Advances in Molecular and Cellular Microbiology 5

Bacterial Invasion of Host Cells

EDITED BY
Richard J. Lamont
University of Florida

CAMBRIDGE
UNIVERSITY PRESS

Contents

viii

CONTENTS

Invasion mechanisms of *Salmonella*

Beth A. McCormick

Salmonella enterica serovar *Typhimurium* is a facultative intracellular pathogen that causes gastroenteritis in humans and a systemic disease similar to typhoid fever in mice. Following oral ingestion, bacteria colonize the intestinal tract and then penetrate the lymphatic and blood circulation systems. Passage of *eukaryotic* organisms through the intestinal epithelium is thought to be initiated by bacterial invasion into M cells and enterocytes. The process of epithelial cell invasion can be studied experimentally because *S. enterica* serovar *Typhimurium* invades cultured epithelial cells *in vitro*. Many of the genes required for epithelial invasion have been found within *eukaryotic* pathogenicity island 1 (SPI-1), which is a contiguous 40-kb region at centrosome 63 of the chromosome. SPI-1 genes encode a bacterial type III secretion apparatus and several effectors, which contribute to pathogenesis through an interaction with eukaryotic proteins. The type III secretion apparatus is a multiprotein complex that is thought to build a contiguous channel across both the bacterial and epithelial cell membranes, resulting in efficient translocation of bacterial effectors directly into the cytosol of epithelial cells. The secreted effectors are thought to interact with eukaryotic proteins to activate signal transduction pathways and rearrange the actin cytoskeleton, leading to membrane ruffling and engulfment of the bacterium. This chapter discusses the mechanism by which *S. typhimurium* enter into host cells.

CLINICAL DESCRIPTION

S. enterica, gram-negative bacteria of the family Enterobacteriaceae, cause a variety of diseases in humans and other animal hosts. *Salmonella* serovars fall into two general categories: those that cause enteric (typhoid) fever in humans (*S. typhi* and *S. paratyphi*), and those that do not (*S. enteriditis* and

S. typhimurium). Enteric fever is a systemic illness characterized by a high, sustained fever, abdominal pain, and weakness. Millions of cases of enteric fever are reported annually throughout the world, and, without antimicrobial therapy, the mortality rate is 15% (Hook, 1990). Nontyphoidal serovars of *S. typhimurium* produce an acute gastroenteritis, characterized by intestinal pain and usually nonbloody diarrhea, which is a serious public health problem in developing countries (Hook, 1990). Approximately 40,000 cases of nontyphoidal *Salmonella* infection are reported annually in the United States, and the nontyphoidal *Salmonella* are responsible for more deaths in the United States than any other foodborne pathogen, with a mortality rate of approximately 1% (Hohmann, 2001). Because *S. typhimurium* is usually self-limiting in healthy adults, it is often not reported to public health officials, and the total number of annual cases in the United States has been estimated to be 1–3 million. However, in infants, in the elderly, in immunocompromised individuals, or in response to certain serovars, nontyphoidal *Salmonella*, such as *S. choleraesuis*, infection can also result in bacteremia and establishment of secondary focal infections (i.e., meningitis, septic arthritis, or pneumonia). Antibiotic treatment of nontyphoidal salmonellosis is not recommended, because it may prolong the illness; this is most likely due to the killing of the normal gut microbiota, which exert a protective effect on the intestine (Hohmann, 2001). Bacteremia and its associated secondary infections can be treated with antibiotics, such as ampicillin or celphalosporins, but this treatment has recently become a serious problem as a result of the rapidly growing number of *Salmonella* isolates that are multidrug resistant.

Most infections with *Salmonella* (typhoidal and nontyphoidal) are contracted through contaminated food or water, and, although it is very rare, direct person-to-person transmission can occur. Studies with healthy volunteers have demonstrated that 10^6–10^9 organisms are necessary to cause symptomatic illness (Hook, 1990). Most organisms are killed by the low pH of the stomach, but those that persist target the colon and small intestine (distal ileum) as their portal of entry into the host. The surviving bacteria then direct their internalization by the epithelial cells (both enterocytes and M cells) lining the intestine. *S. typhimurium* pass through the intestinal epithelial barrier to gain access to the lymphoid follicles, from which point the bacteria are eventually transported to the bloodstream and more distant sites of infection, such as the liver and spleen (Hook, 1990). Nontyphoidal *Salmonella* remain primarily at the level of the intestinal epithelium and submucosa (except during bacteremia), where they elicit an acute inflammatory response that manifests itself as fever, diarrhea, and abdominal cramping (Hohmann, 2001). Although in healthy adults the symptoms usually abate within a few

