the authors observed an accumulation of VSVG on the SCV. This re-routing of VSVG towards the SCV is induced by S. Typhimurium since chloramphenicol treatment, which inhibits bacterial protein synthesis, decreases that accumulation. Moreover the SPI-2 effectors SifA, SseF and SseG are involved in the recruitment of VSVG to the vacuole. More recently the small GTPase Rab9 involved in the retrograde transport of cargo from endosomes to the Golgi (Lombardi et al., 1993) has been shown to be recruited to the SCV and Sifs (Smith et al., 2007). The expression of a dominant-negative construct of Rab9 inhibits the formation of Sifs suggesting that the transport of cargo from the vacuole to the Golgi is important for S. Typhimurium.

8. SCV and cholesterol

The maturation of the vacuole and the formation of Sifs induce the accumulation of cholesterol on bacterial associated membranes and modify levels of host cholesterol biosynthetic intermediates (Brumell et al., 2001, Catron et al., 2002). Further analysis of the interaction between S. Typhimurium and the cholesterol biosynthetic pathway showed that inhibition of the first steps of the sterol biosynthetic pathway using lovastatin, which blocks the conversion of 3-hydroxy-3methylglutaryl coenzyme reductase into mevalonate, inhibits intracellular bacterial proliferation in macrophages as well as in a mouse model (Catron et al., 2004). The SPI-2 effector SseJ is a protein with a deacylase activity and is homologous to a family of enzymes called glycerophospholipid-cholesterol acyltransferases (Ohlson et al., 2005). SseJ has been found to localize to the vacuole as well as along Sifs. Recently it has also been shown that SseJ esterifies cholesterol during infection of S. Typhimurium and increases the formation of lipid
droplets in infected cells (Nawabi et al., 2008). It also modifies the association of SifA with cholesterol structures and Lamp1 positive vesicles. The modification of cholesterol levels by S. Typhimurium seems to be a novel strategy to interfere with the trafficking of the host cell to promote bacterial proliferation.

9. SCV and antigen presentation

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) and are at the junction between innate and adaptive immune responses. DCs have the ability to activate T cells by presenting at their cell surface peptides derived from lysosomal degradation via the major-histocompatibility-complex II (MHC-II). This activation of the immune system is a particularly interesting target for intracellular pathogens such as S. Typhimurium to escape an adaptive immune response. In human or murine DCs, it has been shown that infection by WT S. Typhimurium decreases the capacity of DCs to activate T cells as well as their capacity to present antigens (Cheminay et al., 2005, Mitchell et al., 2004, Tobar et al., 2006). S. Typhimurium infection does not affect the viability of DCs, their maturation or antigen-uptake capability (Cheminay et al., 2005), but instead it decreases the presentation of peptides at the infected cell plasma membrane via MHCII. Further investigations in human cells showed that the biosynthesis of the class II complex as well as the loading of peptides was not affected by S. Typhimurium invasion (Mitchell et al., 2004). In contrast, the authors observed an accumulation of mature molecules in the cytoplasm during S. Typhimurium infection. Different effectors of S. Typhimurium have been implicated in this inhibition of the antigen presentation by DCs. SifA, in human cells (Mitchell et al., 2004), and the effectors SpiC and SpiA, in murine DCs (Tobar et al., 2006), are thought to be involved in interfering with MHC-II antigen presentation. However the precise mechanism by which S. Typhimurium modulates the host machinery to block the trafficking of the complex from the cytoplasm to the plasma membrane remains unknown.
The SopD Effector and its role in *Salmonella* pathogenesis

SopD is an effector that can be translocated into host cells by the SPI-1 and SPI-2 T3SSs (Brumell *et al*., 2003). The *sopD* gene is present in the last common ancestor of all contemporary *Salmonella* species and is conserved in all ‘modern’ *Salmonella* lineages (Mirold *et al*., 2001). SopD does not exhibit sequence homology to any other protein in the non-redundant sequence database except to SopD2 (41% identity), a *Salmonella* effector translocated by the SPI-2 type III secretion system (Brumell *et al*., 2003). SopD2 associates with late endocytic compartments upon delivery into host cells (Brumell *et al*., 2003) and contributes to the formation of *Salmonella*-induced filaments (Sifs) (Jiang *et al*., 2004), a phenotype associated with rapidly replicating *S.* Typhimurium (Birmingham *et al*., 2005). Unlike SopD2, SopD has been reported to localize to the cytosol of fixed HeLa cells and does not contribute to Sif formation (Brumell *et al*., 2003, Jiang *et al*., 2004). However, both SopD and SopD2 promote replication of *S.* Typhimurium in mouse macrophages and contribute to virulence (Jiang *et al*., 2004) and persistence of bacteria in a mouse model of systemic infection (Lawley *et al*., 2006). In addition to promoting systemic disease, SopD was shown to act in concert with SopB to induce fluid secretion and inflammation (neutrophil influx) in a bovine ligated ileal loop model of infection (Jones *et al*., 1998). The mechanism by which SopD contributes to gastroenteritis *in vivo* remains unclear. In principal, SopD may activate pro-inflammatory signaling pathways, as proposed for SopB (Steele-Mortimer *et al*., 2000), and localization of SopD to the cytosol might be consistent with this notion. Alternatively, SopD may play a role in invasion. Indeed, it was recently suggested that SopD contributes to *S.* Typhimurium invasion of polarized T84 human colon carcinoma cells (Raffatellu *et al*., 2005). Thus, despite the importance of SopD in many aspects of *S.* Typhimurium virulence, its role remains undetermined.

The SopB Effector and its role in *Salmonella* pathogenesis

SopB (also known as SigD) is an effector, encoded by SPI-5, and translocated by the SPI-1 T3SS. It plays a role in enteropathogenesis, being required for fluid secretion
and neutrophil influx into infected calf ileal loops (Galyov et al., 1997). Although it is translocated into the host cell early during invasion, it can be maintained within the cell following bacterial internalization for up to 12 h post infection (p.i.) (Drecktrah et al., 2005, Marcus et al., 2002) and may participate in late stages of systemic infection in mice (Giacomodonato et al., 2007). SopB is an inositol phosphate phosphatase and is a homologue of the *Shigella flexneri* effector IpgD, sharing with it two motifs similar to ones found in mammalian inositol 4-phosphatases (Norris et al., 1998). *In vitro*, SopB can dephosphorylate a number of soluble inositol polyphosphates and inositol phospholipids (Feng et al., 2001, Norris et al., 1998, Zhou et al., 2001). Although the extent of SopB’s true *in vivo* targets is not clear (Table 1-3), currently PI(4,5)P$_2$ is emerging as a key substrate of this effector (Mallo et al., 2008, Mason et al., 2007, Terebiznik et al., 2002) and it is becoming apparent that via its lipid modifying activity SopB affects many processes during *S. Typhimurium* infection, including: 1) host-cell nuclear responses, 2) the invasion process itself and 3) SCV maturation.

1. Nuclear Responses

SopB is an important mediator of nuclear responses of host cells. By controlling phosphoinositide signaling during invasion it can activate a prosurvival serine-threonine kinase, Akt, thereby protecting infected epithelial cells from apoptosis (Knodler et al., 2005a, Steele-Mortimer et al., 2000). Redundantly with two other *S. Typhimurium* effectors, SopE and SopE2, SopB can also induce nuclear responses mediated by Cdc42 activation, such as the production of a proinflammatory cytokine, IL-8 (Patel et al., 2006). While SopE/E2 mimic guanine nucleotide exchange factors (GEFs) and can activate Rho GTPases like Cdc42 directly (Friebel et al., 2001), SopB is thought to rely on its ability to affect phosphoinositide levels to activate its host-cell targets. In fact, SopB’s only identified host cell protein binding partner is Cdc42 (Rogers et al., 2008b). It has therefore been proposed that the interaction between Cdc42 and SopB could serve to localize SopB to areas of Cdc42 concentration where the effector can act on phosphoinositides (Rogers et al., 2008b).
Table 1-3: Proposed SopB substrates

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Evidence</th>
<th>Proposed Effect</th>
<th>Reference(s)</th>
</tr>
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<tbody>
<tr>
<td><strong>Direct Substrates:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdc42</td>
<td>• direct binding demonstrated by IP</td>
<td>• binding does not activate Cdc42 directly, therefore may serve to localize SopB</td>
<td>(Rogers et al., 2008b)</td>
</tr>
</tbody>
</table>
| PI(4,5)P₂ | • *in vitro* assay  
• significant changes observed *in vivo* (WT versus ΔsopB infection) using HPLC  
• loss of plasma membrane localization of PLCδ-PH-mRFP during invasion | • actin remodeling at invasion site  
• suggested that PI(5)P produced leads to generation of PI 3-kinase products by unknown mechanism | (Mallo et al., 2008, Marcus et al., 2001, Mason et al., 2007, Terebiznik et al., 2002) |
| Ins(1,3,4,5,6)P₅ | • *in vitro* effect of GST-SopB  
• *in vivo* effect | • creation of Ins(1,4,5,6)P₅ proposed to indirectly activate Cdc42  
• Ins(1,4,5,6)P₅ antagonizes PIP₃-mediated closing of cellular chloride channels (increased chloride secretion) | (Norris et al., 1998, Zhou et al., 2001) |
| Ubiquitin | • coIP | • not involved in promoting proteasomal degradation  
• ubiquitination alters localization of SopB from plasma membrane to SCV | (Marcus et al., 2002, Rogers et al., 2008b, Patel et al., 2009) |
| **Indirect Substrates:** | | | |
| AKT | • no phosphorylation in absence of SopB during infection of epithelial cells | • leads to anti-apoptotic effects | (Knodler et al., 2005a, Steele-Mortimer et al., 2000) |
| SGEF (GEF for RhoG) | • identified in an siRNA screen  
• ΔsopB SCV positioning complemented by constitutively active mutant of RhoG | • active RhoG promotes actin rearrangements at invasion site  
• RhoG can activate ROCK/MLC signaling leading to myosin II activation and altered SCV positioning | (Patel et al., 2006, Wasylnka et al., 2008) |
| Cdc42 | • binding of SopB to Cdc42 doesn’t activate it  
• Ins(1,4,5,6)P₅ is an indirect activator of Cdc42  
• ΔsopB SCV positioning complemented by constitutively active mutant of Cdc42 | • stimulation of nuclear responses such as IL-8 transcription  
• Cdc42 can activate ROCK/MLC signaling leading to myosin II activation and altered SCV positioning | (Patel et al., 2006, Rogers et al., 2008b, Wasylnka et al., 2008) |
Table 1-3: Proposed SopB substrates (cont’d).

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Evidence</th>
<th>Proposed Effect</th>
<th>Evidence Against</th>
<th>Reference(s)</th>
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<tbody>
<tr>
<td>Proposed but unlikely substrates:</td>
<td></td>
<td></td>
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<tr>
<td>PI(3,5)P₂</td>
<td>• in vitro assay</td>
<td>• speculated that this may alter trafficking of SCVs and lead to accumulation of PI(3)P on them</td>
<td>• SopB promotes PI(3)P formation via Rab5/Vps34 recruitment • changes in PI(3)P too large to be accounted for by dephosphorylation of relatively tiny pool of PI(3,5)P₂ • no significant change observed by HPLC during WT versus ΔsopB infections</td>
<td>(Mallo et al., 2008, Marcus et al., 2001)</td>
</tr>
<tr>
<td>PI(3)P</td>
<td>• in vitro effect of GST-SopB</td>
<td>• -</td>
<td>• in vitro assay • also see above counterarguments for PI(3,5)P₂</td>
<td>(Marcus et al., 2001, Norris et al., 1998)</td>
</tr>
<tr>
<td>PI(4)P</td>
<td>• in vitro assay</td>
<td>• -</td>
<td>• significant changes not observed in vivo (WT versus ΔsopB infection) using HPLC</td>
<td>(Marcus et al., 2001, Mason et al., 2007)</td>
</tr>
<tr>
<td>PI(5)P</td>
<td>• in vitro assay</td>
<td>• -</td>
<td>• increase, not decrease, observed in vivo (WT versus ΔsopB infection) using HPLC</td>
<td>(Marcus et al., 2001, Mason et al., 2007)</td>
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<tr>
<td>PI(3,4)P₂</td>
<td>• in vitro effect of GST-SopB • in vitro assay</td>
<td>• -</td>
<td>• PI(3,4)P₂ levels are increased during WT but not ΔsopB infection</td>
<td>(Mallo et al., 2008, Marcus et al., 2001, Norris et al., 1998)</td>
</tr>
<tr>
<td>PI(3,4,5)P₃</td>
<td>• in vitro effect of GST-SopB • in vitro assay</td>
<td>• degradation increases chloride secretion</td>
<td>• PI(3,4,5)P₃ levels are increased during WT but not ΔsopB infection</td>
<td>(Mallo et al., 2008, Marcus et al., 2001, Norris et al., 1998)</td>
</tr>
<tr>
<td>Ins(1,3,4)P₃</td>
<td>• in vitro effect of GST-SopB</td>
<td>• -</td>
<td>• not observed in vivo</td>
<td>(Norris et al., 1998, Zhou et al., 2001)</td>
</tr>
<tr>
<td>Ins(1,3,4,5)P₄</td>
<td>• in vitro effect of GST-SopB</td>
<td>• -</td>
<td>• not observed in vivo</td>
<td>(Norris et al., 1998, Zhou et al., 2001)</td>
</tr>
</tbody>
</table>
2. Invasion

During *S. Typhimurium* invasion, a set of effectors is delivered via the SPI-1 T3SS into the host-cell cytosol and their concerted action leads to generation of prominent ruffles at the cell surface and spacious vacuole (macropinosome) formation due to extensive actin and membrane rearrangements. Although not strictly essential for *S. Typhimurium* entry, SopB, like SopE/E2, mediates actin cytoskeleton rearrangements to promote bacterial invasion in a Rho-dependent manner (Zhou *et al.*, 2001). A recent RNAi approach revealed that SopE mediated actin rearrangements in a Rac1-, but not Cdc42-dependent manner (Hardt *et al.*, 1998) and SopB remodeled actin independently of Rac1 (Patel *et al.*, 2006). SopB and SopE were also found to activate RhoG during infection. SopE acting as a GEF could do so directly, but SopB activated SGEF, a ubiquitously expressed GEF for RhoG, potentially via phosphoinositide fluxes. Activation of RhoG by SopB seemed to account for most of SopB’s actin remodeling activity (Patel *et al.*, 2006).

At the time of invasion when actin remodeling occurs, SopB also hydrolyzes PI(4,5)P₂ at the host-cell plasma membrane. Another invasion-associated *S. Typhimurium* effector, SipC, was found to cluster PI(4,5)P₂ in the plasma membrane and could in this way enhance phospholipid availability to SopB at bacterial invasion sites (Cain *et al.*, 2008). As SopB-mediated disappearance of PI(4,5)P₂ coincides with vesicle fission, it may facilitate sealing of membranes by removing actin and associated proteins from the base of bacterially induced membrane ruffles (Terebiznik *et al.*, 2002). Alternatively, products of PI(4,5)P₂ catabolism may alter membrane curvature and/or its fusion properties. Counter intuitively, PI(4,5)P₂ hydrolysis leads to an enrichment of PI(3,4,5)P₃ and phosphatidylinositol-3,4-bisphosphate [PI(3,4)P₂] at the invasion site (Mallo *et al.*, 2008) (Figure 1-8 and 1-11). Although the mechanism of this event is not known, it is possible that PI(5)P, which is the product of SopB-mediated PI(4,5)P₂ hydrolysis (Mason *et al.*, 2007) can activate a PI3-K and/or inhibit a specific lipid phosphatase, allowing for the generation of PI3-K products (Pendaries *et al.*, 2006).
3. SCV maturation

In addition to affecting phosphoinositide levels at the plasma membrane, SopB was also found to be required for the formation of PI(3)P on nascent SCVs (Hernandez et al., 2004). SopB together with another S. Typhimurium effector, SopD, is responsible for macropinosome formation during invasion (Bakowski et al., 2007, Hernandez et al., 2004) and it has been suggested that these are formed through homotypic fusion of PI(3)P positive endosomes (Hernandez et al., 2004). Although it has been proposed that SopB directly contributes to the formation of PI(3)P on the SCVs via hydrolysis of PI(3,4,5)P$_3$ and PI(3,4)P$_2$ (Hernandez et al., 2004), a recent study by Mallo et al suggests otherwise (Mallo et al., 2008). Here, the authors found that the formation of PI(3,4,5)P$_3$ and PI(3,4)P$_2$ can be uncoupled from the formation of PI(3)P. Instead, SopB was found to recruit Rab5 to the SCVs, which in turn recruited Vps34, a PI3-K that phosphorylated PI to form PI(3)P (Figure 1-12). The mechanism of Rab5 recruitment to invasion associated membranes is not known, but evidence suggests that effects on membrane charge brought about by hydrolysis of PI(4,5)P$_2$ (reviewed in (Yeung et al., 2007)) may serve to recruit this GTPase (Mallo et al., 2008).

The effect of SopB on early SCV composition via recruitment of Rab5 and PI(3)P, as compared to a model phagosome, indicates that SopB plays a role in directing the SCV down a maturation pathway towards a replicative niche. Indeed, mounting evidence indicates that SopB controls the trafficking of the SCV. First, Hernandez et al found that sopB mutant SCVs acquired less LAMP1 than WT, and sopB mutant growth in macrophages was impaired (Hernandez et al., 2004). Infection with WT bacteria also inhibited epidermal growth factor receptor (EGFR) degradation, while infection with the sopB mutant did not, implying that SopB blocks the normal endocytic maturation pathway (Dukes et al., 2006). As already mentioned, SNX1 tubular-based re-modeling of the SCVs is SopB-dependent (Bujny et al., 2008).

Lastly, the activation of Akt by SopB was proposed to prevent phagosomal maturation by deactivating AS160, a Rab14 GAP (Kuijl et al., 2007). In the human breast cancer cell line MCF7, activation of Rab14 on the SCV via this mechanism led to an increase in intracellular growth of bacteria by potentially delaying SCV-lysosome fusion
Figure 1-12: Phosphoinositide transformations during *Salmonella* invasion mediated by the SopB effector. SopB hydrolyzes PI(4,5)P$_2$ at the plasma membrane leading to formation of PI(3,4)P$_2$ and PI(3,4,5)P$_3$. SopB also recruits Rab5 to the SCV, which in turn recruits a PI3-K, leading to PI(3)P accumulation. Differences between maturation of SCVs containing WT (left) and ΔsopB mutant bacteria (right) are highlighted.
(Kuijl et al., 2007). However, in previous work using HeLa cells, no recruitment of GFP-Rab14 to the WT SCV was observed (Smith et al., 2007). Differences in cell types used for each study may have contributed to these discrepant results and the effect of Rab14 on SCV maturation needs to be explored further. Regardless, the effect of SopB on SCV maturation cannot be disputed and we are just beginning to elucidate the mechanisms involved in this process.

**THESIS SUMMARY**

In this thesis I investigate the functions of the SopD and SopB effectors during bacterial infection, both of which contribute significantly to bacterial virulence. My examination of the role of SopD revealed that this effector associates with host-cell membranes during invasion, a previously unrecognized phenotype. This discovery prompted studies into the involvement of SopD in membrane-associated invasion events, establishing SopD’s contribution to membrane sealing and macropinosome formation. In contrast we found no evidence for stimulation of signaling cascades by SopD. This data indicates that SopD contributes to *S. Typhimurium* infection by stimulating bacterial invasion.

SopB is a phosphoinositide phosphatase that promotes *S. Typhimurium* invasion and activates pro-survival and pro-inflammatory signals in infected cells. The importance of membrane charge during phagocytosis has been recently investigated, which prompted our interest in potential effects of SopB, as a lipid phosphatase, on membrane charge. We have discovered that SopB indeed affected membrane charge, and by dephosphorylating PI(4,5)P₂ reduced the negative membrane charge of nascent SCVs. This had a radical influence on subsequent SCV trafficking, decreasing the incidence of lysosome fusion with SCVs. Furthermore, we provide evidence that targeting of Rabs via electrostatic interactions allows SopB to remodel the SCV into an excellent replicative niche.

Chapter 1 of this thesis contains a published review paper. Chapter 3 contains a published paper, the abstract and introduction of which has been shortened into a
Summary at the beginning of the chapter. Published supplementary figures have been incorporated into chapter figures. Materials and Methods for Chapter 3 and Chapter 4 have been combined in Chapter 2. Chapter 4 contains unpublished data: Virginie Braun contributed Figure 4-1B, while data presented in Figure 4-3 was gathered with the help of Grace Y. Lam. A discussion for this thesis can be found in Chapter 5.
CHAPTER 2
MATERIALS AND METHODS

Bacterial Strains

WT S. Typhimurium SL1344 (Hoiseth et al., 1981) was used in this study. Wild-type-expressing mRFP was previously described (Birmingham et al., 2006). The isogenic ΔsopD (Jiang et al., 2004), ΔsopB (Steele-Mortimer et al., 2000), ΔsopB + psopB, ΔsopB + psopB* (the C462S catalytically inactive mutant of sopB)(Steele-Mortimer et al., 2000), and ΔinvA/pRI203 (ΔinvA/inv) 14028S(Steele-Mortimer et al., 2002) were previously described. The ΔsopB mutant complemented with the S. flexneri chaperone ipgE and effector ipgD (ΔsopB+pipgD), or the catalytically inactive (C439S) mutant of ipgD (ΔsopB+pipgD*) was previously described (Marcus et al., 2001). The ΔsopB+GFP strain was constructed by transforming the ΔsopB mutant with a plasmid expressing EGFP under the rpsM promoter, pFPV25.1 (Valdivia et al., 1996).

To construct the double ΔsopBΔsopD mutant (strain NB43) pΔsopD was transferred from E. coli SM10 λpir (Miller et al., 1988) into ΔsopB via conjugation and presumptive merodiploid clones were selected as being resistant to streptomycin and chloramphenicol. These clones were then grown without chloramphenicol selection and plated on LB agar lacking NaCl and containing 5% sucrose, and incubated at 30°C overnight. The resulting chloramphenicol-sensitive clones were genotyped by PCR to confirm the presence of a ΔsopD allele and a lack of a wild-type sopD allele.

Tissue Culture

HeLa (human epithelial cell line) cells and Henle-407 (human embryonic intestinal cell line) were obtained from ATCC. Cells were maintained in high glucose DMEM (HyClone) supplemented with 10% FBS (Wisent) at 37 °C in 5% CO₂ without antibiotics. Cultures were used between passage numbers 5-25. HeLa cells were seeded (on 1 cm or 2.5 cm coverslips, except for cells destined for Western analysis) in 24-well tissue culture plates at 2.5 x 10⁴ cells/well or in 6-well tissue culture plates at 10 x 10⁴
cells/well 40-45 h before use. Henle-407 cells were seeded on 1 cm coverslips in 24-well tissue culture plates at 4 x 10^4 cells/well or on 2.5 cm coverslips in 6-well tissue culture plates at 16 x 10^4 cells/well 40-45 h before use. For DQ-BSA assays cells were seeded at 1.6 x 10^4 cells/well in 8-well coverglass chambers (Lab-Tek™ Chambered Coverglass, Nunc) 40-45 h before use or at 3.2 x 10^4 cells/well 20-24 h before use.

**Plasmids**

The psoD-2HA plasmid used to express SopD-2HA in ΔsopD mutant S. Typhimurium was previously described (Brumell et al., 2003). The low copy plasmid pACYC184 (NCBI accession #X06403) was used to express SopD in ΔsopD mutant S. Typhimurium. Primers JB099 (5’-CGC GGA TCC TTA TGT CAG TAA TAT ATT ACG ACT GCA-3’) and JB0100 (5’-ACG CGT CGA CCG CCT TTT CAA CAG GAA GCG CTC A-3’) were used to amplify sopD and its upstream promoter. The PCR product was digested with BamHI and SalI (sites underlined in JB099 and JB0100) and cloned into corresponding sites in pACYC184.

The SopD-GFP and SopD2-GFP constructs have been previously described (Brumell et al., 2003). Amino-terminus, carboxy-terminus, and 1-270 truncation mutants of SopD fused to GFP were generated by PCR amplification from the pSopD-GFP construct using the following primers: for SopD amino-terminus (amino acids 1-150), MAB015 (5’-CTA GAA TTC TGA GCA TGC CAG TCA CTT TAA GC-3’) and MAB016 (5’-CGG GGA TCC AAT ATT TGT AAA AAT AAT TGT CTC TC-3’); for SopD carboxy-terminus (amino acids 150 – 317), MAB013 (5’-CGA GAA TTC TGA GCA TAT GTG AGG TGA TTG G-3’) and MAB014 (5’-CAT GGA TCC AAT GTC AGT AAT ATA TTA CGA CTG C-3’); for SopD1-270 (amino acids 1 – 270), MAB015 described above and MAB038 (5’-CGC GGA TCC CGA AAA CCA GAA TTT GTT GGT TTA AAC-3’). After digestion with EcoRI and BamHI (sites underlined above), the PCR products were cloned into the multiple cloning region of the pEGFP-N1 N-terminal protein fusion vector from Clontech (Palo Alto, CA, USA). The GFP-SopD construct (with GFP at the amino-terminus of SopD) was generated by PCR amplification from the pSopD-GFP construct using MAB011 (5’-CGC GAA TTC TAT GCC AGT
CAC TTT A-3’) and MAB012 (5’-CGA GGA TCC TTA TGT CAG TAA TAT ATT AC-3’) primers. After digestion with EcoRI and BamHI (sites underlined above), the PCR products were cloned into the multiple cloning region of the pEGFP-C1 carboxy-terminal protein fusion vector from Clontech (Palo Alto, CA, USA). To generate W37A and F44A point mutations in SopD, QuikChange Site-Directed Mutagenesis (Stratagene) was performed according to manufacturers directions with the following primers: for SopDW37A, MAB033 (5’-GCA ATC CAT ATG GGG GGT GCC GAT AAA GTC CAG GAT C-3’) and MAB034 (5’-GAT CCT GGA CTT TAT CGG CAC CCC CCA TAT GGA TTG C-3’) primers; for SopDF44A, MAB035 (5’-GAT AAA GTC CAG GAT CAT GCC AGA GCG GAA AAA AAG GAC C-3’) and MAB036 (5’- GGT CCT TTT TTT CCG CTC TGG CAT GAT CCT GGA CTT TAT C-3’) primers. The SopD-RFP and SopD2-RFP constructs were made by PCR amplification of mRFP from the pBR-RFP.1 construct (Birmingham et al., 2006) using primers RFP5newA (5’-CGC GGA TCC GGT TAT GGC CTC CTC CGA GGA CGT CAT CAA GG-3’) and RFPnew (5’-ATA AGA ATG CGG CCG CTT AGG CGC CGG TGG AGT GGC GGC GGC C-3’). After digestion with BamHI and NotI (sites underlined above), the PCR product was cloned into the corresponding sites in SopD-GFP and SopD2-GFP, thereby exchanging the GFP encoding sequence in these constructs for the mRFP encoding sequence.

Rab5A-GFP was a gift from the lab of Dr. Craig Roy (Yale University). GFP-Rab7 and LAMP1-GFP were a gift from Dr. Jean-Pierre Gorvel (Centre d’Immunologie de Marseille-Luminy). Rab9-GFP was a gift from Dr. Suzanne Pfeffer (Stanford University School of Medicine) (Barbero et al., 2002). RFP-LC3 was a gift from Dr. Walter Beron. GFP fused to myristoylation and palmitoylation sequences from Lyn (PM-GFP) (Teruel et al., 1999), PLCδ-PH-mRFP (Yeung et al., 2006b) were described previously and 2xFYVE-mRFP, consisting of two tandem FYVE domains from EEA1 conjugated to mRFP, was a generous gift from Dr. Gustavo Mallo and Dr. Sergio Grinstein (The Hospital for Sick Children, Toronto). FuGene6 transfection reagent (Roche) was used for transient transfection of cells with plasmid DNA according to the manufacturer's instructions and cells were used between 12 and 16 h after transfection.

The plasmid encoding RpreRed and LactC2-GFP were previously described (Yeung et al., 2007), as were GFP-Rab8B, 13, 23, and 35 (Smith et al., 2007) and CFP-
Rab35, CFP-CT, CFP-CTΔGG, CFP-CTcaax, and CFP-CTcaaxΔpB (Heo et al., 2006). CFP-Rab7 (Barbero et al., 2002), GFP-Rab22A (Weigert et al., 2004), and GFP-Rac1 (Michaelson et al., 2001) were previously described. GFP-Rab14 was from J. Casanova (University of Virginia). inp54-YFP is a plasmid encoding the amino-terminal 331 amino acids of Inp54p, a yeast inositol polyphosphate 5-phosphatase(Suh et al., 2006). This fragment retains Inp54p’s full phosphatase activity and is fused to a domain from FK506 binding protein (FKBP) (Suh et al., 2006) and YFP. Lyn11-FRB (LDR) is a plasma membrane targeted FKBP12-rapamycin binding domain of mTOR, described previously (Suh et al., 2006). GeneJuice transfection reagent (Oncogene Research Products) was used for transient transfection of cells with plasmid DNA according to the manufacturer’s instructions and cells were used between 12 and 16 h after transfection.

For FLAsH labeling experiments SopD tagged with the optimized tetra cysteine (4C) motif (AGSFLNCCPGCCMEPGGR) (Enninga et al., 2005) was generated by PCR amplification from the p sosD plasmid used in this study. Primers MAB024 (GGT TGT TGT ATG GAA CCA GGT GGT CGT TAA GGA TCC ACA GGA CGG GTG TGG) and MAB 025 (AGG GCA GCA GTT TAG AAA AGA ACC GGC GGC CGC TGT CAG TAA TAT ATT ACG ACT GCA CC) were used to amplify the entire plasmid, adding half of the 4C sequence onto each end of the PCR product. The PCR product was kinase treated and ligated together, resulting in the full 4C sequence at the carboxy-terminus of SopD. Primers MAB026 (CCA GAA TTC ACC ATG CCA GTC ACT TTA AGC TTC G) and MAB027 (CCG TCT AGA TTA ACG ACC ACC TGG TTC C) were used to amplify the SopD-4C sequence. The PCR product was digested with EcoRI and XbaI (sites underlined above) and cloned into corresponding sites in pBAD24 (Guzman et al., 1995). The generated p sosD-4C plasmid was transformed into ΔsopD S. Typhimurium.

Bacterial Infections

Late-log S. Typhimurium cultures were used for infecting cells and prepared using a method optimized for bacterial invasion (Steele-Mortimer et al., 1999). Briefly, wild-type and mutant bacteria were grown for 16 h at 37°C with shaking and then subcultured (1:33) in LB without antibiotics for 3 h. Bacterial inocula were prepared by pelleting at
10,000 x g for 2 min, diluted 1:100 in PBS, pH 7.2, and added to cells for 10 min at 37°C. After infection, extracellular bacteria were removed by extensive washing with PBS and 100 µg/mL gentamicin was added to the medium at 30 min p.i. Following 90 min of bacterial infection, the gentamicin concentration was decreased to 10 µg/mL.

**Pharmacological Agents**

LY 294002 was used at final concentration of 100 µM. ATP depletion was carried out by incubating cells with 10 mM 2-deoxy-d-glucose (Sigma) and 200 nM antimycin A (Sigma) in PBS. Folimycin (concanamycin A) (Sigma) was used at a final concentration of 1 µM. All treatments were at 37°C. To detect acidic compartments in SopD-GFP transfected cells, the cells were incubated in RPMI-1640 in the presence of 75 nM LysoTracker (Molecular Probes) for 30 min prior to imaging. Rapamycin (Cedarlane, Biomol) was used at a final concentration of 10 µm.

**Immunofluorescence**

Cells were fixed in 2.5% paraformaldehyde in PBS pH 7.2 for 10 min at 37°C and immunostained to differentiate between intracellular and extracellular bacteria. Fixed cells were washed twice with PBS and blocked by treatment with PBS containing 10% normal goat serum (S-PBS) for 30–60 min. Primary and secondary antibodies were overlaid on coverslips in S-PBS for 30 min to 1h, followed by three washes with PBS. Cells were then permeabilizedblocked by treatment with 0.2 % saponin (Calbiochem, San Diego, CA) in PBS containing 10 % normal goat serum (SS-PBS) for 30–60 min. Primary and secondary antibodies were overlaid on coverslips in SS-PBS for 30 min to 1h, followed by three washes with PBS. Coverslips were mounted onto 1 mm glass slides using Fluorescent Mounting Medium (DakoCytomation) and colocalization quantifications were performed by direct visualization on a Leica DMIRE2 epifluorescence microscope. Bacteria were considered to be positively colocalizing with a fluorescent marker when >50 % of the bacterial circumference was surrounded by a signal recognizable by eye as brighter than background. Strongly transfected, very bright
cells were avoided during quantification. At least 100 bacteria were counted per condition in each experiment, and at least three independent experiments were performed. The mean ± S.E.M are shown in the figures.

**Antibodies**

Rabbit polyclonal antibodies to *S. Typhimurium* O antiserum Group B were obtained from Difco. Murine monoclonal anti-GFP antibody 49005A was obtained from Invitrogen. Murine monoclonal anti-human LAMP-1 antibodies (clone H4A3) developed by T. August were obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Secondary antibodies used for immunofluorescence were all Alexa conjugated and obtained from Molecular Probes.

For Western blot analysis the following mouse monoclonal antibodies were used: anti-phospho-p44/42 MAPK (Thr202/Tyr204) E10, anti-phospho-p38 MAPK(Thr180/Tyr182), anti-phospho-c-Jun(Ser73), anti-phospho-Akt (Ser473) (all from Cell Signaling Technology, Danvers, MA), and anti-phospho PKCδ (Thr505) (Calbiochem, San Diego, CA), and anti-β-actin Clone AC-15 (Sigma). Secondary antibody was the anti-murine HRP (Jackson ImmunoResearch, West Grove, PA).

**NHS-647 and FITC Labeling of Bacteria**

NHS-647 (Alexa Fluor 647 carboxylic acid, succinimidyl ester; Molecular Probes) spectrally identical to the Cy5 dye, was used to label live bacteria prior to infection and imaging. Briefly, 200 µL of late log bacterial suspension was extensively washed with PBS and incubated with 0.3 mg/mL NHS-647 for 5 min at 37°C, washed in PBS, and resuspended in 200 µL PBS. This suspension was diluted 1:10 in RPMI-1640 media (supplemented with L-glutamine, HEPES, no bicarbonate; Wisent) and 2 mL used for infection of cells in 6-well plates.

To label bacteria with FITC (fluorescein isothiocyanate; Sigma), 200 µL of late log bacterial suspension was extensively washed with PBS and incubated with 0.5
mg/mL FITC for 10 min at 37°C, washed in PBS, and resuspended in 200 µL PBS. This suspension was diluted 1:10 in RPMI-1640 and 2 mL used for infection of cells in 6-well plates. For DQ-BSA experiments in 8-well coverglass chambers, the suspension was diluted 1:20 in PBS for all infections except for the ΔinvA/inv strain, where it was diluted 1:10.

**Live Cell Imaging – Chapter 3**

For live cell imaging experiments cells were grown on 2.5 cm coverslips, transfected as indicated and pre-incubated with RPMI-1640 media (supplemented with L-glutamine, HEPES, no bicarbonate; Wisent) with 10% FBS at 37°C for 20 min. Except where indicated, coverslips were mounted in a chamber with media onto a heated stage at 37°C to be imaged either by a Leica DMI8 inverted fluorescence microscope with a 100x oil immersion objective and equipped with Openlab 3.1.7 software (Improvision) or a Quorum spinning disk microscope also with a 100x oil immersion objective (Leica DMI8 inverted fluorescence microscope equipped with a Hamamatsu Back-Thinned EM-CCD camera, spinning disk confocal scan head and Volocity 3.7.0 acquisition software (Improvision)).

To image live cell infections bacterial inocula were prepared as described above and 50 µL of bacterial suspension in RPMI-1640 was added directly to the heated chamber. For three-dimensional representations, confocal Z-stacks were deconvolved and assembled with Volocity software (Improvision). All images were imported into Adobe Photoshop in RGB format and assembled in Adobe Illustrator.

**Live Cell Imaging – Chapter 4**

For the DQ-BSA assay cells seeded in 8-well coverglass chambers were incubated for 1 h in 0.5 mg/mL DQ BSA Red (Molecular Probes), washed with PBS, and further incubated in growth medium for 4 h. Cells were infected with FITC labelled bacteria. Following a 2 h infection the media was changed to RPMI-1640 and cells imaged as described below.
For RpreRed, PLC\&-PH-mRFP, and LactC2-GFP live-cell imaging experiments
cells were grown on 2.5 cm coverslips and transfected 12 to 16 h before invasion with the
appropriate construct. After a 20 min pre-incubation with RPMI-1640 cells were infected
for 10 min with fluorescently labelled \textit{S. Typhimurium}, extracellular bacteria removed by
extensive washing with PBS, cells incubated for additional 20 min in RPMI-1640,
followed by addition of 100 \(\mu\)g/mL gentamicin. At appropriate times cells were washed
with ice-cold PBS and extracellular adherent bacteria stained with rabbit anti-\textit{Salmonella}
antibodies (Difco) followed by Cy5- or Alexa568-conjugated secondary antibodies
(Jackson ImmunoResearch) in ice-cold RPMI-1640. Cells were washed, kept cold and
imaged as described below.

For the above experiments 0.3 \(\mu\)m confocal \(z\)-stacks of the cells were imaged using
a Quorum spinning disk microscope with a 63x oil immersion objective (Leica DMIRE2
inverted fluorescence microscope equipped with a Hamamatsu Back-Thinned EM-CCD
camera, spinning disk confocal scan head and Volocity 3.7.0 acquisition software
(Improvision)). Confocal \(z\)-stacks were analyzed with Volocity software, and fraction of
bacteria that colocalized with dequenched DQ-BSA signal or fluorescent constructs were
directly quantified.

All images were imported into Adobe Photoshop in RGB format and assembled in
Adobe Illustrator.

\textbf{\textit{Salmonella}-containing vacuole fission assay}

\textit{Salmonella}-containing vacuole fission was measured as previously described
(Terebiznik \textit{et al.}, 2002). Briefly, cells were grown on 2.5 cm coverslips and transfected
12 to 16 h before invasion with PM-GFP. After a 20 min pre-incubation with RPMI-
1640 cells were infected for 10 min with \textit{S. Typhimurium}, extracellular bacteria removed
by extensive washing with PBS, and cells incubated for additional 10 min in RPMI-1640.
Cells were then washed with ice-cold PBS and extracellular adherent bacteria stained
with rabbit anti-\textit{Salmonella} antibodies (Difco) followed by Cy5-conjugated secondary
antibodies (Jackson ImmunoResearch) in ice-cold RPMI-1640 medium. Cells were then
washed and bathed in 20 \(\mu\)m FM 4-64 (Molecular Probes) in ice-cold medium. Live
samples were kept cold during analysis by confocal microscopy. Confocal sections were taken using a Zeiss Axiovert confocal microscope (100X oil immersion objective) with LSM 510 software and imported into Volocity 3.7.0 software for quantification of fluorescence areas. Areas containing PM-GFP positive vacuoles were manually selected in Volocity 3.7.0 software, excluding the plasma membrane ruffles and extracellular bacteria. Within these regions, the areas containing red (FM4-64) and green (PM-GFP) signal were automatically calculated using the same threshold function for the FM4-64 signal, and a threshold function that was adjusted based on the brightness (strength of transfection) of the PM-GFP signal to select PM-GFP positive membranes. Based on this data the ratio of FM4-64 positive area to PM-GFP positive area was calculated. Images were imported into Adobe Photoshop in RGB format and assembled in Adobe Illustrator. For Figure 1E, the number of determinations performed at 20 min were 39 (WT), 48 ($\Delta$ sopD), 41 ($\Delta$ sopB), and 33 ($\Delta$ sopB$\Delta$ sopD), at 40 min were 52 (WT), 58 ($\Delta$ sopD), 50 ($\Delta$ sopB), and 56 ($\Delta$ sopB$\Delta$ sopD), and at 60 min were 61 (WT), 57 ($\Delta$ sopD), 56 ($\Delta$ sopB), and 38 ($\Delta$ sopB$\Delta$ sopD). The mean ± s.e.m. are shown in the figures.