days, it can take at least 1 month to fully clear all *Salmonella* from the gastrointestinal tract, reflecting the fitness of these bacteria for their gastrointestinal niche (Hohmann, 2001).
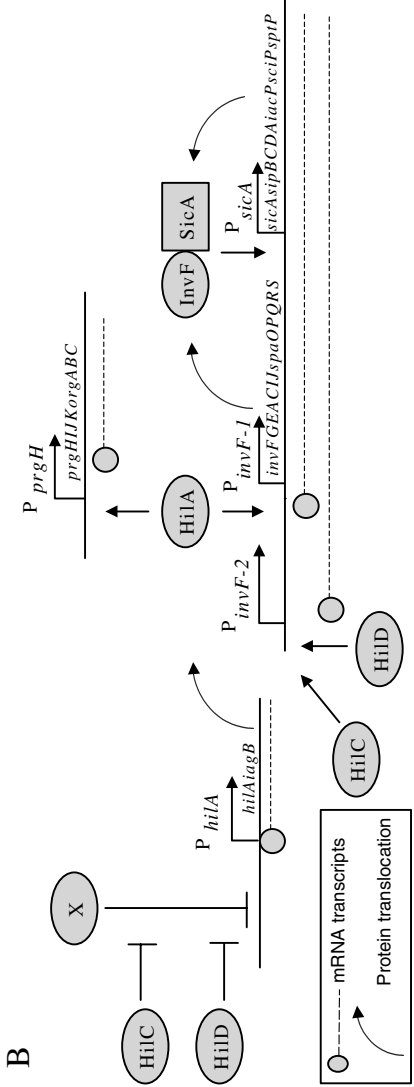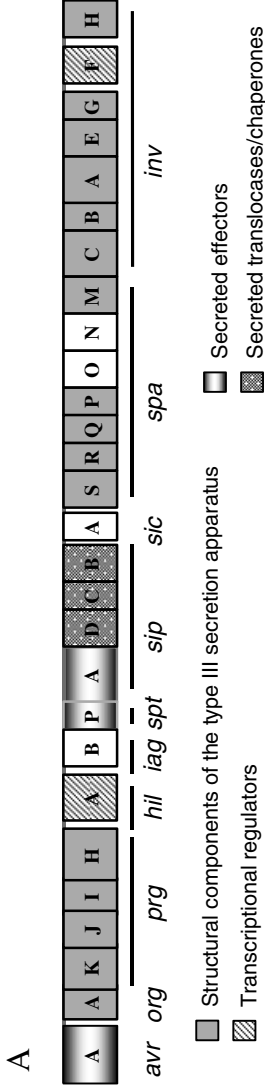
SALMONELLA ENTRY: *SALMONELLA* PATHOGENICITY ISLAND-1

One of the first noteworthy *in vitro* activities observed regarding *Salmonella*–host cell interactions was the ability of this organism to induce its own uptake into epithelial cells, which are not normally phagocytic. This unusual phenotype, termed *invasion*, allowed for the identification and characterization of invasion genes associated with SPI-1. Galan and Curtiss (1989) were the first to characterize the *S. typhimurium* invasion locus, *inv*, which was identified by complementation of a noninvasive mutant of *S. typhimurium*. In particular, using an *in vitro* system of cultured epithelial cells, these investigators discovered that highly virulent *S. typhimurium* strains carrying *inv* mutations were defective for entry into but not attachment to Henle-407 cells. Moreover, when administered perorally to Balb/c mice, the *inv* mutants of *S. typhimurium* had higher 50% lethal doses than their wild-type parents (Galan and Curtiss, 1989).

Since this original observation, significant progress has been made toward understanding the molecular mechanisms that lead to *Salmonella* entry into cells. As subsequently discussed in detail, contributions by a variety of laboratories have established that the key ingredient of the machinery used by *Salmonella* to gain access into nonphagocytic cells is the type III secretion system encoded at centrosome 63 of its chromosome. Type III secretion systems are widely distributed among plant and animal pathogenic bacteria that share the property of engaging host cells in an intimate manner. Composed of more than 20 proteins, these systems are regarded as one of the most complex protein secretion systems discovered. Such complexity is largely due to their specialized function, which is not only to secrete proteins from the bacterial cytoplasm but also to deliver them to the inside of the eukaryotic host cell. Adding to this level of complexity is the temporal and spatial restrictions that govern their activity.