**Macropinosome Assay**

Cells were seeded on 2.5 cm coverslips in 6-well tissue culture plates at 20 x 10$^4$ cells/well 16-24 h before use. Assay was performed in the presence of RPMI-1640 media. Bacterial inocula were prepared as previously described (Steele-Mortimer et al., 1999) and cells were infected for 10 min with a 1:100 dilution of prepared bacteria at 37°C. After infection, extracellular bacteria were removed by extensive washing with PBS and addition of 100 µg/mL gentamicin to the medium. Following 20 min of bacterial infection coverslips were mounted onto a heated stage and a Leica DMIRE2 fluorescence microscope was used to enumerate directly (using DIC) the presence of macropinosomes in infected cells. However, when cells were transfected with SopD-GFP or GFP constructs, following 30 min of invasion cells were bathed in ice-cold RPMI to stop the invasion process and were kept cold during the more time demanding quantification procedure. For each bacterial strain at least 100 cells were counted in each experiment, and at least three independent experiments were performed. The mean ±
Macropinosomes were defined as distinct circular/oval structures and invasion sites were identified by membrane ruffling and presence of bacteria that appeared as dark rods within the cell. Although direct measurements were not performed, vacuoles <2 µm in diameter were not easily distinguished among ruffles stimulated by the bacteria and were not counted as macropinosomes.

**Western Blot Analysis**

Protein extracts from WT or sopD mutant S. Typhimurium infected HeLa cells in 24-well tissue culture plates were prepared by lysis in Laemmli buffer at indicated times post infection, separated in 12.5% SDS-PAGE gels, and transferred to PVDF membrane. Monoclonal antibodies against indicated targets and anti-murine HRP as a secondary antibody (Jackson ImmunoResearch, West Grove, PA) were used to probe the blots. Immunoreactive proteins were visualized using chemiluminescence and signals were captured by exposure to film (Amersham Biosciences).

**Plasmid construction for FLAsH labeling and invasion**

The FLAsH (or Lumio; Invitrogen) labeling procedure was performed essentially as described (Enninga et al., 2005). For details of generation of the SopD-4C-expressing ΔsopD S. Typhimurium refer to the Plasmids section of Materials and Methods. Late-log S. Typhimurium cultures were used for infections. The bacteria were grown for 16 h at 37°C with shaking and then subcultured (1:33) in LB without antibiotics, with 0.2% arabinose to induce expression of SopD-4C. After 2 h, LB was exchanged for fresh LB with 0.2% arabinose, 1 µM TCEP-HCl and 5 µM FLAsH. The bacteria were incubated for an additional 1 h in the dark, at 37°C with shaking. Bacterial inocula were prepared by washing bacteria extensively with PBS++ with 40 µM EDT (a FLAsH reagent antidote), labeling with NHS-647 and resuspending in 300 µL RPMI. The entire 300 µL was added to cells mounted in a chamber on a heated stage assembly of a spinning disk confocal microscope where the infection process was imaged.
Statistics

Colocalization quantifications were performed by direct visualization on a Leica DMIRE2 epifluorescence microscope (except when confocal z slices were used for Fig. 1b,c, 4 a, c, e, g-i, k). At least 100 bacteria were counted for each condition in each experiment except where indicated, and three independent experiments were performed. The mean ± s.e.m. is shown in the figures, and P values were calculated using two-way ANOVA with Bonferroni post hoc test (Fig. 2 a-g, 4 a, c, e, Supplementary Fig. 3), a one-way ANOVA with a Dunnett’s Multiple Comparison test, comparing values to WT (Fig. 1b, c, 3d, e, 4 g-i), or a two-tailed two-sample equal variance Student’s t-test (Fig. 4 k). A P value of less than 0.05 was determined to be statistically significant.
CHAPTER 3
SOPD ACTS COOPERATIVELY WITH SOPB DURING S. TYPHIMURIUM INVASION

SUMMARY

The work from this chapter has been published in Bakowski et al. (2007) Cellular Microbiology 9(12), 2839-2855.

To invade and replicate in host cells S. Typhimurium subvert host molecular machinery using bacterial proteins, called effectors, which they translocate into host cells using specialized protein delivery systems. One of these effectors, SopD, contributes to gastroenteritis, systemic virulence and persistence of S. Typhimurium in animal models of infection. Recently, SopD has been implicated in invasion of polarized epithelial cells and here we investigate the features of SopD-mediated invasion. We show that SopD plays a role in membrane fission and macropinosome formation during S. Typhimurium invasion, events previously shown to be mediated by the SopB effector. We further demonstrate that SopD acts cooperatively with SopB to promote these events during invasion. Using live cell imaging we show that a SopD-GFP fusion does not localize to HeLa cell cytosol as previously described, but instead is membrane associated. Upon S. Typhimurium infection of these cells, SopD-GFP is recruited to the invasion site, and this recruitment required the phosphatase activity of SopB. Our findings demonstrate a role for SopD in manipulation of host-cell membrane during S. Typhimurium invasion and reveal the nature of its cooperative action with SopB.

RESULTS

SopD contributes to membrane fission during S. Typhimurium invasion.

SopD has recently been shown to be important for S. Typhimurium invasion of polarized but not non-polarized T84 colon carcinoma cells (Raffatellu et al., 2005) and we decided to further investigate the role of SopD in this process. Invasion of non-polarized epithelial Henle-407 cells by ΔsopD deficient S. Typhimurium was not
attenuated when measured using a standard gentamicin protection assay consistent with previous findings (Raffatellu \textit{et al.}, 2005, Jones \textit{et al.}, 1998). In this assay bacteria that remain extracellular following a brief incubation with cells are killed by addition of the antibiotic gentamicin that is unable to penetrate eukaryotic cells. The infected cells are then lysed releasing bacteria that were intracellular and therefore protected from the gentamicin treatment. This lysate is plated on solid medium plates and colonies resulting from growth of surviving bacteria are enumerated. However, the gentamicin protection assay is relatively insensitive, as it requires time for gentamicin to kill extracellular bacteria. Therefore, to measure the contribution of SopD to \textit{S. Typhimurium} invasion, we used the \textit{in vitro} membrane fission assay described by Terebiznik \textit{et al.} (Terebiznik \textit{et al.}, 2002). In the fission assay, the membrane of the SCV becomes inaccessible to FM 4-64, a membrane-impermeant dye that partitions exclusively to the outer leaflet of the plasmalemma, after the SCV has become “sealed” and fission from the plasma membrane occurs. Fission is determined by the degree of accessibility of extracellular FM 4-64 to membranes accumulating at the base of invasion ruffles marked by expression of a GFP fusion targeted to the plasma membrane by myristoylation and palmitoylation (PM-GFP). Using this assay, Terebiznik \textit{et al.} demonstrated that \textit{ΔsopB} mutant \textit{S. Typhimurium} displayed delayed invasion (Zhou \textit{et al.}, 2001) due to a defect in the fission of SCVs from the plasma membrane (Terebiznik \textit{et al.}, 2002). To test a role for SopD in SCV fission we observed the effect of SopD on the kinetics of sealing of the intracellular vesicles generated during \textit{S. Typhimurium} invasion of Henle-407 cells.

After 20 min of invasion with wild type (WT) \textit{S. Typhimurium}, the majority of membranes that accumulated at the base of invasion ruffles were inaccessible to FM 4-64 (Figures 3-1A and 3-1E), implying that they have formed sealed intracellular vesicles. In accordance with the previous study, the \textit{ΔsopB} mutant bacteria exhibited impaired membrane fission during invasion, and membranes generated 20 min after the \textit{ΔsopB} mutant \textit{S. Typhimurium} invasion were \textasciitilde50\% more accessible to FM 4-64 (Figures 1B and 1E) than ones generated during WT \textit{S. Typhimurium} invasion. The \textit{ΔsopD} mutant and the double \textit{ΔsopBΔsopD} mutant bacteria had impaired membrane fission as well, with levels closely matching that of \textit{ΔsopB} mutant \textit{S. Typhimurium} (\textasciitilde50\% more accessible to FM 4-64 than WT) (Figures 3-1C and 3-1E). This defect in host cell
Figure 3-1: SopD contributes to membrane fission during S. Typhimurium invasion. Henle-407 cells transfected with PM-GFP were infected with WT or mutant S. Typhimurium strains and 20 min, 40 min, or 60 min after infection cooled and labeled with FM 4-64 (red). Bacteria were stained with antibodies against Salmonella followed by Cy5-conjugated secondary antibodies (magenta). Representative images of cells 20 min after infection with WT (A), ΔsopB mutant (B), ΔsopD mutant (C), and double ΔsopBΔsopD mutant (D) S. Typhimurium. Size bar = 5 µm. E, Extent of vesicle fission in cells infected with the four strains of S. Typhimurium. The area labeled with FM 4-64 to that labeled with PM-GFP is an index of sealing of the invaginated areas of the ruffles. Data are means ± S.E.M. of at least 33 and up to 61 determinations (see Experimental Procedures). Asterisks indicate a statistically significant difference from WT (P-value < 0.05). F, Vesicle fission measurements in cells 20 min after infection with WT, mutant, and complemented mutant strains of S. Typhimurium. Data are means ± S.E.M. P-values (P) and numbers of cells used for measurements (n) are indicated in the figure.
membrane sealing at 20 min post invasion caused by individual sopB and sopD deletions could be complemented by expression of the corresponding effectors in trans from a plasmid (Figure 3-1F). By 40 min post invasion membranes generated during invasion of cells with WT, ΔsopB, or ΔsopD mutant were mostly sealed, with ΔsopD infected cells showing statistically significant but minor differences from WT infected cells (Figure 3-1E). However, the double ΔsopBΔsopD mutant displayed a more severe delay in membrane fission since, at this time, significantly less membrane sealing occurred in cells infected with the ΔsopBΔsopD mutant than with the other bacterial strains (Figure 3-1E). Even at 60 min post invasion the double mutant did not effectively seal host cell membranes at sites of invasion. Notably, during invasion with ΔsopBΔsopD mutant S. Typhimurium, large PM-GFP positive vesicles (macropinosomes) formed less frequently (Figure 3-1D) than during WT (Figure 3-1A), ΔsopB (Figure 3-1B), or ΔsopD (Figure 3-1C) mutant S. Typhimurium invasions. These results suggest that the SopD and SopB effectors may act cooperatively to encourage host cell membrane internalization and sealing during S. Typhimurium invasion.

**SopD contributes to macropinosome formation during S. Typhimurium invasion.**

The SopB effector has been previously shown to promote macropinosome formation in intestinal Henle-407 cells during S. Typhimurium invasion (Hernandez et al., 2004). Since we observed a striking decrease in membrane internalization during invasion with the double ΔsopBΔsopD mutant S. Typhimurium (Figure 3-1D), we investigated whether SopD played a role in macropinosome formation as well. Henle-407 cells were infected with WT, ΔsopD, ΔsopB, or double ΔsopBΔsopD mutant S. Typhimurium. Twenty minutes after infection large macropinosomes were observed in ~73% of WT S. Typhimurium infected cells, using DIC microscopy (Figure 3-2A, B). During infection with the ΔsopD or ΔsopB mutant, significantly less macropinosomes formed than with WT S. Typhimurium (56% and 50% respectively), and the defect due to ΔsopD mutation could be complemented by expression of SopD from a plasmid (Figure 3-2B). However, during infection with the double ΔsopBΔsopD mutant bacteria only 17% of infected cells
Figure 3-2: SopD contributes to macropinosome formation following S. Typhimurium invasion. A, WT S. Typhimurium invasion of Henle-407 cells is accompanied by formation of macropinosomes (arrows) readily observed using DIC microscopy. In contrast the ΔsopBΔsopD mutant makes macropinosomes infrequently (invasion site ruffles marked by arrowheads). Size bar = 10 µm. B, Henle-407 cells were infected with S. Typhimurium strains indicated and 30 min post invasion formation of macropinosomes in infected cells was quantified using DIC microscopy. Data are means ± S.E.M. of three independent experiments. Asterisks indicate a statistically significant difference from WT (P-value < 0.05) and individual P-values (P) are indicated in the figure. C, Henle-407 cells were transiently transfected with GFP (black bars) or SopD-GFP (gray bars), and infected with either WT or ΔsopBΔsopD S. Typhimurium. Macropinosome formation in transfected and infected cells was quantified as above. Data are means ± S.E.M. of three independent experiments. Asterisks indicate statistically significant differences (P-value < 0.05).
contained macropinosomes (Figure 3-2A, B). This indicates that SopD, like SopB, contributes to macropinosome formation and that both effectors act cooperatively to promote this process during S. Typhimurium invasion.

The SopD-GFP fusion construct transiently transfected into HeLa cells was previously used to study the localization of SopD in host cells (Brumell et al., 2003). To determine if SopD-GFP expressed in mammalian cells retains the activity of bacterially expressed SopD, we investigated whether overexpression of SopD-GFP in Henle-407 cells could complement in trans the substantial macropinosome formation defect of the double ΔsopBΔsopD mutant S. Typhimurium. Henle-407 cells were transfected with SopD-GFP or with GFP alone as a negative control. After 30 min of infection with either WT or ΔsopBΔsopD mutant bacteria, the percent of transfected and infected cells that possessed macropinosomes was determined. Unexpectedly, transfecting cells with GFP alone resulted in an overall increase in macropinosome formation as a result of infection compared to untransfected cells (Figure 3-2B,C). Despite this, GFP transfected cells infected with the ΔsopBΔsopD mutant still produced significantly less macropinosomes compared to WT infected GFP expressing cells (39% and 84%, respectively), and during infection with the ΔsopBΔsopD mutant significantly less macropinosomes formed in GFP compared to SopD-GFP transfected cells (39% and 67% respectively) (Figure 3-2C). Also, no significant difference in macropinosome formation existed between SopD-GFP transfected cells infected with either WT or ΔsopBΔsopD S. Typhimurium (P=0.070). This suggests that overexpression of SopD-GFP in trans can rescue the macropinosome formation defect of ΔsopBΔsopD S. Typhimurium and implies that the SopD-GFP fusion construct expressed in mammalian cells retains activity of the endogenous, bacterially expressed and translocated SopD effector.

**SopD-GFP localizes to membranes in live HeLa cells.**

To investigate the localization of the SopD effector in host cells we observed live HeLa cells transfected with the SopD-GFP fusion construct. Surprisingly, in live cells SopD-GFP did not localize exclusively to the host cell cytosol as previously described in fixed cells (Brumell et al., 2003), but also localized to vesicular structures (Figure 3-3A,
Figure 3-3: SopD-GFP localizes to membranes in live HeLa cells. A, HeLa cells were transiently transfected with SopD-GFP. Live cells were examined using an inverted fluorescence microscope with a heated stage assembly. SopD-GFP associated with endosomes (left panel), however fixation with 2.5% paraformaldehyde (PFA) disrupted this association (right panel). B, HeLa cells were co-transfected with SopD2-RFP and SopD-GFP. In order to limit endosomal movement, live cells were cooled by washing them with ice-cold PBS and imaged using a spinning disk confocal microscope in the presence of cold media. Throughout the cell SopD2-RFP and SopD-GFP are found on endosomes together (arrow) and individually (arrowheads). Size bar = 10 µm.
left panel). Upon treatment of cells with 2.5% PFA (a standard fixation procedure), the membrane binding of SopD-GFP was rapidly disrupted (Figure 3-3A, right panel), explaining the previously reported cytoplasmic localization of this protein. Therefore, the localization of a transfected effector protein can be affected by standard fixation protocols. An alternate method of fixation using cold MeOH was somewhat, but not entirely, successful at preserving localization of SopD-GFP in transfected cells (Figure 3-4A).

Since SopD is 41% identical to SopD2, a SPI-2 effector that has previously been shown to be membrane bound (Brumell et al., 2003), we investigated whether these two effector proteins are associated with the same membrane compartments. HeLa cells were co-transfected with SopD-GFP and SopD2-RFP fusion constructs and their localization was observed in live cells using spinning disk confocal microscopy. Cells were cooled prior to imaging to limit endosomal movements and facilitate colocalization studies. As shown in Figure 3B, SopD-GFP and SopD2-RFP occasionally localized to the same compartment (arrow) but were also found individually (arrowheads), indicating that SopD and SopD2 occupy interacting but largely distinct membrane compartments within epithelial cells.

To determine the nature of SopD positive compartments within live cells we co-expressed the SopD-RFP protein together with various GFP tagged markers of the endocytic machinery. SopD-RFP showed partial colocalization with both early (Rab5A) and late (Rab7, Rab9) endosomal markers, and with lysosome associated membrane protein 1 (LAMP1)-GFP (Figure 3-5). Also, many SopD-GFP vesicles were acidic as determined by treatment of cells with an acidotropic dye, LysoTracker Red (Figure 3-5). However, SopD-GFP showed no colocalization with an autophagy marker RFP-LC3, and rapamycin induced autophagosomes (Figure 3-6). The partial colocalization of SopD-RFP with both early and late endosomal markers indicates that the association of SopD-RFP with membranes is either specific but not restricted to a single endosomal compartment, or the observed pattern may be nonspecific as a consequence of SopD-RFP overexpression. However, these results indicate that at least some SopD positive vesicles have characteristics of late endosomes, consistent with the partial colocalization with SopD2, which localizes to late endocytic compartments (Brumell et al., 2003).
Figure 3-4: Cold MeOH fixation partially preserves SopD localization in host cells. **A**, Henle-407 cells were transiently transfected with SopD-GFP and fixed in cold MeOH. Transfected cells were examined using an inverted fluorescence microscope. SopD-GFP remained associated with a number of vesicles (arrow) after fixation. **B**, Henle-407 cells were transiently transfected with SopD-GFP, infected with WT S. Typhimurium, and fixed in cold MeOH 30 min p.i. Cells immunostained for LAMP1 and *Salmonella* were examined as above. SopD-GFP could be seen on macropinosomes associated with invading bacteria (arrowhead) as well as SCVs (arrow in inset).
Figure 3-5: Characterization of endosomes targeted by SopD-GFP. Live HeLa cells co-transfected with fluorescent SopD fusion constructs and endosomal markers (Rab5A-GFP (A), Rab7-GFP (B), Rab9-GFP (C) and LAMP1-GFP (D)) or treated with LysoTracker (E) were examined using a spinning disk confocal microscope with a heated stage assembly. 3D z-stack reconstructions are shown in A-C, E and a single plane of focus image is shown in D. Colocalization of SopD and an endosomal marker is indicated with arrows and lack of colocalization is indicated with arrowheads. Size bar = 10 µm.
Figure 3-6: SopD-GFP does not co-localize with LC3-RFP, nor rapamycin induced autophagosomes. **A**, Live HeLa cells transiently co-transfected with SopD-GFP and LC3-RFP were examined using a spinning disk confocal microscope with a heated stage assembly. A single plane of focus is shown. Lack of colocalization between LC3-RFP and SopD-GFP is indicated with arrowheads in the inset. **B**, Live HeLa cells transiently co-transfected with SopD-GFP and LC3-RFP were treated with 25 µg/mL rapamycin for 3 hours to induce autophagy, and examined as above. Lack of colocalization between LC3-RFP on an autophagosome and SopD-GFP is indicated with an arrowhead in the inset. Size bar = 10 µm.
SopD association with endosomes is ATP-dependent but not dependent on PI3-K activity and acidification of endosomes.

We wanted to further characterize the labile nature of SopD-GFP interaction with host cell membranes. To determine if association of SopD with host cell membranes was energy dependent, we depleted SopD-GFP-expressing cells of ATP using inhibitors of both glycolysis (2-deoxy-D-glucose) and mitochondrial respiration (antimycin A). Since the ongoing formation of phosphoinositides (i.e. their phosphorylation) can be inhibited by depletion of cellular ATP, we used the PLCδ-PH-mRFP construct (a specific probe for PI(4,5)P₂, found largely at the plasma membrane) (Varnai et al., 1998) as a positive control for ATP depletion. First, ATP was depleted in HeLa cells co-transfected with SopD-GFP and PLCδ-PH-mRFP. As shown in Figure 3-7A, after 30 min of ATP depletion, the PH domain of PLCδ partially detached from the plasma membrane, indicating partial loss of PI(4,5)P₂. This was also accompanied by complete detachment of SopD-GFP from the membrane of endosomes, indicating that SopD-GFP membrane binding is very sensitive to intracellular ATP levels. Conversely, as shown in Figure 3-7B, the SopD2-RFP binding to endosomes remained unaltered during ATP depletion, suggesting that SopD and SopD2 bind host cell membranes by distinct mechanisms.

Since phosphatidylinositol 3-kinase (PI3-K) has been shown to act in the development of the Salmonella-containing vacuole (SCV) (Scott et al., 2002), we determined if PI3-K inhibition had an effect on SopD-GFP membrane binding. One of the products of PI3-K, PI(3)P, is found predominantly on early endosomes within cells and is recognized with great specificity by the FYVE domain (Gaullier et al., 1998). Therefore, we used the 2xFYVE-mRFP construct to monitor levels of intracellular PI(3)P during treatment with LY294002, a specific PI3-K inhibitor. HeLa cells cotransfected with SopD-GFP and 2xFYVE-mRFP were treated with LY294002 and observed using spinning disk confocal microscopy. After 5 min of treatment with LY294002, we observed that 2xFYVE-mRFP detached from endosomal membranes (Figure 3-7C). However, membrane binding of SopD-GFP was unaffected by PI3-K inhibition. Likewise, the membrane binding of SopD2-RFP remained unaltered.
Figure 3-7: SopD association with endosomes is ATP-dependent and does not require PI3-K activity or the acidification of endosomes. Spinning disk confocal microscope with a heated stage assembly was used to examine HeLa cells co-transfected with SopD-GFP and PLCδ-PH-mRFP (A) or SopD-RFP and SopD2-GFP (B) before and after 30 min of ATP depletion using 10 mM 2-deoxy-d-glucose and 200 nM antimycin. HeLa cells co-transfected with SopD-GFP and 2xFYVE-mRFP (C) or SopD-RFP and SopD2-GFP (D) were imaged as above, before and after treatment with LY294002. Also imaged were HeLa cells transfected with SopD-GFP and incubated with LysoTracker Red for 30 min without (E), or following a 1 h treatment with 1 μm Folimycin (F). In (F), Folimycin was also present during the 30 min LysoTracker incubation. The exposure conditions used to image LysoTracker Red in (E) and (F) were identical.
during treatment with LY294002 (Figure 3-7D). This indicates that both SopD and SopD2 association with membranes is independent of PI3-K activity.

SopD-GFP partially co-localized with many markers of the late endosomal machinery and many SopD-GFP positive vesicles were found to be acidic based on LysoTracker Red staining (Figure 3-5). Therefore, it is possible that acidification of endosomes is required for SopD-GFP membrane binding or that SopD-GFP is degraded in lysosomes. Hence, we investigated the effect of inhibition of endosomal acidification on SopD-GFP localization and expression levels. HeLa cells were transiently transfected with SopD-GFP and treated for 1 hour with 1 µm Folimycin, a specific inhibitor of V-ATPase. Following Folimycin treatment LysoTracker Red was added to the media, cells were incubated for a further 30 min, and imaged live using spinning disk confocal microscopy. Although endosomes enlarged due to inhibition of acidification, membrane binding of SopD-GFP was unaffected (Figure 3-7F) as compared to the Folimycin untreated control cells (Figure 3-7E). Inhibition of endosome acidification with Folimycin had no discernable effect on the stability of SopD-GFP as determined by Western blot (data not shown).

**SopD-GFP is recruited to the SCV and macropinosomes during *S. Typhimurium* infection.**

Since we found SopD-GFP to be bound to membranes in live cells and Δ*sopD* mutant *S. Typhimurium* was deficient in membrane fission and macropinosome formation during invasion, we investigated SopD-GFP distribution during *S. Typhimurium* invasion using spinning disk confocal microscopy. HeLa cells were transfected with the SopD-GFP encoding plasmid and infected with WT *S. Typhimurium* expressing the red fluorescent protein (RFP). As seen in Figure 3-8A, SopD-GFP is recruited to the *S. Typhimurium* invasion site, the macropinosomes (arrow), and the SCV (arrow head). Similar results were obtained during invasion with the Δ*sopD* mutant bacteria (data not shown), indicating that this recruitment does not require bacterial production of SopD. In addition, the initial precise recruitment of SopD-GFP to
**SopD Constructs:**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Start</th>
<th>Stop</th>
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<tbody>
<tr>
<td>SopD</td>
<td>1</td>
<td>317</td>
</tr>
<tr>
<td>SopDNT</td>
<td>1</td>
<td>150</td>
</tr>
<tr>
<td>SopDCT</td>
<td>150</td>
<td>317</td>
</tr>
<tr>
<td>SopDW37A</td>
<td>1</td>
<td>317</td>
</tr>
<tr>
<td>SopDF44A</td>
<td>1</td>
<td>317</td>
</tr>
<tr>
<td>SopD1-270</td>
<td>1</td>
<td>270</td>
</tr>
</tbody>
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*Conserved Motif:

SopD 37-44: W E K (I/M)x F F

SopD 37-44: W D K V Q D H F
Figure 3-8: SopD-GFP is recruited to the SCV and macropinosomes during S. Typhimurium infection. A. HeLa cells were transfected with the SopD-GFP and invasion with WT S. Typhimurium expressing RFP was examined using a spinning disk confocal microscope with a heated stage assembly. During invasion, SopD-GFP localized to the invasion site including the newly formed SCVs (arrowhead) and macropinosomes (arrow). B. Schematic diagram of SopD-GFP fusions (GFP at the carboxy-terminus of SopD). The conserved W(E/D)(K/R)xxFF motif (asterisk), the putative coiled coil region (CC), and sites of point mutagenesis (arrowheads) are indicated. The SopD sequence in the conserved motif is shown with amino acids mutated to alanines underlined. C, D. HeLa cells were co-transfected with SopDNT-GFP (SopD 1-150) (C) or SopDCT-GFP (SopD 150-317) (D) and full length SopD-RFP and invaded with WT S. Typhimurium labeled with NHS-647. Unlike the full length SopD-RFP, neither truncated SopD construct localized to membranes within cells, nor the S. Typhimurium invasion site. E, F, G. HeLa cells were transfected with SopD-GFP fusion constructs (W37A (E), F44A (F), and αααα1-270 (G)), and infected with WT S. Typhimurium expressing RFP as described above. All three constructs did not localize to bacterial invasion sites including macropinosomes (arrows) and SCVs (arrowheads). W37A and F44A fusion constructs were exclusively cytosolic, while αααα1-270 was found, although very infrequently, in aggregates inside cells as shown in (G). Size bars = 10 µm.
the invading bacteria occurs predominantly from the cytosolic pool of this protein with little recruitment via fusion of SopD-GFP positive vesicles with the plasma membrane (Figure 3-9). Like the localization of SopD-GFP to endosomal membranes (Figure 3-4A), at 30 min post invasion the localization of SopD-GFP to host cell membranes at the invasion site of *S*. Typhimurium and on SCVs could be partially conserved using cold MeOH fixation (Figure 3-4B). However, fixation of the endogenous SopD tagged with two HA epitopes and expressed by *ΔsopD* mutant *S*. Typhimurium were unsuccessful (data not shown).

To determine if a particular segment of SopD is responsible for membrane binding during invasion we generated SopD amino- and carboxy-terminal truncations (SopD 1 – 150 and SopD 150 – 317, called SopDNT and SopDCT respectively) fused to GFP. As shown in Figure 3-8C and 3-8D, neither SopDNT-GFP nor SopDCT-GFP is recruited to the invasion site of *S*. Typhimurium, while the co-expressed full length SopD-RFP construct is recruited. Additionally, in uninfected cells neither construct associated with membranes.

To further investigate the nature of SopD binding to membranes additional SopD mutants fused to the GFP protein were created and their localization observed in live cells using fluorescence microscopy. We generated a SopD truncation mutant lacking the predicted coiled-coil domain (Brumell *et al.*, 2003) (SopD1-270). We also generated mutations in the WEK(I/M)xxFF motif of SopD, a sequence conserved within the amino-termini of members of the *Salmonella* translocated effector (STE) family, which appears to be essential for effector translocation (Miao *et al.*, 2000). This conserved motif is also required to target the related SopD2 effector to endosomal membranes within host cells (Brown *et al.*, 2006b). We mutated the conserved W37 and F44 residues in SopD, singly and together, to alanines. Surprisingly, all of the mutant constructs (SopDW37A (Figure 3-8E), SopDF44A (Figure 3-8F), SopDW37AF44A (data not shown), and SopD1-270 (Figure 3-8G)) localized almost exclusively to the cytosol of transfected cells, even in cells infected with WT *S*. Typhimurium. Some aggregation of SopD1-270 could be observed in select cells, however unlike the full length SopD-GFP, SopD1-270 was not recruited to the bacterial invasion site (Figure 3-8G). Placing GFP at the amino-terminus
Figure 3-9: SopD-GFP is recruited to the *S. Typhimurium* invasion site. Three-dimensional representation of deconvolved confocal Z-stacks. HeLa cells were transiently transfected with SopD-GFP (green) and infected with WT *S. Typhimurium* (unmarked). Infection of cells by bacteria was imaged live using spinning disk confocal microscopy, with images taken every 1 min. Representative frames are shown with different SopD-GFP positive SCVs indicated with coloured arrows. A SopD-GFP positive SCV interacting with SopD-GFP positive vesicles is indicated with an arrowhead in the 18 min frame.
of SopD interfered with membrane binding of this protein (data not shown) possibly by affecting the interaction of WEK(I/M)xxFF motif with membranes. These findings suggest that the WEK(I/M)xxFF motif as well as the predicted coiled-coil region of SopD are both necessary to target SopD to host cell membranes, consistent with the fact that neither SopDNT nor SopDCT is sufficient to target GFP to membranes (Figure 3-8C, D). This is in contrast to SopD2, whose amino-terminus is sufficient to mediate late endosome binding (Brumell et al., 2003).

**SopB mediates recruitment of SopD to the S. Typhimurium invasion site.**

Since our data and a previously published study indicated that SopD acts cooperatively with SopB (Jones et al., 1998), we examined the localization of SopD-GFP in cells infected with ΔsopB mutant S. Typhimurium. HeLa cells were co-transfected with SopD-GFP and PLCδ-PH-mRFP expressing plasmids. The PLCδ-PH-mRFP fusion protein specifically binds PI(4,5)P₂, a phospholipid degraded by the phosphatase activity of SopB (Terebiznik et al., 2002). In uninfected cells PLCδ-PH-mRFP is localized to the plasma membrane where PI(4,5)P₂ concentration is highest in the cell. Upon S. Typhimurium invasion, PLCδ-PH-mRFP was shown to be observed on the ruffles generated by the bacterial invasion, however it rapidly disappears from ruffles due to SopB-mediated dephosphorylation of PI(4,5)P₂ (Terebiznik et al., 2002). Based on previous studies, we used PLCδ-PH-mRFP as a marker of bacterial invasion (bacteria were unmarked) and as a read-out of SopB phosphatase activity. The co-transfected cells were infected with the WT, the ΔsopB mutant or the ΔsopB mutant strain complemented with a plasmid encoding SopB C462S (the phosphatase inactive mutant of SopB). As expected, PLCδ-PH-mRFP initially seen on the plasma membrane and invasion ruffles generated by WT S. Typhimurium disappeared from these membranes within 20 min of bacterial invasion (Figure 3-10A). Concurrently, a strong and immediate recruitment of SopD-GFP to the bacterial invasion site including the SCVs (Figure 3-10A, arrows) and the invasion ruffles (Figure 3-10A, arrowhead) was observed. The recruitment on SCVs and surrounding membranes was maintained even after PI(4,5)P₂ turnover. In contrast, during ΔsopB mutant S. Typhimurium invasion
Figure 3-10: SopB mediates recruitment of SopD to the S. Typhimurium invasion site. A-C, HeLa cells co-transfected with SopD-GFP and PLCδ-PH-mRFP were infected with WT or mutant S. Typhimurium strains and invasions imaged using a spinning disk confocal microscope with a heated stage assembly. 3D z-stack reconstructions are shown. Asterisk indicates site of initial invasion. The 0 min time corresponds to the image prior to detection of a PLCδ-PH-mRFP positive ruffle generated by invading bacteria. A, WT S. Typhimurium invasion. SopD-GFP is seen on SCVs (arrows) and on base of ruffles formed during invasion (arrowhead). Accumulation of SopD-GFP is seen at the invasion site a minute following detection of a PLCδ-PH-mRFP positive invasion ruffle (2 min image). B, ΔsopB S. Typhimurium invasion. C, ΔsopB + psopB C462S S. Typhimurium invasion. Size bar = 5 μm.
PLCδ-PH-mRFP was maintained on the plasma membrane and ruffles generated by the invading bacteria up to 44 min post invasion and no recruitment of SopD-GFP to the invasion site was observed (Figure 3-10B), suggesting SopB is required for SopD-GFP recruitment. Furthermore, during invasion with *S.* Typhimurium expressing the phosphatase inactive SopB C462S, PLCδ-PH-mRFP remained on the plasma membrane near the invasion site as expected and SopD-GFP was not recruited (Figure 3-10C). These observations indicate that while SopD-GFP requires the SopB effector for recruitment to the *S.* Typhimurium invasion site, SopB does not recruit SopD-GFP via a direct protein-protein interaction but rather by its modification of phospholipids during invasion.

In addition to PI(4,5)P₂ turnover, SopB has also been reported to be required for the formation of PI(3)P at the site of bacterial invasion immediately after infection (Hernandez *et al.*, 2004). To investigate the possibility that SopD-GFP is recruited to the bacterial invasion site through localized generation of PI(3)P we treated HeLa cells transfected with SopD-GFP and 2xFYVE-mRFP (as a positive control of PI3-K inhibition) with LY294002 prior to invasion with bacteria labeled with NHS-647 (an amine-reactive Alexa Fluor 647 carboxylic acid, succinimidyl ester dye). Within 5 min of LY294002 treatment no 2xFYVE-mRFP positive endosomes remained, demonstrating effective PI(3)-K inhibition by the drug. Despite inhibition of PI3-K, SopD-GFP was recruited to the invasion site (Figure 3-11), indicating that its recruitment to the invasion site is PI3-K independent, similar to its ability to bind endosomes in a PI3-K independent mechanism (Figure 3-7C and 3-7D).

**SopD does not contribute to LAMP1 acquisition by the SCV.**

Another phenotype attributed to SopB during infection of Henle-407 cells is maturation of the SCV, as measured by LAMP1 recruitment to this compartment (Hernandez *et al.*, 2004). LAMP1, a lysosomal glycoprotein, is rapidly acquired by the SCV and maintained on this compartment throughout infection (Steele-Mortimer *et al.*, 1999). We compared the levels of colocalization of LAMP1 with WT and ΔsopB
Figure 3-11: SopD-GFP is recruited to S. Typhimurium invasion site in the presence of PI3-K inhibitor, LY294002. HeLa cells co-transfected with SopD-GFP (green) and 2xFYVE-mRFP (red) were pretreated with LY294002 for 5 min, infected with WT S. Typhimurium labeled with NHS-647 (blue) and imaged using a spinning disk confocal microscope with a heated stage assembly every minute. 3D z-stack reconstructions are shown. At a time designated as beginning of invasion (0 min) a bacterium was observed associated with the host cell surface (arrowhead in 0 min panel). It was found in the same location 1 min later and SopD-GFP was recruited to it at that time despite PI3K inhibition (arrowhead in 1 min panel). SopD-GFP and 2xFYVE-mRFP positive (yellow) endosome before LY294002 treatment (-10 min) is indicated with an arrow. Red signal in remaining panels is the background 2xFYVE-mRFP signal found in the host cell nucleus. Size bar = 5 µm.
Figure 3-12: SopD does not promote LAMP1 acquisition by the SCV.

Henle-407 cells were infected with S. Typhimurium strains indicated, fixed and immunostained for LAMP1, total and outside bacteria. Percent of intracellular LAMP1 associated bacteria was enumerated. Data are means ± S.E.M. of three independent experiments. Asterisks indicate a statistically significant difference of the ΔsopB strain (black) or the ΔsopBΔsopD strain (gray) from WT (P-value < 0.05).
mutant S. Typhimurium to that of ΔsopD and double ΔsopBΔsopD mutant strains in infected Henle-407 cells. Only intracellular bacteria were scored for colocalization with LAMP1. As seen in Figure 3-12, SopB, but not SopD, contributes to LAMP1 delivery to the SCV and the significance of the partial SopD-RFP co-localization with LAMP1-GFP positive vesicles (Figure 3-5) is currently unknown. This indicates that while SopD acts cooperatively with SopB during S. Typhimurium invasion, unlike SopB it does not contribute to LAMP1 acquisition by the SCV.

DISCUSSION

The Salmonella effector SopD, in concert with the SopB effector, promotes fluid secretion and inflammation (neutrophil influx) in a bovine model of Salmonella infection (Jones et al., 1998). This, combined with its previously described cytoplasmic localization in fixed cells, led to speculation that SopD plays a role in inflammatory signal transduction pathways initiated upon infection by S. Typhimurium. In the current study we investigated this possibility (Figure 3-13) and found no involvement of SopD in known signaling events associated with S. Typhimurium invasion. These included the previously described activation of ERK1/2 (p42/44) (Rosenshine et al., 1994, Uchiya et al., 2004b), JNK, Akt/PKB (Steele-Mortimer et al., 2000), Protein Kinase C (PKC) isoforms (Silva et al., 2004), and p38 MAP kinase cascades (Hobbie et al., 1997, Uchiya et al., 2005, Uchiya et al., 2004a). Although it is possible that SopD may induce other undescribed signaling events that lead to pro-inflammatory cytokine release, recently Raffatellu et al. presented an alternative explanation for SopD’s contribution to inflammation in vivo. These authors showed that SopD contributes to invasion of polarized but not nonpolarized T84 cells, suggesting that SopD might only be required for the invasion of cells with a brush border such as that found in polarized intestinal epithelia (Raffatellu et al., 2005). They further proposed that by augmenting S. Typhimurium invasion, SopD may contribute to proinflammatory responses by enhancing delivery of pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and flagellin (Zeng et al., 2003), to Toll-like receptors (TLRs)
Figure 3-13: SopD does not play a role in previously characterized signal transduction events initiated by S. Typhimurium invasion. Protein extracts were prepared from HeLa cells infected with WT (left) or sopD (right) mutant S. Typhimurium at the indicated time points post infection. Western blot analysis was with phospho-specific antibodies (upper panels) after which the blots were stripped and probed with anti-b-actin antibodies as a loading control (lower panels).
found on the basolateral membrane of epithelial cells. TLRs recognize specific PAMPs expressed on infectious agents and initiate a signaling cascade leading to activation of NF-κB, cytokine production, and inflammatory responses (reviewed by (Yamamoto et al., 2004). Our observations complement those of Raffatellu et al., and further strengthen the argument that inflammatory responses elicited in vivo by SopD are due to its contribution to S. Typhimurium invasion, rather than a direct effect on host cell signaling pathways.

Previously, upon translocation into host cells, SopD was believed to localize to the cytoplasm of host cells not only on the basis of localization of SopD-GFP in fixed cells but also based on results of mechanical fractionation studies, which indicated that SopD translocated by S. Typhimurium localized exclusively to the cytosolic fraction of host cell lysates (Brumell et al., 2003). Therefore, the unstable association of SopD-GFP with membranes in live cells was an unexpected finding. As fixation with paraformaldehyde rapidly creates stable protein crosslinks, the disruption of SopD association with membranes by this fixation method suggests that SopD may bind membranes through an interaction with a labile molecule. Upon fixation or cell lysis, the rapid turnover of the target molecule could release SopD-GFP from membranes and allow its diffusion within the cell. Accordingly, SopD-GFP membrane binding is acutely sensitive to ATP levels inside host cells. Phosphoinositides are rapidly degraded by phosphoinositide phosphatases when ATP levels fall (eg. see Figure 3-8A). SopD-GFP is maintained stably on membranes in cells following treatment with PI3-K inhibitor LY294002, indicating that SopD does not bind to host cell membranes via 3’-phosphoinositides. Furthermore, we did not detect binding of SopD-GST to any phospholipids (LPA, LPC, PI, PI(3)P, PI(4)P, PI(5)P, PE, PC, S1P, PI(3,4)P2, PI(3,5)P2, PI(4,5)P2, PI(3,4,5,)P3, PA, PS) using commercially available phospholipid dot blots (PIP strips, Echelon Biosciences Inc.) in an overlay assay (data not shown). These results indicate that SopD does not bind host membranes via interaction with phosphoinositides, but by another ATP-dependent mechanism. Importantly, disruption of SopD-GFP binding to membranes by fixation highlights the point that studying the localization of effectors in live cells should be considered to avoid basing conclusions on unexpected
fixation artifacts. It will be interesting to see if other effectors (from *Salmonella* or other pathogens) have this unique membrane-binding characteristic.