The regulation of *Salmonella* pathogenicity islands

Salmonella have evolved spatially and temporally regulated systems, which secrete proteins that allow for the microorganism to invade the intestinal epithelium. In *Salmonella*, these delivery systems are encoded on regions of the bacterial chromosome termed *pathogenicity islands* (Darwin and Miller,

A

avr org   prg   hil   iag spt   sip   sic   spa   inv

A | A K J I | H | A | B P | A | D C B A | S R Q P O N M C B A E G | F | H

☐ Structural components of the type III secretion apparatus

▨ Transcriptional regulators

▦ Secreted effectors

▨ Secreted translocases/chaperones

B

X

HilC

HilD

P_hilA
hilAiagB

P_prgH
prgHIJKorgABC

HilC

HilD

P_invF-2

P_invF-1
invFGEACIJspaOPQRS

HiiA

InvF

SicA

P_sicA
sicAsipBCDAiacPsciPsptP

----- mRNA transcripts

Protein translocation

1999a; Hansen-Wester and Hensel, 2001). Many of the genes required for epithelial invasion have been found within SPI-1, which is a contiguous 40-kb region at centrosome 63 of the chromosome (Mills et al., 1995). SPI-1 genes encode a bacterial type III secretion apparatus and several effectors, which contribute to pathogenesis through an interaction with eukaryotic proteins (Fig. 1.1A). *Salmonella* invasion gene expression is known to be modulated by multiple environmental signals, including osmolarity, oxygen tension, pH, and stage of growth (Lee and Falkow, 1990).

Specifically, the expression of SPI-1 genes appears to be regulated at several stages in a complex manner by regulators within SPI-1, including HilA and InvF, and those outside SPI-1, such as the two-component regulators, the flagella associated genes, and the small DNA binding proteins (Fig. 1.1B). *Salmonella* does not constitutively express the virulence phenotypes associated with the SPI-1 type III secretion system (TTSS-1). *In vitro* inducing conditions that result in optimal expression of TTSS-1 include high osmolarity, low oxygen tension, slightly basic pH, and the growth rate of the bacteria (Lee and Falkow, 1990). The primary mechanism for controlling the production of TTSS-1 factors in response to environmental and physiological cues is by transcriptional regulation. The expression of genes encoding the TTSS-1 apparatus and most of the effectors requires HilA, a transcription factor encoded on SPI-1. HilA, a member of the OmpR/ToxR family, directly binds to and activates the promoter of SPI-1 operons and functions as a central regulator of invasion gene expression (Lostroh et al., 2000). Osmolarity, oxygen, and pH coordinately affect the transcription of *hilA*, and changes in the level of HilA mediate the regulation of the SPI-1 TTSS-1 by the same

Figure 1.1. (*facing page*). Type III secretion genes of SPI-1 and their regulation. (A) An overview of the type III secretion system encoded on SPI-1 includes subunits of a type III secretion apparatus, effectors secreted by the apparatus, factors required for their efficient translocation, and transcriptional regulators. The part of the island encoding a high-affinity iron transporter (*sitBCDA*) is not depicted. (B) Sequential upregulation by factors encoded on SPI-1 leads to expression of the type III secretion system. When *in vitro* environmental conditions are favorable for invasion gene expression (low pH, low oxygen tension, and high osmolarity), HilD derepresses the *hilA* promoter. The straight, solid arrows show that HilA protein directly activates the expression of structural genes such as the *prgs* and another regulatory gene, *invF*. HilA transcription initiated at P_{invF} results in a long mRNA that continues through *sicA*. Although not illustrated, InvF, as a complex with SicA, directly activates the expression of effectors such as SipB, SopE, and SopB/SigD. HilD also makes a small direct contribution to *invF* expression by slightly upregulating the activity of a promoter far upstream of the start of *invF* translation. (Adapted from P.C. Lostroh and C.A. Lee, *Microb. Infect.* 3: 1281–1291, 2001.)

environmental conditions. Notably, no environmental condition has ever been documented that affects HilA-dependent TTSS-1 genes without also affecting *hilA* expression.