Thus far, the only identified protein with which SopD shares significant sequence homology is the SPI-2 effector SopD2. While both effectors bind membranes, they do so via different mechanisms. In the present study we showed that the binding of SopD is ATP-dependent, requires the full-length protein, has broad distribution on endosomes, and occurs at the invasion site of *S. Typhimurium*. Conversely, SopD2 binding to membranes is ATP-independent (this study), only the amino-terminus of SopD2 is required for binding, and SopD2 binds exclusively to late endosomes (Brumell *et al.*, 2003). SopD2 is also not present at the *S. Typhimurium* invasion site. Yet, it is conceivable that while SopD and SopD2 are targeted to different membranes and maintained there via different mechanisms, their mode of action at their discrete locations is similar. For example, SopD2 contributes to the formation of Sifs and enhances endosome fusion inside host cells (Brumell *et al.*, 2003), while during invasion SopD plays a role in efficient SCV fission and macropinosome formation presumably by controlling membrane fission and fusion events. Therefore, both effectors are involved in alteration of host cell membrane dynamics.

SopD-GFP expressed in cells prior to bacterial invasion was rapidly recruited to the invasion site of WT *S. Typhimurium*, and was detected at the base of invasion ruffles a minute following their generation (Figure 3-10) as well as a minute following bacterial association with the cell surface in LY294002 treated cells (Figure 3-11). We have shown that the phosphatase activity of the *S. Typhimurium* SopB effector is required for the recruitment of SopD to the invasion site of the bacteria (Figure 3-10). As an inositol and phosphoinositide phosphatase, SopB has been implicated in a variety of cellular responses during infection, including Cdc42, PLC and Akt activation at the plasma membrane and modulation of chloride secretory responses (Marcus *et al.*, 2001, Zhou *et al.*, 2001, Norris *et al.*, 1998, Galyov *et al.*, 1997, Eckmann *et al.*, 1997, Feng *et al.*, 2001). Our data suggests that a product of the metabolism of molecular targets by SopB is necessary for the recruitment of SopD to membranes. However, while SopB has been shown to act on many phosphoinositide and inositol phosphate substrates *in vitro*, the actual substrates *in vivo* are not clear. One of the reported products of SopB metabolism
in cultured cells is PI(3)P. SopB is responsible for maintaining high levels of PI(3)P in the membrane at the site of bacteria-host cell interaction immediately after infection (Hernandez et al., 2004). Also, heterologously expressed SopB was found to localize almost exclusively to endosomes containing PI(3)P (Dukes et al., 2006). However, we have shown that the recruitment of SopD-GFP to membranes and S. Typhimurium invasion site is not PI3-K dependent and SopD-GFP does not colocalize with the PI(3)P probe 2xFYVE-mRFP (data not shown). Consequently other SopB mediated changes in character or composition of membranes at the invasion site are likely responsible for SopD recruitment. In uninfected cells, fluorescent SopD fusions colocalized with many endocytic markers within cultured cells including late endosomal markers (Figure 3-5). Recruitment of SopD-GFP to plasma membrane and nascent SCVs during invasion may therefore seem counterintuitive, since late endocytic compartments do not fuse with SCVs during the first 3 hrs post invasion (Brumell et al., 2001). We speculate that SopD binds to a molecular target that is generated early during invasion in a SopB dependent manner, which would allow a rapid recruitment of SopD at this time. In the absence of SopB, steady state levels of this target could accumulate in endosomal compartments (including late endosomes) yielding the observed broad distribution of the SopD fusion construct. Alternatively, SopD may bind two targets, one present on an endosomal compartment in resting cells and the other generated in a SopB-dependent manner at the site of invasion. Interestingly, SopB is required for recruitment of SopD-GFP to the invasion site, yet membrane sealing and macropinosome formation during invasion with ΔsopB mutant bacteria is not impaired to the same degree as during invasion with the double ΔsopBΔsopD mutant bacteria. It is therefore possible that despite the absence of SopB, low levels of the locally delivered SopD protein may form transient associations with invasion site membranes, sufficient to enhance macropinocytosis and membrane sealing during bacterial entry.

In conclusion, we have shown that SopD plays a role in membrane dynamics that occur at the invasion site of S. Typhimurium. Furthermore, the SopD and SopB effectors act cooperatively to enhance membrane fission and to promote macropinocytosis during S. Typhimurium invasion. Our findings further support the idea that the contribution of SopD to gastroenteritis and systemic disease during Salmonella infection is due to its role
in bacterial invasion. Certainly, more insight will be gained into the complex process of *Salmonella* invasion with the discovery of SopD host-cell target(s).
CHAPTER 4
A SALMONELLA PHOSPHOINOSITIDE PHOSPHATASE INHIBITS SCV-LYSOSOME FUSION

SUMMARY

Avoidance of phagosome-lysosome fusion is critical for survival in host cells by intracellular pathogens, but the mechanisms by which this is accomplished is unclear for most. S. Typhimurium can invade multiple cell types and grow within them in a vacuolar compartment called the Salmonella-containing vacuole (SCV), a process essential for virulence and disease. The bacteria actively control trafficking of the SCV by inhibiting SCV-lysosome fusion, thereby ensuring their intracellular survival. We found that the S. Typhimurium invasion-associated effector protein SopB that is delivered into the host cell helps bacteria avoid SCV-lysosome fusion. The phosphoinositide phosphatase activity of SopB reduces levels of negatively charged lipids found on the SCV. This change in membrane charge globally affects SCV localization of host-cell endocytic trafficking proteins that rely on electrostatic interactions for their associations with membrane surfaces, resulting in dissociation of proteins previously shown to promote phagosome-lysosome fusion, including Rab35. Our results reveal a previously unrecognized mechanism used by an intracellular pathogen to evade the lysosomal degradation pathway.

RESULTS

SopB inhibits SCV-lysosome fusion

To determine the role of SopB in inhibiting lysosome-SCV fusion we labelled degradative intracellular compartments with a self-quenching BODIPY TR-X dye conjugate of BSA (DQ-BSA). Upon delivery to a degradative compartment DQ-BSA is cleaved, generating fluorescence. DQ-BSA was pulse-chased into Henle-407 epithelial cells. At 2 h post infection (p.i.) ~30% of wild-type (WT) S. Typhimurium colocalized with fluorescent (ie. hydrolyzed) DQ-BSA. As a positive control for SCV-lysosome
fusion we used the ΔinvA/inv strain of S. Typhimurium. These bacteria do not assemble the SPI-1 T3SS and enter cells via the invasin protein of Yersinia pseudotuberculosis. Following uptake, infected cells are treated with a cell permeant antibiotic tetracycline, blocking protein synthesis of internalized ΔinvA/inv bacteria and ensuring their trafficking to a degradative compartment (Smith et al., 2007). As expected, lysosomes marked with DQ-BSA fused with ~70% of phagosomes containing ΔinvA/inv S. Typhimurium. Importantly, we found that the ΔsopB mutant SCVs also fused with degradative compartments (Figure 4-1A, B). The inability to block early SCV-lysosome fusion depended on the lipid phosphatase activity of SopB, as the defect could be complemented by expression of the SopB effector from a plasmid, but not its C462S phosphatase-inactive mutant (Figure 4-1B). Similarly, expression of IpgD, a homologous effector from Shigella flexneri (S. flexneri) (Marcus et al., 2001), but not its C439S phosphatase-inactive mutant (Figure 4-1C), could complement the SopB defect. Although IpgD shares only 30% homology with SopB, they are both phosphatases containing a mammalian inositol 4-phosphatase motif (Norris et al., 1998).

**SopB alters Rab recruitment to SCVs**

Rabs are small GTPases involved in membrane trafficking events within eukaryotic cells. We investigated whether SopB affected recruitment of Rabs that could lead to the observed differences in SCV-lysosome fusion. We focused on Rabs that we previously observed to be excluded from the WT SCVs but are retained on degradative phagosomes containing the ΔinvA/inv mutant bacteria (Rabs 8B, 13, 23, and 35) (Smith et al., 2007) (Figure 4-2A). Rab23 and Rab35 were of special interest as both were shown to promote phagosome-lysosome fusion (Smith et al., 2007). We found that Rabs 8B, 13, 23 and 35 were retained at higher levels on the ΔsopB SCVs than on WT SCVs (Figure 4-3 A-D). The phosphatase activity of SopB (Figure 4-4 D) as well as IpgD (Figure 4-4 E) could prevent Rab35 localization to ΔsopB SCVs. Other Rabs tested as controls, including Rab7, 14, and 22A did not share this pattern of association (Figure 4-3 E-G), and were not extensively associated with the phagosomes containing the ΔinvA/inv mutant bacteria (Figure 4-2A).
**Figure 4-1: SopB inhibits SCV-lysosome fusion.** Henle-407 epithelial cells were pulse chased with DQ-BSA to label degradative compartments and infected with FITC-labelled *S. Typhimurium*. At 2 h p.i. 0.3 µm confocal z-stacks of infected cells were imaged and the fraction of bacteria colocalizing with dequenched DQ-BSA signal counted. A, Representative single focal plane images of cells infected with WT *S. Typhimurium* showing no colocalization between bacteria and DQ-BSA (arrowhead) or ΔsopB *S. Typhimurium* showing colocalization (arrow). Scale bar = 10 µm. B, C, Colocalization of DQ-BSA with *S. Typhimurium*. The phosphatase-inactive mutants *sopBC462S* and *ipgDC439S* are indicated as *sopB* and *ipgD*, respectively. Mean ± S.E.M. for three independent experiments. At least 73 and up to 185 bacteria were enumerated per strain in each individual experiment. **P value < 0.01.
Figure 4-2: Characterization of the $\Delta$invA/inv phagosome interactions with endosomal machinery. A, Henle-407 cells were transfected with GFP or CFP tagged Rabs or GFP-Rac1 and infected with $\Delta$invA/inv bacteria. Colocalization between these proteins and the bacteria was determined for at least 100 bacteria in each individual experiment, except at the 15 min time point where at least 50 bacteria were counted. Mean ± S.E.M. for three independent experiments is shown. B, Henle-407 cells were transfected with CFP-Rab35 or CFP-CTcaax, and infected with $\Delta$invA/inv bacteria for 1 h. Colocalization between CFP-Rab35 or CFP-CTcaax and the bacteria was determined for at least 100 bacteria in each individual experiment. Mean ± S.E.M. for three independent experiments is shown. C, Colocalization between RpreRed (open circle), PLC$\delta$-PH-mRFP (square), LactC2-GFP (triangle) and $\Delta$invA/inv bacteria was quantified. Mean ± S.E.M. for three independent experiments.
Figure 4-3: SopB alters Rab recruitment to SCVs. Henle-407 cells were transfected with Rab fluorescent protein fusions and infected with WT or ΔsopB S. Typhimurium. Colocalization between WT (blue squares) and ΔsopB (red circles) intracellular bacteria and A, GFP-Rab8B, B, GFP-Rab13, C, GFP-Rab23, D, GFP-Rab35, E, CFP-Rab7, F, GFP-Rab14 and G, GFP-Rab22A are shown. Fluorescence microscope images for each construct are shown for 10 or 60 min p.i., where a significant difference (if any) was observed. Colocalization of bacteria with Rabs is indicated in insets with arrows and lack of colocalization with arrowheads. Scale bar = 10 µm. Mean ± S.E.M. for three independent experiments. At least 100 bacteria were enumerated per strain in each individual experiment. * P value < 0.05, ** P value < 0.01.
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**% colocalization**

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E

**GFP-Rab35**

![Graph](image6)
Figure 4-4: Rab recruitment to SCVs is mediated by electrostatic interactions. A, Henle-407 cells were transfected with full length Rab35 or mutants of its carboxy-terminus fused to CFP. B, Arrow indicates CFP-Rab35 positive ΔsopB bacteria 1 h p.i. and arrowhead indicates lack of CFP-Rab35 around WT bacteria. C, Arrow indicates CFP-CTcaax positive ΔsopB bacteria 1 h p.i. and arrowhead indicates lack of CFP-CTcaax around WT bacteria. D, Henle-407 cells were transiently transfected with CFP-Rab35 or CFP-CTcaax and infected for 1 h with the indicated strains. The percentage of CFP-Rab35 (black bars) and CFP-CTcaax (white bars) were quantified. The phosphatase-inactive mutant sopBC462S is indicated as sopB*. Scale bar = 10 µm. E, Colocalization of GFP-Rab35 and S. Typhimurium at 1 h p.i.. The phosphatase-inactive mutant ipgDC439S is indicated as ipgD*. Mean ± S.E.M. for three independent experiments. At least 100 bacteria were enumerated per strain in each individual experiment. ** P value < 0.01.
**Rab recruitment to SCVs is mediated by electrostatic interactions**

Rab 8B, 13, 23, and 35 have a polybasic-prenyl plasma membrane targeting motif in their carboxy-terminus that was previously shown to be responsible for their recruitment to the negatively charged plasma membrane (Heo et al., 2006). Therefore we tested if the same mechanism targeted these Rabs to the SCV. To this end, Henle-407 cells were transfected with several mutants of cyan fluorescent protein (CFP)-tagged carboxy-termini of Rab35, or full length Rab35 (Figure 4-4A). We found that only the carboxy-terminus of Rab35 that contained both the polybasic residues and the prenylation motif (CFP-CTcaax) was sufficient for recruitment to the ΔsopB SCVs (Figure 4-4C, D, 4-5) and the ΔinvA/inv phagosome (Figure 4-2B). Levels of CFP-CTcaax recruitment to ΔsopB SCVs was indistinguishable from that of the full length CFP-Rab35, indicating that the polybasic-prenyl plasma membrane targeting motif of Rab35 is responsible for SopB-mediated Rab35 exclusion from WT SCVs (Figure 4-4B). Additionally, a constitutively active mutant of Rab35 (CFP-Rab35Q67L) was not targeted to WT SCVs, indicating that the activation state of Rab35 does not direct its targeting to membranes (Figure 4-5D). Rac1 is another protein involved in endocytic trafficking that contains the polybasic-prenyl plasma membrane targeting motif. It too was found less frequently on WT SCVs as compared to ΔsopB SCVs (Figure 4-6) and the ΔinvA/inv phagosome (Figure 4-2A).

**SopB alters SCV surface charge by altering levels of PI(4,5)P_2 and PS on the SCV**

We hypothesized that SopB, through its phosphatase activity, is altering the charge of the SCV membrane and consequently affecting recruitment of proteins, such as Rab35, that rely on electrostatics for interactions with membranes (Heo et al., 2006, Yeung et al., 2008, Yeung et al., 2007). To determine the membrane charge of SCVs, we transfected Henle-407 cells with a cationic membrane probe, RpreRed, prior to S. Typhimurium infection. RpreRed is a red fluorescent protein (RFP)-tagged derivative of the K-Ras tail with all serine and threonine residues mutated to alanine to avoid phosphorylation and
Figure 4-5: Polybasic prenylated carboxy-terminus of Rab35 is necessary for its recruitment to ΔsopB SCVs. Representative images of Henle-407 cells transfected with A, CFP-CT, B, CFP-CTΔGG, or C, CFP-CTcaaxΔpB and infected with either WT or ΔsopB bacteria for 1h. Scale bar = 10 μm. D, Henle-407 cells were transiently transfected with CFP-Rab35 or a constitutively active mutant CFP-Rab35Q67L (Rab35CA) and infected for 1 h with the indicated strains. The percentage of CFP-Rab35 (white bars) and CFP-Rab35CA (black bars) colocalizing with intracellular bacteria were quantified. Mean ± S.E.M. for three independent experiments. At least 100 bacteria were enumerated per strain in each individual experiment. ** P value < 0.01.
Figure 4-6: SopB alters Rac1 recruitment to SCVs. Henle-407 cells were transfected with GFP-Rac1 and infected with the indicated *S*. *Typhimurium* strains. The phosphatase-inactive mutant sopBC462S is indicated as sopB*. At least 100 bacteria were enumerated per strain in each individual experiment. Mean ± S.E.M. for three independent experiments.
lysine residues mutated to arginine to avoid ubiquitination of the probe (Yeung et al., 2007). Expressed in cells, RpreRed localized to the highly negatively charged plasma membrane, but not to intracellular WT S. Typhimurium (Figure 4-7A, B). Conversely, the probe colocalized strongly with ΔsopB and ΔinvA/inv bacteria (Figure 4-2, 4-7 A, B), indicating their compartments retain a strong negative membrane charge following invasion. Furthermore, the phosphatase inactive SopB and the S. flexneri phosphatase inactive IpgD were unable to complement the ΔsopB phenotype (Figure 4-7 A, B, G), suggesting that SopB uses its lipid phosphatase activity to alter charge of the SCV membrane.

Phosphoinositides, specifically the relatively abundant and negatively charged PI(4,5)P$_2$, and the phospholipid phosphatidylserine (PS) have both been implicated in significant changes in plasma membrane charge during phagocytosis (Yeung et al., 2007). We examined the levels of PI(4,5)P$_2$ and PS in the SCV membrane using fluorescent probes against each lipid, the PLCδ-PH-mRFP and LactC2-GFP, respectively. We found no detectable PI(4,5)P$_2$ associated with the WT SCVs following invasion, while the PI(4,5)P$_2$ on the ΔsopB SCVs persisted until 60 min p.i. (Figure 4-7 C, D). Additionally, using a rapamycin-induced dimerization strategy to translocate a yeast Inp54p 5-phosphatase to the plasma membrane to dephosphorylate PI(4,5)P$_2$ (Suh et al., 2006) (Figure 4-8) prior to infection with ΔsopB bacteria, we found that elimination of PI(4,5)P$_2$ at the plasma membrane can inhibit SCV-lysosome fusion following invasion (Figure 4-8 C, D). Levels of PS were also affected by the phosphatase activity of SopB and were significantly higher on the ΔsopB than WT SCVs at 60 and 120 min p.i. (Figure 4-7 E, F). The S. flexneri IpgD could functionally complement the ΔsopB mutation with respect to PI(4,5)P$_2$ and PS acquisition.

**DISCUSSION**

The S. Typhimurium invasion-associated effector SopB is a phosphoinositide phosphatase that, by dephosphorylation of PI(4,5)P$_2$ at the plasma membrane, contributes to invasion and nascent SCV biogenesis (Hernandez et al., 2004, Mallo et al., 2008, Mason et al., 2007, Terebiznik et al., 2002). Since ΔsopB bacteria have impaired growth
Figure 4-7: SopB alters SCV surface charge by altering levels of PI(4,5)P2 and PS on the SCV. A, Henle-407 cells were transfected with RpreRed, infected with S. Typhimurium and colocalization between the RpreRed and intracellular WT (blue squares), ΔsopB (red circles), ΔsopB + psopB (filled in triangles) and ΔsopB + psopB* (empty triangles) bacteria was determined using live cell imaging at indicated times p.i.. Mean ± S.E.M. for three independent experiments. At least 100 bacteria were enumerated per strain in each individual experiment. B, Confocal images taken at 20 min p.i. showing lack of colocalization of RpreRed with WT bacteria (arrowhead) and colocalization with ΔsopB bacteria (arrow). C, Henle-407 cells were transfected with PLCδ-PH-mRFP and colocalization determined as in A. D, Confocal images taken at 20 min p.i. showing lack of colocalization of PLCδ-PH-mRFP with WT bacteria (arrowhead) and colocalization with ΔsopB bacteria (arrow). E, Henle-407 cells were transfected with LactC2-GFP and colocalization determined as in A. F, Confocal images taken at 60 min p.i. showing lack of colocalization of LactC2-GFP with WT bacteria (arrowhead) and colocalization with ΔsopB bacteria (arrow). G, Colocalization between RpreRed at 20 min p.i. with WT, ΔsopB, ΔsopB + ppgD, or ΔsopB + ppgD* bacteria. Mean ± S.E.M. for three independent experiments. At least 100 bacteria were enumerated per strain in each individual experiment. H, Colocalization between PLCδ-PH-mRFP and bacteria as in G. I, Colocalization between LactC2-GFP and bacteria as in G, except at 60 min p.i. The phosphatase-inactive mutants sopBC462S and ipgDC439S are indicated as sopB* and ipgD*, respectively.
**Figure 4-8: Plasma membrane targeting of the 5-phosphatase domain of inp54 to the plasma membrane results in rapid dephosphorylation of PI(4,5)P$_2$.**

A, Henle-407 cells were transfected with inp54-YFP, LDR, and PI(4,5)P$_2$ probe, PLCδ-PH-mRFP. Upon addition of 10µM of rapamycin inp54-YFP heterodimerizes with plasma membrane-localized LDR, and so is recruited to the plasma membrane (arrow) where it dephosphorylates PI(4,5)P$_2$ resulting in loss of plasma membrane localization of PLCδ-PH-mRFP (arrowhead). 

B, Henle-407 cells were transfected with inp54-YFP, LDR, and the PS probe, LactC2-RFP. Upon addition of 10µM of rapamycin inp54-YFP is recruited to the plasma membrane (arrow) but no alteration in LactC2-RFP localization is observed.

C, Henle-407 cells were transfected with inp54-YFP and LDR, loaded with DQ-BSA, and infected for 2 h with ΔsopB+GFP bacteria either without rapamycin (top panels) or with rapamycin (bottom panel). Representative images are shown, indicating colocalization of bacteria with DQ-BSA (arrow) or lack of colocalization (arrowhead). The left-most panel has increased brightness showing redistribution of inp54-YFP from the cytoplasm (magenta arrowhead) to the plasma membrane (magenta arrow) in the presence of rapamycin.

D, Colocalization of DQ-BSA with ΔsopB+GFP S. Typhimurium at 2 h p.i.. Mean ± s.e.m. for three independent experiments. At least 100 bacteria were enumerated per strain in each individual experiment.

E, Same as in C, except infected with FITC-labeled ΔinvA/inv bacteria.

F, Colocalization of DQ-BSA with FITC-labeled ΔinvA/inv S. Typhimurium at 2 h p.i.. Mean ± s.e.m. for three independent experiments. At least 100 bacteria were enumerated per strain in each individual experiment. Scale bar = 10 µm.
in bone marrow derived macrophages as well as altered trafficking, it has been speculated that SopB may also contribute to the S. Typhimurium avoidance of SCV-lysosome fusion (Hernandez et al., 2004). As the mechanisms of SCV-lysosome fusion avoidance by S. Typhimurium are not well understood, we investigated the involvement of SopB in this process.

Previous work on SCV-lysosome fusion has yielded controversial results. Many studies found that S. Typhimurium blocks lysosome fusion (Hang et al., 2006), yet others contest these results. Using fluorescent Dextran to label specific endosomal compartments it was found most recently that S. Typhimurium interacts extensively with the host-cell endosomal system and with lysosomes themselves even as early as 30 min p.i. (Drecktrah et al., 2007) However, it is possible that due to long over-night pulse and relatively short 3-hour chase times in these experiments, in addition to thorough lysosome labeling, Dextran labeling of other endosome populations could occur. Furthermore, it is difficult to differentiate between late endosomes and lysosomes since they share similar markers and morphology. In the current study we attempt to make this distinction by using the DQ-BSA reagent, which only becomes fluorescent upon hydrolysis in a degradative compartment, the lysosome (Reis et al., 1998). Using DQ-BSA to label lysosomes we found that WT bacteria colocalized infrequently with DQ-BSA, unlike ΔsopB bacteria, which showed frequent colocalization (Figure 4-1 A, B).

As a positive control for lysosome fusion we employed the ΔinvA/inv strain of S. Typhimurium, which following entry traffics to the phagolysosome (Smith et al., 2007). Unexpectedly, the level of association of ΔsopB mutant SCVs with lysosomes was similar to that of ΔinvA/inv phagosomes, despite major differences between the two strains. Mainly, the ΔinvA/inv S. Typhimurium strain is deficient in SPI-1 T3SS-mediated effector secretion and enters cells via β-integrin mediated phagocytosis. These bacteria are also killed with antibiotic treatment following uptake ensuring that other bacterial factors such as SPI-2 T3SS secreted effectors are not expressed in the host cell. In contrast, the ΔsopB bacteria have the potential to express all of S. Typhimurium’s other virulence factors, which we believe highlights the importance of SopB in blocking early lysosome fusion with the SCV. We speculate that SopB-mediated early lysosome avoidance protects S. Typhimurium from degradation until other bacterial virulence factors involved in this process are induced in the SCV, including the SPI-2 T3SS delivered effector(s), such as SpiC (Uchiya et al., 1999), and as yet unidentified gene(s)
regulated by the PhoPQ system (Garvis et al., 2001). Recently, ΔsopB mutant recombinant attenuated vaccine strains of Salmonella enterica were found to be highly immunogenic compared to isogenic controls (Li et al., 2008). It’s an intriguing possibility that this may occur due to increased SCV-lysosome fusion in the absence of SopB, which leads to improved antigen presentation in infected cells.

Upon investigation of the mechanism of SopB-mediated lysosomal avoidance we found that a group of Rabs (specifically 8B, 13, 23, 35) was absent from WT SCVs, but was retained on the ΔsopB SCVs and on the ΔinvA/inv-containing phagosomes (Figure 4-2 A, 4-3). Importantly, Rab23 and 35 were previously shown to play a role in promoting phagosome-lysosome fusion (Smith et al., 2007). As both the ΔsopB SCVs and ΔinvA/inv-containing phagosomes fuse extensively with lysosomes within 2 h p.i., this specific pattern of Rab association indicates a pathway biased towards lysosomal fusion and degradation. Additionally, the effect of SopB on Rab localization was specific. For example, Rab14 and Rab22A were recruited equally to WT and ΔsopB SCVs (Figure 4-3 F, G). Rab14 was previously implicated in contributing to S. Typhimurium lysosome avoidance in a SopB-dependent manner (Kuijl et al., 2007). However, these experiments were performed in a breast cancer cell line and our work in Henle-407 intestinal epithelial cells, which may account for the differences in recruitment observed. Rab7, which is normally recruited to the WT SCVs and responsible for lysosome-associated protein 1 (LAMP1) accumulation on the SCV (Meresse et al., 1999), was recruited significantly less to the ΔsopB SCVs (Figure 4-3 E), consistent with previous finding that ΔsopB SCVs acquire less LAMP1 (Hernandez et al., 2004). This seems contradictory as LAMP1, among other intracellular locations, is found on lysosomes. However, it has been proposed that WT SCVs acquire Rab7 and LAMP1 from a different intracellular compartment other than lysosomes and these data support our findings (Meresse et al., 1999).

How could SopB achieve such specific yet broad effect on Rab localization to the SCV? Significantly, all four Rabs, as well as Rac1, whose localization was regulated by SopB contained a polybasic-prenyl plasma membrane targeting motif. We found that this motif was necessary and sufficient to target Rab35 to ΔsopB SCVs (Figure 4-4). Additionally the activation state of Rab35 did not impact on its recruitment to the SCVs
(Figure 4-5 D), indicating that only the prenylation and the polybasic domain mediated its differential recruitment. Notably Rab22A, which like Rab8B, 13, 23, and 35, contains a polybasic-prenyl plasma membrane targeting motif, was not removed from the SCV in a SopB-dependent manner (Figure 4-3 G). This indicates that although SopB can have an extensive effect on host-cell protein localization through the exploitation of their polybasic-prenyl plasma membrane targeting motifs, other factors can be important determinants of protein recruitment.

Since SopB is a phosphoinositide phosphatase and we found that it controlled the recruitment of several proteins through a motif containing polybasic residues it was an attractive possibility that SopB could achieve a broad effect on a group of proteins by controlling the charge of the SCV membrane. To measure intracellularly the charge of the SCV membrane we employed the fluorescent cationic protein probe called RpreRed, which is recruited to negatively charged membranes in cells (Yeung et al., 2006b). The effect of surface potential rather than specific lipid species on the affinity of Rpre for pure lipid bilayers was previously assessed \textit{in vitro}, this affinity decreasing with increasing ionic strength (Yeung et al., 2006b). Using RpreRed we found that SopB was indeed significantly affecting SCV membrane charge. While WT SCVs lost the strong negative charge found at the plasma membrane following invasion, \textit{ΔsopB} SCVs retained the negative charge much longer (Figure 4-7 A, B).

\textit{PI(4,5)P}_2 is a tetravalent anion at physiological pH and it could contribute significantly to the SCV membrane charge. In accordance with previous findings (Terebiznik et al., 2002) and consistent with \textit{PI(4,5)P}_2 being the proposed main \textit{in vivo} target of SopB (Mallo et al., 2008, Mason et al., 2007), using PLCδ-PH-mRFP we found that SopB was reducing the levels of \textit{PI(4,5)P}_2 on WT SCVs (Figure 4-7 C, D). PS is another anionic lipid, abundant in the plasma membrane and endosomal membranes in eukaryotic cells. A fluorescent protein probe specific for PS, the C2 domain of lactadherin (LactC2), was recently developed and shown to bind this lipid specifically, and not to bind PI, \textit{PI(4,5)P}_2, or PA, based on \textit{in vitro} assays (Yeung et al., 2008). Additionally, no membrane binding of LactC2-GFP was observed when it was expressed in a yeast strain deficient for PS production, further illustrating the specificity of this probe (Yeung et al., 2008). Using LactC2-GFP we found that \textit{ΔsopB} SCV membranes
contained more PS than WT SCVs (Figure 4-7 E, F). It is unclear how SopB is reducing PS levels on the SCV but we speculate it may do so by influencing recycling pathways of the host-cell (Bujny et al., 2008). Additionally, since PS is found on lysosomes, it is possible that increased fusion of lysosomes with ΔsopB SCVs is responsible for delivering PS to this compartment.

As the phosphatase activity of SopB was important for the effects described above, we wanted to determine if we could complement SopB function by the expression of another phosphoinositide phosphatase. The IpgD effector is a SopB homologue from Shigella flexneri (S. flexneri) (Marcus et al., 2001). We found that S. flexneri IpgD could functionally complement SopB activity, including modifying SCV charge by altering PI(4,5)P$_2$ and PS levels on the SCV (Figure 4-7 G-I), affecting Rab35 recruitment, and decreasing SCV-lysosome fusion (Figure 4-1 C). As IpgD and SopB are effectors from microbial pathogens adapted to different intracellular lifestyles (cytosol versus vacuole adapted, respectively) this indicates that different pathogens may manipulate phagosome membrane charge during entry into cells to protect against lysosomal degradation early during infection, either prior to escape into the cytosol (S. flexneri) or extensive remodeling of the vacuoles they inhabit (S. Typhimurium).

The contribution to overall SCV membrane charge of other phosphoinositides such as PI(3)P and PI(3,5)P$_2$ cannot be altogether discounted. PI(3)P is produced in the SCV in a SopB-dependent manner (Mallo et al., 2008) and it has been hypothesized that SopB could dephosphorylate PI(3,5)P$_2$ to alter SCV trafficking. However, reliable probes for detection of PI(3,5)P$_2$ are not currently available and it was found that PI(3)P does not significantly affect the membrane charge of phagosomes during their maturation (Yeung et al., 2008). Also we were able to significantly impair ΔsopB SCV-lysosome fusion and ΔinvA/inv phagosome-lysosome fusion by reducing levels of PI(4,5)P$_2$ at the plasma membrane prior to bacterial invasion indicating that SopB, mainly by hydrolysis of PI(4,5)P$_2$ is promoting the trafficking of the SCV away from the degradative pathway (Figure 4-8).

Finally, some observations regarding the trafficking of the ΔinvA/inv model phagosome and WT S. Typhimurium are hard to reconcile with respect to what is known about FcγR-mediated phagocytosis. Firstly, the ΔinvA/inv model phagosomes fuse with
lysosomes but relatively few recruit Rab5 (Smith et al., 2007) or Rab7 (Figure 4-2A) (Smith et al., 2007). Secondly, the model phagosomes appear to be sealed but maintain high levels of PI(4,5)P₂ and negative charge even at 2 h p.i. (Figure 4-2C). In contrast, during FcγR-mediated phagocytosis Rab5 and Rab7 are recruited sequentially to phagosomes (Scott et al., 2003), and PI(4,5)P₂ and the negative membrane charge disappear shortly after phagosome closure (Yeung et al., 2006a, Yeung et al., 2006b).

Thirdly, WT SCVs avoid fusion with lysosomes yet they resemble the trafficking of phagosomes taken up by FcγR-mediated phagocytosis with respect to PI(4,5)P₂, negative membrane charge dynamics (Figure 4-7A-D), and lack of Rab8B, 13, 23, 35 recruitment (Figure 4-9). In fact, the loss of PI(4,5)P₂ and negative membrane charge (whether through the action of SopB or inducible PI(4,5)P₂ dephosphorylation) is important for reduction of SCV-lysosome fusion as well as the ΔinvA/inv model phagosome-lysosome fusion (Figure 4-1A, B, 4-8C-F). Clearly, resolving these incongruencies requires further study. However, collectively our data suggest that ΔsopB S. Typhimurium triggered phagocytosis resembles β-integrin-mediated internalization more than FcγR-mediated phagocytosis. Therefore, the action of SopB is necessary to divert SCVs away from the fate of lysosomal degradation of particles taken up by a process similar to β-integrin-mediated phagocytosis. These data also suggest that phagosomes generated by different mechanisms (FcγR- versus β-integrin-mediated internalization) maintain different membrane and protein markers but are nonetheless targeted for fusion with lysosomes, presumably also by diverse mechanisms. These distinct pathways of phagosome maturation, despite culminating in phagosome-lysosome fusion, may have other functional consequences, for example with respect to antigen processing/presentation and/or activation of cell signaling events in response to phagocytosis.

In conclusion our study shows that S. Typhimurium can control SCV maturation by altering the membrane charge of the vacuole. This has a global effect on the recruitment of host-cell proteins involved in endocytic trafficking, such as Rab35, and helps the bacteria contained in SCVs to avoid lysosomal degradation (see model in Figure 4-10). We speculate that manipulation of an entire class of host proteins via membrane charge may be a universal strategy adopted by diverse intracellular pathogens to evade host-cell innate and adaptive defense mechanisms.
Figure 4-9: Rab8B, 13, 23, 35 colocalization with ΔinvA/inv phagosomes, WT and ΔsopB SCVs. ΔinvA/inv model phagosomes, WT and ΔsopB SCVs were scored for their association with Rab8B, 13, 23 and 35 at indicated times following internalization into RAW647.1 macrophages (RAW) or Henle-407 epithelial cells, as indicated. The degree of shading represents the percent of phagosomes or SCVs colocalizing with each Rab GTPase according to the scale shown (0 to 100%). A. Data replotted from Figure 4-2A. B. Data taken from Smith et al., 2007. C. Data taken from Smith et al., 2007. ΔinvA mutant bacteria not expressing the Y. pseudotuberculosis invasin protein were coated with IgG and taken up by RAW 647.1 macrophages via FcyR-mediated phagocytosis. D, E. Data replotted from Figure 4-3A-D.
Figure 4-10: Model of SopB-mediated avoidance of SCV-lysosome fusion. During WT *S. Typhimurium* invasion the phosphatase activity of SopB promotes SCV maturation by driving an early recruitment of Rab5 and Vps34 PI3-K to the SCV (Mallo *et al.*, 2008). The SCV then acquires lysosomal glycoproteins (eg. LAMP1) in a Rab7 dependent manner from a poorly defined late endosomal compartment containing very low amounts of cathepsin D (CathD in figure) and which is independent of lysosomes (Meresse *et al.*, 1999). Such trafficking of the SCV precludes its fusion with lysosomes and helps bacteria establish a niche permissive for their replication. Here we present new evidence that the SopB phosphatase activity additionally alters the membrane charge of the nascent SCV resulting in displacement of specific Rabs and other host-cell proteins that rely on a strong negative charge for their membrane recruitment, some of which are involved in promoting phagosome-lysosome fusion. Hence, in the absence of SopB, the ΔsopB mutant is not only unable to acquire Rab5 and PI(3)P to promote proper SCV maturation, but it also cannot reduce the negative membrane charge on the nascent SCV, leading to retention of specific host-cell proteins that promote SCV-lysosome fusion. This in turn can result in increased bacterial degradation and perhaps antigen presentation by ΔsopB *S. Typhimurium*-infected cells.
CHAPTER 5
DISCUSSION

ROLE OF SOPD IN S. TYPHIMURIUM INFECTION

Role of SopD in invasion

SopD is a *Salmonella* effector with no known homologues with the exception of the *Salmonella* effector SopD2. In 1998, early on in the investigation of *Salmonella* effectors (the first secreted *Salmonella* effector, SopE, was identified in 1996 (Wood *et al*., 1996)) Jones *et al* established SopD’s importance in infection (Jones *et al*., 1998). SopD was found to be important in fluid secretion and inflammation in the infection model of cow gastroenteritis. It was also shown to act together in these same phenotypes with another effector discovered nearly at the same time, SopB (Galyov *et al*., 1997). Since the discovery of both effectors, a plethora of research papers on the many functions of SopB have been published, compared to the relative draught of publications on the subject of SopD. This can be partly explained by the fact that SopB taps into a principal eukaryotic cell regulatory pathway, the regulation of signaling cascades by phosphoinositides, and from that vantage point is able to orchestrate a multitude of host-cell responses. The function of SopD, even more than ten years after this protein’s discovery, remains more enigmatic, although its importance in gastroenteritis and systemic forms of *Salmonella* infection has been well recognized.

Our research has attempted to address SopD’s function and mechanism of action during *S. Typhimurium* infection. The initial hypotheses that SopD affects host-cell signaling cascades were unsupported by our data (Figure 3-13). However, a great surprise arose from my investigation of SopD. A SopD-GFP fusion, although cytosolic in fixed cells, was found also on endocytic vesicles within live cells. The standard fixation with PFA caused a redistribution of SopD-GFP to the cytosol, explaining the previously observed cytosolic location of this construct (Brumell *et al*., 2003).
The association of SopD-GFP with vesicles prompted us to look at its cellular distribution during the course of S. Typhimurium infection. Importantly, I found that SopD-GFP was recruited to the bacterial invasion site and that the phosphatase activity of SopB was required for this recruitment. Subsequently we found that SopD participated in membrane fission and macropinosome formation during bacterial internalization into Henle-407 epithelial cells, phenotypes originally associated with SopB. Despite these observations, no defect in invasion of ΔsopD mutants could be observed in this cell type (data not shown). However, the location of SopD at the invasion site and its modulation of membrane dynamics fell in line with a study by Raffatelu et al, in which the authors indicated that SopD participated in bacterial invasion of a specific epithelial cell line, the polarized T84 intestinal epithelial carcinoma cells, albeit in a highly redundant manner (Raffatellu et al., 2005). In this study, SopD contributed to invasion of bacteria only in the absence of other invasion-associated effectors and only when the T84 cells were polarized. Furthermore, the invasion of the other polarized cell type tested was not affected. This indicates that certain S. Typhimurium effectors have roles restricted to cell types such as polarized epithelial cells encountered by bacteria throughout their infectious life cycle and points out the insufficiency of standard tissue culture models in exploring certain phenotypes. It also suggests that effectors can contribute to subtle phenotypes, which nonetheless play a significant role in the overall fitness of the pathogen.

**SopD in polarized cells?**

Based on Raffatelu et al’s data, SopD contributes to invasion of polarized, but not non-polarized cells (Raffatellu et al., 2005). The reasons for this could be many, since the function and structure of the apical and basolateral membranes of intestinal epithelial cells are quite different. While the basolateral membrane is similar to that of non-polarized cells, the apical membrane is organized into a brush border. It contains a dense forest of microvilli (up to 3000/cell), which are projections of the plasma membrane supported by actin cytoskeleton bundles (Danielsen et al., 2006). Microvilli are defined by their actin-based cytoskeleton in the core of the microvillus and short, actin-binding
cross filaments connected transversely with the cytoplasmic leaflet of the microvillus membrane and the core filaments (Danielsen et al., 2006). Actin cross-linking proteins such as fimbrin and villin are compartmentalized in the microvilli at relatively high concentrations (Heintzelman et al., 1992, Louvard et al., 1992). Below the microvilli is the terminal web region, which is a myosin-rich filamentous structure extending up to 1 µm into the cell. Deep membrane invaginations between adjacent microvilli that span the terminal web, called apical tubules, are membrane domains possibly specialized in exo/endocytic transport. Also likely to be of significance to bacterial invasion, the composition of lipid rafts at the intestinal brush border is quite different compared to other epithelial cells. While cholesterol and sphingomyelin compose the majority of lipids in rafts of kidney proximal tubule cells, they contribute only ~15% to rafts found in the small intestinal brush border, with glycolipids the predominant constituent, making up >30% of the raft (Danielsen et al., 2006). Therefore, due to its complex structure and distinct composition, disruption of the intestinal brush border and induction of membrane ruffling by S. Typhimurium may require the function of effectors, such as SopD, whose mode of action cannot be uncovered in traditional tissue culture models of infection.

In addition to structural differences between the apical surfaces of polarized and non-polarized cells, different signaling cascades are relevant in each area. In fact, it has been shown that different signaling cascades are activated upon bacterial entry via the apical versus the basolateral pole of polarized cells, perhaps due to different sub-cellular distributions of Rho GTPases (Criss et al., 2001). While Rac1 activation is necessary for S. Typhimurium to enter polarized MDCK cells from the apical side, both Rac1 and Cdc42, although activated are not necessary for basolateral invasion (Criss et al., 2001). Since intracellular bacteria effectors often act by targeting small GTPases to elicit engulfment, it is possible that SopD could be acting in this manner also, whether as a GEF, a GAP or a GTPase mimic, whose signaling was specific to the brush border of intestinal epithelial cells.

Unfortunately, although undoubtedly bound to be instructive, due to technical difficulties we failed to examine the distribution of SopD-GFP in polarized T84 cells and the phenotypes associated with invasion of this cell line. Therefore, we were unable to determine how SopD controls membrane dynamics during invasion. However our work
builds on that of Raffatelu et al.’s and provides a foundation for discovering the mechanism of SopD function in bacterial entry.

**SopD and lipid substrates?**

A further possibility is that SopD could be contributing to specific lipid modifications at the invasion site. This is a particularly attractive possibility because of the properties of binding of the over-expressed SopD-GFP construct we observed and the contribution of SopD to membrane fusion. SopD-GFP membrane binding was extremely sensitive to PFA fixation and ATP depletion, indicating that SopD-GFP is likely targeted to membranes by virtue of a tenuous interaction with a labile factor, sensitive to phosphorylation events, such as a phospholipid. This idea is further supported by the fact that recruitment of SopD-GFP to the \( S. \) Typhimurium invasion site was SopB-dependent. SopB is a phosphoinositide phosphatase with a well-documented effect on phospholipids at the invasion site. It is therefore an intriguing possibility that SopD is directly interacting with a lipid by-product of SopB’s catalytic activity. Such an interaction could have several consequences. First of all, it could simply recruit SopD to an appropriate location, focusing its activity. For example, SopD could act as a membrane deformation protein at the invasion site, akin to BAR domain proteins. Secondly, the hypothetical lipid could also serve as a SopD substrate. SopD-mediated alterations to membrane composition could contribute to regulation of GTPases, have consequences in host-cell protein recruitment, or they could also affect the physical properties of membranes, such as curvature, facilitating membrane reorganizations. Despite these appealing predictions, we were unable to identify a phospholipid as a GST-SopD binding partner \textit{in vitro}, possibly due to a requirement of additional features for SopD’s recognition of its lipid substrate or SopD’s recognition of more general membrane features, such as electrostatic charge, imparted by membrane composition (as discussed later). In another scenario, SopD may be recognizing a protein, such as a GTPase that becomes recruited and activated by SopB-mediated phospholipid alterations.
**SopB and SopD working together**

SopB and SopD have been shown to act in concert during infection of bovine ilea to elicit fluid secretion and PMN influx into the ileal lumen (Jones *et al.*, 1998). In my work we have uncovered the basis of their cooperative action, their joint contribution to membrane rearrangements at the bacterial invasion site, presumably resulting in efficient bacterial internalization leading to increased proinflammatory signaling.

At 20 min following invasion SopD’s contribution to host-cell membrane sealing was similar to that previously shown for SopB and was not additive in nature, with the double ΔsopBΔsopD mutant showing the same defect as either one of the single mutants (Figure 3-1E, F). However, at 40 – 60 min following invasion the ΔsopBΔsopD mutant showed a stronger sealing defect than either one of the single mutants (Figure 3-1E). Both effectors also contributed to macropinosome formation in a cooperative manner, with the double ΔsopBΔsopD mutant showing the most severe phenotype, a 77% loss of ability to form macropinosomes compared to only a 23% and 30% decrease for ΔsopD and ΔsopB mutant, respectively (Figure 3-2B). These data suggest that SopD and SopB use different pathways/mechanisms to promote bacterial invasion. It has been postulated that ΔsopB’s delay in membrane sealing is due to the rigidity of the plasma membrane imparted by the underlying actin cytoskeleton mesh that is not efficiently disassembled in the presence of SopB’s substrate, PI(4,5)P₂ (Terebiznik *et al.*, 2002). However, the contribution of membrane alterations driven by SopB cannot fully be discounted since SopB-driven macropinosome formation has been proposed to occur due to phosphoinositide alterations that promote homotypic membrane fusion (Hernandez *et al.*, 2004). It remains to be determined whether SopD promotes invasion by driving further unrelated endocytic membrane fusion events or by promoting actin rearrangements unrelated to PI(4,5)P₂ hydrolysis.

**SopD and SCV maturation?**

We, and others, have identified SopD’s contribution to invasion. However, SopD is a protein that can be translocated by both SPI-1 and SPI-2 T3SSs and has been shown
to play a significant role in persistence of \textit{S. Typhimurium} in a systemic model of infection. SPI-1 T3SS translocated effectors are increasingly found to play important roles in subsequent SCV trafficking as in the case of SopB (see discussion below). Therefore, could SopD also affect SCV maturation?

In live uninfected and infected cells over-expressing SopD-GFP, we observed this construct localized to dynamic vesicles. However, using colocalization studies with RFP-tagged SopD and GFP-tagged Rab GTPases we failed to identify an indiscriminate marker of SopD positive compartments, although frequent association with GFP-Rab7 was observed and some SopD-GFP compartments were acidic as marked by LysoTracker (Figure 3-5). Despite this, SopD had no effect on LAMP1 acquisition by SCVs (Figure 3-12) and did not contribute to avoidance of SCV-lysosome fusion in Henle-407 cells (data not shown). At late times of invasion SopD-GFP was also localized to \textit{Sifs} but SopD itself played no detectable role in \textit{Sif} formation (data not shown).

Despite these negative results, others in our lab have found that SopD promoted the formation of SopB-dependent SNX3 positive tubules associated with SCVs following invasion. The SNX family of proteins is thought to participate in endocytic recycling and the SNX3 tubule network may effectively remodel the SCV (Carlton \textit{et al.}, 2005, Bujny \textit{et al.}, 2008). Therefore, SopD, perhaps by promoting recycling, may affect SCV biogenesis. Further supporting SopD’s role in SCV maturation, our preliminary experiments revealed that 1 hour following invasion \textit{ΔsopD} mutants acquired significantly more Rab23 (~50% colocalization of GFP-Rab23 with \textit{ΔsopD} SCVs) than WT bacteria (~30% colocalization), similar to \textit{ΔsopB} bacteria (~50% colocalization). The double \textit{ΔsopBΔsopD} mutants showed the greatest (65%) colocalization with GFP-Rab23, indicating that both SopB and SopD contribute to removal of Rab23 from nascent SCVs. Previously Rab23 was implicated in promoting phagosome-lysosome fusion (Smith \textit{et al.}, 2006) but as SopD doesn’t seem to play a role in lysosomal avoidance the significance of these results remain to be discovered. It is possible that SopD may help to specifically remove Rab23 from the SCVs via the SNX3 recycling tubules, but whether it also affects the localization of other endocytic markers is unknown. In contrast SopB was found to affect the targeting of other Rabs in addition to Rab23. The unexpected
mechanism by which SopB affects protein targeting and contributes to SCV maturation is discussed next.

ROLE OF SOPB IN SCV MATURATION

SopB inhibits SCV-lysosome fusion

As previously mentioned, a few SPI-1 T3SS translocated effectors have been recognized to participate in events beyond that of host cell invasion classically attributed to them. Indeed, our investigation of SopB-mediated membrane alterations during invasion led to unexpected and interesting results concerning SCV maturation. We discovered that during invasion SopB was responsible for reducing the negative charge of the nascent SCV membrane, was affecting the array of Rab proteins recruited to the SCV, and was inhibiting the fusion of lysosomes with SCVs. Our results suggest that through control of nascent SCV membrane charge, SopB helps S. Typhimurium steer clear of the degradative pathway of the host cell.

The plasma membrane, due to its specific assortment of anionic phospholipids such as PI(4,5)P$_2$ and PS, is the most negatively charged membrane in eukaryotic cells (Yeung et al., 2007). Using a protein probe designed to measure strong negative membrane charge (Yeung et al., 2006b) we observed that ΔsopB -containing SCVs retained a strong negative charge on their membrane that is normally associated with the inner leaflet of the plasma membrane for up to 2 hours following invasion (Figure 4-7 A, B). In contrast, the WT SCVs showed no retention of negative membrane charge, even at early times (20 min) following invasion (Figure 4-7 A, B). We expected that such dramatic differences in charge would have important consequences in protein recruitment to the SCV, and therefore SCV trafficking. Indeed, we discovered that Rab GTPases normally associated with model phagosomes and not WT bacteria were significantly retained on the ΔsopB SCVs in a charge dependent manner (Figure 4-3, 4-4). Two of these Rabs (Rab23 and Rab35) were previously implicated in promoting phagosome-lysosome fusion (Smith et al., 2007). Furthermore, we were able to show that ΔsopB-containing SCVs were unable to avoid fusion with lysosomes unlike WT-containing
SCVs, at early times following invasion (Figure 4-1 A, B, 5-4). Our observations suggest that SopB by controlling membrane charge of the SCV was removing Rabs from this compartment that would normally promote the delivery of its contents to lysosomes.

We speculate that SopB, by preventing fusion of lysosomes with the SCV immediately following invasion allows S. Typhimurium sufficient time to express genes required for its continued intracellular survival. In this way, SopB helps the bacteria to establish a firm foothold in the infected cell. The involvement of SopB in lysosome avoidance was a very exciting result, as previously no SPI-1 T3SS translocated effector was directly implicated in this phenotype, one classically attributed to the SPI-2 T3SS-translocated effectors. Furthermore, the manipulation of lysosome fusion with SCVs by SopB may have additional consequences on the infected host cell early during infection. Evidence suggests that SopB allows S. Typhimurium to decrease its immunogenicity during infection, and we speculate it may do so by inhibition of lysosome fusion, thereby affecting antigen processing and presentation. Additionally, a recent report indicated that late endosomes are novel platforms for KRas-mediated MAPK signaling (Lu et al., 2009). Therefore SopB, by blocking lysosome fusion with late endosomes could prolong signaling events from these compartments. Whether this specific form of signaling occurs during S. Typhimurium invasion, its consequences for the bacteria, and SopB’s impact on it should be investigated.

**SopB regulation of membrane charge**

SopB is a phosphoinositide phosphatase that hydrolyzes the negatively charged phospholipid PI(4,5)P$_2$. Therefore, it was not too surprising that SopB affected membrane charge during invasion by principally dephosphorylating PI(4,5)P$_2$ at the plasma membrane. Measuring levels of this anionic phospholipid on the SCV using a PI(4,5)P$_2$-specific probe revealed that a significantly higher number of sealed ΔsopB SCVs containing PI(4,5)P$_2$ in their membrane existed 20 min following invasion than WT SCVs (51% and 0%, respectively) in agreement with previous findings (Terebiznik et al., 2002) (Figure 4-7 C, D). Differences in levels of another phospholipid, PS, in SCV membranes were also observed between WT and ΔsopB bacteria, with significantly
higher levels of PS present in ΔsopB SCV membranes 1 and 2 hours following invasion (Figure 4-7 E, F). However, we could rescue ΔsopB SCVs as well as model phagosomes from fusion with lysosomes by artificially dephosphorylating PI(4,5)P₂ at the plasma membrane prior to bacterial invasion (Figure 4-8 C-F). This indicates that PI(4,5)P₂ dephosphorylation is the critical SopB-mediated event that contributes to maturation of the SCV. The significance of increased frequency of PS localization to ΔsopB SCVs compared to WT SCVs is currently waiting to be explored. It may be that PS is more often present on ΔsopB SCVs because of enhanced fusion with PS-rich lysosomes or diminished recycling of membrane from the SCV. Therefore, whether increased levels of PS on ΔsopB SCVs are the consequence of or actually contribute to lysosome fusion remains to be determined.

**SopB regulation of SCV-lysosome fusion**

What is the consequence of SopB’s function that ultimately prevents the progression of the SCV into a mature phagolysosome? Our observed differences between ΔsopB and WT SCVs with respect to Rab association make Rab targeting one of the obvious answers to this question, but not the only possibility. Membrane charge has the potential to affect targeting of a variety of proteins. Furthermore, SopB-mediated lipid modifications in the SCV membrane could more directly dictate the trafficking of this compartment.

1. **Effects of charge**

   The Rabs we found selectively associated with ΔsopB SCVs (Rab8B, 13, 23, and 35) belonged to a group of Rabs previously found associated with a model phagosome destined for fusion with lysosomes but not WT SCVs (Smith et al., 2007). They also shared another common feature, that of the prenylated, polybasic carboxy-terminus that was shown to be responsible for their targeting to the plasma membrane of HeLa and/or NIH3T3 cells (Heo et al., 2006). Furthermore, the specific targeting to the plasma membrane via this motif was dependent on the plasma membrane’s strong negative
charge. This same motif was found necessary and sufficient to target Rabs to ΔsopB SCVs, which like the plasma membrane maintained a strong negative charge (Figure 4-4, 4-5). Based on this data, we speculate that the association of Rabs that promote phagosome-lysosome fusion (Rab 23 and Rab35) with the mutant SCVs predisposes them for lysosome fusion. However, this remains to be proved by further work. Additionally, other small GTPases share the prenylated, polybasic carboxy-terminus motif (Heo et al., 2006). It is conceivable that other proteins, alone or jointly with Rab8B, 13, 23, and 35 contribute to ΔsopB SCV-lysosome fusion.

Rac1 is a small GTPase that regulates actin rearrangements during phagocytosis and S. Typhimurium internalization alike. Rac1 also regulates the activity of the NADPH oxidase, which is responsible for producing ROS in phagosomes and microbial killing (Grandvaux et al., 2007). It does so by helping to assemble the NADPH oxidase complex on the phagosome membrane and importantly, is also recruited to membranes via electrostatic charge and its prenylated, polybasic carboxy-terminus. Upon investigating the association of Rac1 with WT and ΔsopB SCVs we found that it was recruited preferentially to the latter (Figure 4-6). It is therefore possible that Rac1 may promote NADPH oxidase activity on ΔsopB SCVs. This could allow the host cell to more successfully re-route the SCVs containing S. Typhimurium “weakened” by ROS towards fusion with lysosomes. In addition to Rac1 and the Rabs investigated in my work, 32 other GTPases also contain at least the polybasic domain in their carboxy-terminus and are targeted to the negatively charged plasma membrane. Whether they are preferentially targeted to ΔsopB SCVs remains to be investigated.

2. Alternative mechanisms

SopB has been shown to activate the pro-survival kinase Akt by hydrolysis of PI(4,5)P₂ at the plasma membrane, and subsequent generation of PI(3,4,5)P₃ (Knodler et al., 2005a, Marcus et al., 2001, Steele-Mortimer et al., 2000). Recently, a report indicated that Akt activation during S. Typhimurium infection might inhibit SCV fusion with lysosomes (Kuijl et al., 2007). The authors showed that WT bacteria replicated less in a breast cancer cell line treated with an Akt inhibitor. They also made a link between
SopB, Akt activation and Rab14 activation and recruitment to SCVs, implying that Rab14 activation was responsible for a block in SCV maturation into a phagolysosome. However, in our study we failed to observe an increased recruitment of Rab14 to WT versus ΔsopB SCVs, as well as Rab22A, another GTPase associated with viable M. tuberculosis, suggesting that S. Typhimurium and M. tuberculosis avoid lysosome fusion by distinct mechanisms (Figure 4-3). Also, Akt activation during S. Typhimurium invasion peaks at 30-60 min following bacterial internalization, while the authors treated cells with Akt inhibitors 60 min following invasion. Therefore, although it has been established that Rab14 participates in remodeling of the M. tuberculosis-containing phagosomes, the role of this GTPase in SCV maturation is still unclear. Likewise, the role of Akt activation in preventing SCV-lysosome fusion should be further investigated.

Finally, could other lipids whose production is controlled by SopB prevent SCV-lysosome fusion? PI(3)P is formed transiently on the SCV by a SopB-dependent mechanism. SopB recruits Rab5 to the SCV, which in turn recruits Vps34 that is responsible for PI(3)P formation (Mallo et al., 2008). However, inhibition of Vps34 using a PI3-K inhibitor LY294002 caused a significant reduction of lysosome fusion with ΔsopB SCVs (Figure 5-1). This seems counterintuitive since SopB promotes PI(3)P formation as well as avoidance of lysosome fusion (Figure 4-1 A, B). Then again, inhibition of PI3-K causes an arrest of endosome and phagosome maturation in general, and the effect of LY294002 on SCV-lysosome fusion agrees with those observations and probably reflects this more general effect. Another lipid that participates in phagosome maturation and whose formation SopB may affect is PI(3,5)P₂. PI(3,5)P₂ could be formed from PI(3)P on the SCV by the host-cell PI(3)P 5-kinase PIKfyve, and in fact, an accumulation of PIKfyve on SCVs has been reported (unpublished observations of (Bujny et al., 2008)). As a phosphoinositide phosphatase, SopB could also hydrolyze PI(3,5)P₂. DAG is also an important lipid second messenger and indirect evidence suggests that during S. Typhimurium invasion SopE and SopB both could activate PLC leading to production of DAG. During invasion a SopE and SopB dependent accumulation of lower inositol phosphates was observed to occur in excess of that which could be accounted for by dephosphorylation of higher inositol phosphates (Zhou et al., 2001). Therefore, it has been suggested that the increase in lower inositol phosphates
could occur via activation of Cdc42 and Rac by SopE and SopB, which can in turn activate PLC\(\beta\)2 (Illenberger et al., 1998) and bind PLC\(\gamma\), presumably also activating it (Hong-Geller et al., 2000) leading to hydrolysis of phosphoinositides. However, whether DAG and/or PI(3,5)P\(_2\) are formed or degraded on the SCV in a SopB-dependent manner, whether they contribute to SCV maturation, and the significance of PI(3)P formation on the SCV in terms of lysosome avoidance are all interesting questions that remain to be answered.

**Implications for other pathogens**

*S. Typhimurium*’s manipulation of key endocytic regulatory proteins, the Rabs, via membrane charge is an elegant mechanism of subversion of host cell function by an intracellular pathogen. The simplicity and benefit of this mechanism to *S. Typhimurium* implies that other intracellular pathogens may have taken advantage of membrane charge in order to survive inside their hosts. For example, the mechanism of entry of *Brucella* spp. into macrophages affects bacterial survival (Gorvel et al., 2002) and differences in membrane charge during different types of *Brucella* internalization could be responsible. However, no SopB homologue has been identified in *Brucella* spp. or other vacuole-adapted pathogens. The two SopB homologues IpgD and BopB belong to cytosol-adapted pathogens *S. flexneri* and *Burkholderia pseudomallei*, respectively.

We took advantage of the homology between IpgD and SopB to investigate the ability of IpgD, as a phosphoinositide phosphatase, to complement the SCV trafficking phenotypes associated with SopB. We were able to show that IpgD complemented the ability of SopB to lower the negative membrane charge, hydrolyze PI(4,5)P\(_2\) and lower levels of PS on SCVs (Figure 4-7 G, H, I). IpgD translocated by ΔsopB *S. Typhimurium* was also able to complement Rab35 removal from the SCV and lysosome fusion avoidance mediated by SopB (Figure 4-1 C, 4-4 E). The putative effector BopB of *B. pseudomallei* contains, like SopB and IpgD, an amino acid motif conserved in catalytic domains of numerous phosphatases (Stevens et al., 2002) and it is possible that it could affect membrane charge in a manner similar to its homologues. Like SopB, IpgD and BopB stimulate bacterial entry into host cells but because *S. flexneri* and *B. pseudomallei*
Figure 5-1: PI3-K inhibition prevents fusion of ΔsopB SCVs with lysosomes.
Henle-407 epithelial cells were pulse chased with DQ-BSA to label degradative compartments and infected with GFP-expressing S. Typhimurium. White bars indicate untreated cells. Black bars indicate LY294002 treated cells. LY294002 was added to cells 15 min prior to invasion and remained present throughout the course of infection. At 2 h p.i. 0.3 µm confocal z-stacks of infected cells were imaged and the fraction of bacteria colocalizing with dequenched DQ-BSA signal counted. Colocalization of DQ-BSA with S. Typhimurium. Mean ± S.E.M. for four independent experiments. ** P value < 0.01.
escape from their vacuoles shortly after internalization, the significance of vacuole membrane charge potentially controlled by these effectors is unknown.

SPI-2 T3SS translocated effectors and the PhoPQ system are necessary in addition to SopB for *S. Typhimurium* to prevent lysosome-SCV fusion throughout this pathogen’s intracellular habitation. In contrast *S. flexneri* and *B. pseudomallei* escape fusion with lysosomes by escaping into the cytosol. However the cytosol adapted pathogen *L. monocytogenes* has been shown to prevent the fusion of lysosomes with its vacuoles early following internalization and prior to escape into the cytosol (Shaughnessy *et al.*, 2006, Henry *et al.*, 2006a). This may allow bacteria sufficient time to escape the vacuole unharmed. Additionally, a fraction of intracellular population of *L. monocytogenes* has been found in large membrane compartments at late times following bacterial internalization (Birmingham *et al.*, 2008), indicating that other cytosol-adapted pathogens may also reside in phagosomes. It has been suggested that this vacuole bound *L. monocytogenes* population contributes to persistent infection (Birmingham *et al.*, 2008) and, at least in the case of *B. pseudomallei*, infections have been notoriously difficult to treat with relevant antibiotics, with reemergence of infection often occurring following treatment (Chaowagul *et al.*, 1993). It is therefore possible that the cytosol adapted pathogens *S. flexneri* and *B. pseudomallei* also prevent lysosome fusion with their nascent vacuoles prior to escape or in the case of *B. pseudomallei* maybe also later during infection, via the function of IpgD and BopB, respectively. Additionally, the ability of IpgD (and potentially BopB) to control charge and phagosome/endosome maturation in the cell might help the bacteria in suppressing antigen presentation. Further work is necessary to determine if IpgD, BopB, or both have an effect on lysosome fusion and/or bacterial immunogenicity during *S. flexneri* and *B. pseudomallei* infections.
FUTURE DIRECTIONS

SopD phenotypes in polarized cells

SopD’s mechanism of action remains unknown. As it was shown to promote *S. Typhimurium* invasion of polarized but not non-polarized T84 cells, it would be valuable to observe SopD-associated phenotypes uncovered in my work in this cell line. SopD-GFP dissociates from membranes following fixation, indicating that colocalization with potential host-cell targets is weak and transient and best observed in live cells. However, observing live polarized cells by immunofluorescence microscopy is difficult since the translucent filter they must be grown on to establish proper polarity obstructs the light path of the microscope. Ingeniously, growing cells on the underside of a filter support has been used to solve this problem (Wakabayashi *et al.*, 2007). Once a polar monolayer is established, live polarized cells can be imaged on the inverted filter support placed in a glass chamber slide, as depicted in Figure 5-2. Using this technique and spinning disk confocal microscopy the localization of SopD-GFP transiently transfected into polarized T84 cells could be observed. Invasion of these SopD-GFP expressing cells by RFP expressing *S. Typhimurium* delivered apically or basolaterally would allow observation of SopD-GFP distribution during invasion of polarized cells, in the presence or absence of SopB. Effects of SopD on membrane sealing and macropinosome formation could also be studied. It would be interesting to see how our observations made in unpolarized cells would translate to events occurring during invasion of polarized epithelia.

The actin structure associated with the apical side of a polarized cell is very different from that of the basolateral side. As suggested in the discussion, SopD may affect F-actin during invasion. Therefore it would be worthwhile to investigate actin dynamics during invasion of polarized cells by ΔsopD compared with WT bacteria. A new F-actin probe (Lifeact-GFP) has been recently developed that does not interfere with cellular processes and shows a lower background signal compared to actin-GFP (Riedl *et al.*, 2008). Therefore Lifeact-GFP could be used to study effects of SopD on actin dynamics during *S. Typhimurium* invasion of polarized cells. Additionally, *S.*
Typhimurium is known to recruit focal adhesion proteins to the invasion site, and some of them also promote invasion (Finlay et al., 1991, Shi et al., 2006). Therefore observation of fluorescently tagged focal adhesion proteins such as FAK, p130Cas, talin and vinculin during invasion in the presence or absence of SopD could be informative. Collectively, these experiments could provide new insights into SopD function during invasion.

SopD Targeting

In our work we were unable to show how SopB’s phosphatase activity recruits SopD to host-cell membranes at the invasion site. Discovering this would enable us to understand SopD’s mechanism of function and perhaps the nature of its host-cell target(s). Several questions regarding SopD recruitment to membranes remain to be addressed:

Is SopB phosphatase activity sufficient for SopD recruitment, or are other bacterial factors necessary? To answer this question several microscopy-based studies can be used. When expressed ectopically in epithelial cells, SopB causes ruffling and macropinocytosis. Localization of SopD-RFP in SopB-expressing cells to ruffles and large vacuoles would imply that SopB was sufficient to promote SopD recruitment to membranes. It would be interesting to determine if co-expression of the two effectors would affect macropinocytosis driven by SopB. Additionally, inp54 phosphatase-mediated inducible dephosphorylation of PI(4,5)P$_2$ at the plasma membrane could be used. If SopD-RFP is recruited to the plasma membrane following PI(4,5)P$_2$ dephosphorylation, this would suggest that PI(4,5)P$_2$ dephosphorylation is the critical event mediating SopD recruitment. Additionally, determining if SopD can be recruited to macropinosomes formed independently of $S$. Typhimurium invasion (for example following EGFR stimulation) would help determine the requirement for other bacterial or host cell proteins for SopD recruitment.

Is endogenous SopD recruited to the same location as over-expressed fluorescently tagged SopD? This is a technically challenging question due to SopD’s dissociation from membranes following fixation. Also, the fluorescent protein tags (GFP
Figure 5-2: Live cell imaging of polarized cells grown on the underside of a filter insert. A monolayer of polarized cells grown on the underside of a Transwell filter can be imaged live using an inverted microscope by placing the Transwell filter into a glass chamber slide (available from LabTek). Reagents or bacteria can be applied to specific plasma membrane domains (apical or basolateral) during imaging. Image taken from Wakabayashi et al., 2007.
and RFP) that allowed us to observe over-expressed SopD in live cells are too structurally stable to pass through the T3SS, which requires effectors to unfold during translocation. We previously attempted to visualize endogenous SopD delivered by bacteria during invasion using the Lumio (also known as FlAsH tag) method (Invitrogen). Using this method others have successfully visualized the location of *S. flexneri* IpaB and IpaC, which are presumed to form the *S. flexneri* T3SS translocon (Enninga et al., 2005). We, however, were unsuccessful in labeling SopD (Figure 5-3), perhaps due to lower amounts of translocated SopD or its diffuse localization compared to the two *S. flexneri* proteins that are localized to puncta at the site of bacterial translocation (Enninga et al., 2005). Therefore, visualization of endogenous SopD still remains a difficult problem that awaits new technical developments in live cell protein imaging.

**SopD Protein Targets**

Currently we do not know if SopD is binding a protein or lipid on macropinosomes formed during *S. Typhimurium* invasion. Initial studies did not reveal any affinity of SopD for phospholipids. If SopD is interacting with a protein, due to the labile nature of this interaction, it is likely that affinity pull-down of tagged SopD construct transfected into cells may not be a useful approach in identifying its interacting partner(s). Therefore, a first step in the identification of a SopD binding partner or substrate would be the identification of protein and lipid factors present on macropinosomes during WT but not ΔsopB invasion. Good candidates include Rho and Rab GTPases, a fluorescent fusion library of which is currently available in our lab. Proteins that mediate membrane fission, such as dynamin and CtBP1/BARS, whose phosphorylation by PAK1 is required for membrane fission during EGF stimulated macropinocytosis, are also interesting candidates (Liberali et al., 2008). Following identification of proteins absent from ΔsopB invasion sites but present on macropinosomes formed during WT invasion, the effect of SopD on their localization during invasion can be studied. Furthermore, the polarized T84 cells grown on inverted filters would also make a great system for confirming colocalization of fluorescent SopD constructs with potential host cell targets during invasion.
SopD and SCV maturation

We found that SopD and SopB could act cooperatively to prevent the accumulation of Rab23 on SCVs following invasion. Both effectors have been implicated in the formation of a SNX3 positive tubule network early during S. Typhimurium invasion (data not shown) and the role of SopD in SNX1 tubule network formation (Bujny et al., 2008) still remains to be tested. While removal of Rab23 from the SCV by SopB is mediated by electrostatic charge, SopD’s mechanism of Rab23 removal is unknown. Therefore it would be interesting to see if SopD prevented accumulation of Rab23 on SCVs by controlling SNX3 and/or SNX1 recycling events. To this end, GFP-Rab23 colocalization with WT and ΔsopD SCVs could be determined in cells treated with SNX3 and/or SNX1 siRNA and control siRNA. If SopD removes Rab23 from SCVs via SNX3- or SNX1-mediated recycling events then it would be expected that upon knockdown of the SNXs, WT SCVs would retain increased amounts of GFP-Rab23, similar to ΔsopD mutants, and the knockdown of the SNXs would have no further effect on GFP-Rab23 colocalization with ΔsopD SCVs.

The fact that SopD can affect the localization of Rab23 on SCVs following invasion implies that it may control the localization of other proteins. Therefore, if potential SopD targets are identified as described in the above section, their colocalization with WT and ΔsopD SCVs may uncover other ways in which SopD affects SCV trafficking.

Mechanism of inhibition of SCV-lysosome fusion by SopB

The work presented in this thesis describes a model in which SopB, through controlling the charge of the SCV membrane, promotes the removal of specific Rabs from the SCV, thereby helping the SCV avoid fusion with lysosomes. However, this model must be more stringently tested and alternative explanations for SopB-mediated lysosome avoidance fully addressed.
Figure 5-3: FIAsH method of labeling SopD in live cells is not effective in observing the localization of this translocated effector. Henle-407 cells transfected with PLCδ-PH-mRFP were mounted onto a heated stage and infected with doubly labeled (NHS-647 and FIAsH) ΔsopD + psopD-4C (A, B) or ΔsopD + psopD (C) S. Typhimurium. The course of infection was examined using a spinning disk confocal microscope with a heated stage assembly. Representative images at 30 min post infection are shown. Bacteria (arrowheads) as well as unspecific background FIAsH reagent staining of cells (arrows) are indicated. Bacteria that expressed SopD tagged with a FIAsH specific binding sequence, 4C, were stained effectively with this reagent (A, B), while bacteria expressing SopD without the binding sequence were not (C). However, localization of the labeled effector outside of the bacteria was not ascertained due to the high background signal, rapid photo bleaching, and most likely low levels of translocated SopD-4C-FIAsH in the host cell compared to the bacterial cytosol.
To prove that the assortment of Rabs removed from SCVs by SopB-mediated charge alterations prevents SCV-lysosome fusion it would be necessary to see if inhibition of Rab function is able to rescue ΔsopB SCVs from fusing with lysosomes. Inhibition of specific Rab function can be attained by expression of dominant negative constructs or siRNA treatment targeting Rab8B, 13, 23, and 35, singly or together. Also, observing the fusion of SCVs positive for these Rabs with DQ-BSA positive compartments would further support our model. Rac1 is another GTPase that is preferentially retained on ΔsopB SCVs probably via an electrostatic interaction. Rac1 could be recruiting the NADPH oxidase to SCVs and contributing to bacterial damage. The damaged bacteria could then be more prone to fusion with lysosomes. Therefore, it is necessary to test if Rac1 retention and NADPH oxidase activity contributes to SCV fusion with lysosomes in the absence of SopB. Effects on fusion of lysosomes with ΔsopB SCVs in cells treated with chemical NADPH oxidase inhibitors such as Diphenyleneiodonium (DPI) can be observed. Also quantifying the localization of NADPH oxidase components to WT and ΔsopB SCVs could indicate if the oxidase preferentially assembles on ΔsopB SCVs.

These experiments could also yield insight into a recently reported ability of SopB to control SCV positioning via a pathway involving Rho, ROCK, and myosin II. SopB was found to activate this pathway and increase the centripetal movement of SCVs following invasion (Wasylinka et al., 2008). Interestingly, the generation of ROS has an inhibitory effect on the activation of the same pathway (Figure 5-4) (Nimnual et al., 2003). It would be very interesting to determine if in the absence of SopB, the greater amount of Rac1 on the SCVs brought there by the strong negative membrane charge led to increased generation of ROS that inhibited myosin II activation. To test this idea, the distance of ΔsopB SCVs from the nucleus following invasion could be measured in the presence and absence of DPI, to inhibit NADPH oxidase activity and ROS generation.

Recent data suggest that active Akt may be involved in SCV trafficking (Kuijl et al., 2007). To determine if activation of Akt contributes to SopB-mediated avoidance of lysosome fusion the extent of DQ-BSA positive compartment colocalization with SCVs
in cells treated with an Akt inhibitor (AKTi1/2, Calbiochem) should be measured. If SopB-mediated Akt activation prevents SCV-lysosome fusion, the WT SCVs from AKTi1/2 treated cells will fuse with lysosomes as often as \( \Delta sopB \) SCVs, while no difference will be observed between the \( \Delta sopB \) SCVs from inhibitor treated and untreated cells.

The role of lipids in SCV maturation should be studied further. Of particular interest is the role of PI(3,5)P\(_2\) and PS. HPLC experiments could allow us to quantify the differences in PI(3,5)P\(_2\) levels in cells infected with WT and \( \Delta sopB \) S. Typhimurium at 90 min post infection, a time when a first significant difference is observed with respect to lysosome-SCV fusion (Figure 5-5). Additionally, effects of dominant negative PIKfyve expression on SCV-lysosome fusion of WT and \( \Delta sopB \) SCVs could be measured.

To determine if PS acquisition is a cause or a consequence of lysosome avoidance, lysosome fusion with \( \Delta sopB \) SCVs could be inhibited (for example by using LY294002 or the rapamycin inducible PI(4,5)P\(_2\) dephosphorylation system) and PS colocalization with SCVs quantified. If PS colocalization with \( \Delta sopB \) SCVs is lowered by these techniques this would suggest that PS accumulation on \( \Delta sopB \) SCVs is a consequence rather than cause of SCV-lysosome fusion. However, PS could also be removed from WT SCVs in a SopB- and SNX1 or SNX3-dependent manner (Bujny et al., 2008). Therefore, the colocalization of PS with WT SCVs in cells treated with SNX1 and/or SNX3 siRNA should be quantified. Increased levels of PS on WT SCVs in siRNA treated cells would indicate that normally PS levels on these SCVs are lowered because of increased recycling driven by SopB, SNX1 and/or SNX3. This would further suggest that PS might play a role in SCV trafficking. Determining if PI(3,5)P\(_2\) levels and PS localization to the SCVs are controlled by SopB, and if these lipids contribute to lysosome avoidance, would shed more light on this complicated process.

**SCV-lysosome fusion by SopB in macrophages**

The macrophage is a professional phagocyte adept at destroying microbial invaders and a cell type that is highly relevant in systemic disease caused by *Salmonella enterica* serovars. Also, SopB confers to *S. Typhimurium* a survival advantage in
Figure 5-4: Inhibition of Rho activity by Rac signaling. Activation of Rac can inhibit Rho activity in various cell types. For example, in fibroblasts, activation of Rac leads to a three to four-fold decrease in the levels of active GTP-bound Rho (Nimnual et al., 2003). This occurs independently of Rac’s ability to remodel the actin skeleton or stimulate the JNK kinase pathway (Sander et al., 1999, Nimnual et al., 2003). Firstly, Rac can stimulate PAK1, which in turn inhibits acto-myosin contractility by phosphorylation of myosin–II heavy chain and myosin light chain kinase (Sanders et al., 1999, van Leeuwen et al., 1999). Secondly, the Rac insert region (small helix composed of residues 124-135 of Rac) is responsible for p190Rho-GAP activation that is necessary to down regulate Rho activity (Nimnual et al., 2003). Surprisingly this occurs via ROS production. Rac activates the NADPH oxidase by bringing p67-phox to membrane-associated components of the oxidase complex, which p67phox can then activate. Additionally, the Rac insert region can control oxidase activity by interacting directly with the catalytic flavoprotein of the NADPH oxidase and regulating electron flow (Bokoch et al., 2002). ROS production was shown to down regulate Rho activity in fibroblasts by ROS-mediated inactivation of the tyrosine phosphatase LMW-PTP (low-molecular weight protein tyrosine phosphatase) through direct oxidation of reactive cysteines in its catalytic pocket. The p190Rho-GAP is normally phosphorylated by LMW-PTP and in this way its function is inhibited. Therefore the inhibition of LMW-PTP activity by ROS results in increased phosphorylation of p190Rho-GAP and inactivation of Rho. (Figure taken from Caron, 2003).
Figure 5-5: Time course of SCV-lysosome fusion during S. Typhimurium invasion. Henle-407 epithelial cells were pulse chased with DQ-BSA to label degradative compartments and infected with GFP-expressing S. Typhimurium. At 20 min, 40 min, 60 min, 90 min, 120 min and 240 min p.i. 0.3 μm confocal z-stacks of infected cells were imaged and the fraction of bacteria colocalizing with dequenched DQ-BSA signal counted. Mean ± S.E.M. for four independent experiments. More than 100 bacteria were counted for each strain and time point during each experiment. * P value < 0.05, ** P value < 0.01.
macrophages (Hernandez et al., 2004). Therefore, it should be determined if SopB-mediated lysosome avoidance also occurs in this cell type and whether it occurs via SCV membrane charge alterations. Also, the mechanism of internalization of S. Typhimurium by macrophages has been found to affect the replication capacity of this pathogen. Hence, the macrophage would be a great system to study the effects of different internalization processes and whether survival of bacteria following each relates to phagosome membrane charge differences. The extent of lysosome fusion with WT and ΔsopB SCVs can be measured by the DQ-BSA assay in RAW264.7 macrophages. The colocalization of the RpreRed construct with bacteria following invasion would uncover any charge differences between WT and ΔsopB SCVs. Impact on SCV membrane charge and fusion with lysosomes can be studied during SPI-1 T3SS mediated invasion of macrophages or macrophage phagocytosis of non-opsonized bacteria, bacteria opsonized by IgG or by complement. Interestingly, S. Typhimurium replicates better following SPI-1 T3SS mediated invasion and phagocytosis of complement opsonized bacteria rather than following phagocytosis of IgG-opsonized or non-opsonized bacteria (Drecktrah et al., 2006). It would be very interesting to determine if charge differences during different types of phagocytosis are contributing to greater pathogen survival.

Other Bacteria and Membrane Charge

Do other bacteria expressing proteins with homology to SopB manipulate membrane charge to their advantage? Observing the localization of RpreRed (negative charge probe) during S. flexneri (WT and ΔipgD) and B. pseudomallei (WT and ΔbopB) invasion could answer this question. Although both pathogens escape their vacuoles shortly following invasion, it is possible that not all bacteria escape efficiently. Perhaps the activity of IpgD and BopB prevent the fusion of lysosomes with vacuoles of bacteria that are slower to (or do not) escape (see Discussion). A DQ-BSA assay could be used to see if at least some S. flexneri and B. pseudomallei fuse with lysosomes and if fusion is enhanced in the absence of IpgD and BopB, respectively.

Bacteria may also take advantage of membrane charge alterations without expression of a specific phosphatase, perhaps by triggering an internalization process that
results in the loss of negative membrane charge on nascent phagosomes. The fate of *Brucella* spp. in macrophages is highly dependent on the method of internalization (phagocytosis of IgG or complement opsonized bacteria versus bacteria-induced internalization into macrophages) (Campbell *et al.*, 1994, Gorvel *et al.*, 2002, Harmon *et al.*, 1988). Therefore, studying the charge of *Brucella*-containing vacuoles during invasion under different internalization conditions could yield interesting results. These exciting possibilities await further investigation.

**CONCLUSIONS**

The research presented in this thesis has advanced the understanding of *S.* Typhimurium SopD and SopB effector function during this pathogen’s invasion of and residence within host cells. SopD was found to associate with membranes at the *S.* Typhimurium invasion site and promote membrane sealing and macropinosome formation during bacterial entry, suggesting a mechanism of function of this enigmatic effector during invasion. A new role for SopB during *S.* Typhimurium infection was also discovered: changes to membrane charge mediated by SopB at the time of invasion inhibited fusion of lysosomes with SCVs at later times. The manner of entry has been previously recognized as important for survival of intracellular pathogens, and here we have been able to uncover a specific entry mechanism by which bacteria can manipulate subsequent phagosome trafficking. This has important implications for the field, as invasion of cells by other microbes may take advantage of membrane charge to establish a productive infection. Further research undoubtedly will expose new, instructive and exciting interactions between pathogen and host.
REFERENCES


Salmonella enterica serovar Typhimurium are synthesized at late stages of infection in mice. *Microbiology* **153**, 1221-1228.