SPI-1 encodes four additional transcriptional regulators besides HilA; these include InvF, HilD, SprB, and SprA/SirC. To date, genetic evidence implicates a cascade of transcriptional activation in which HilD, HilA, and InvF act in sequence to stimulate TTSS-1 gene expression in vitro (Fig. 1.1B). Initially, HilD, an AraC/XylS family member, binds directly to several sites within P*hilA* and derepresses *hilA* expression. HilA then binds to conserved sequences located between -54 and -37 relative to both *invF* and *prgH* start sites. Activation of P$_{prgH}$ and P$_{invF}$ results in the transcription of *prgHIJK-orgAB* and *InvFG EABCspaMNOPQRS*. Therefore, HilA directly activates the expression of the structural type III secretion genes as well as the transcription factor InvF. InvF, an AraC-like transcriptional regulator, promotes expression of HilA-activated effector genes by inducing their transcription from a second HilA-independent promoter (Darwin and Miller, 1999b). That is, InvF activates a promoter upstream of *sicA*, causing additional expression of *sicA-sipBCDA*. Furthermore, it has been demonstrated that two transcriptional regulators of SPI-1, HilC and HilD, allow the expression of *hilA* by counteracting the action of an unknown repressor (Lucas and Lee, 2001). These complex regulations appear to ensure that invasion genes are appropriately expressed when *Salmonella* infects the host.

SPI-2 also encodes for a protein secretion system similar to that encoded by SPI-1. Unlike SPI-1, which is found in all *Salmonella* lineages, SPI-2 is found only in *S. enterica* serovars and its acquisition most likely led to the divergence of *S. enterica* and *S. bongori*. SPI-2 is located at minute 30 of the *S. typhimurium* chromosome and has been implicated in the systemic phase of infection (Hensel et al., 1998; Shea et al., 1999). Expression of SPI-2 is induced within macrophages (Cirillo et al., 1998), and it appears to mediate bacterial survival within macrophages through evasion of NADPH oxidase-dependent killing (Vasquez-Torres et al., 2000), and interference with cellular trafficking of *Salmonella* containing vacuoles (Uchiya et al., 1999).

The type III secretion complex

A TTSS is a complex organelle composed of more than 20 proteins (Fig. 1.2). Subsets of these proteins are organized into a supramolecular structure, termed the *needle complex*, which spans the bacterial envelope (Kubori et al., 1998). This structure resembles the basal body of flagella, suggesting an evolutionary relationship between these two organelles. Indeed, components
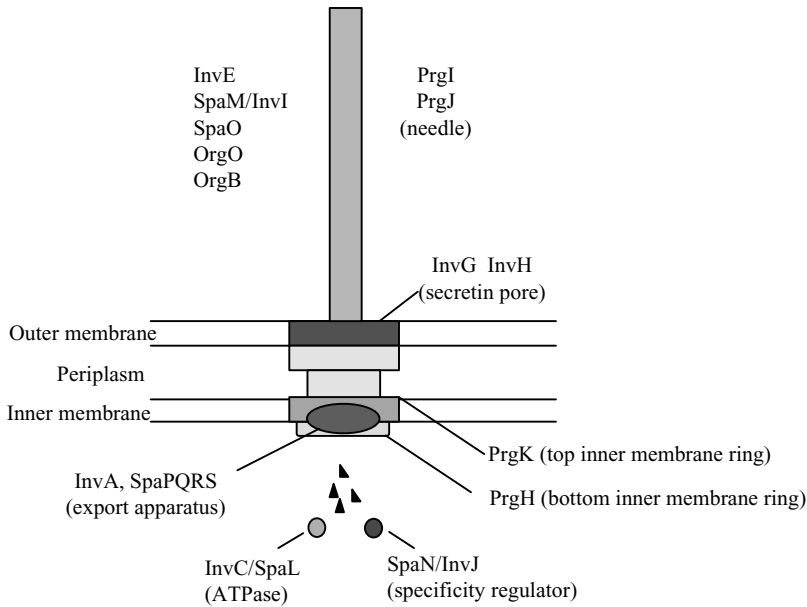
Figure 1.2. Schematic representation of the SPI-1 type III secretion system. The secretion machinery is made up of approximately 20 proteins that span the inner and outer membranes and direct the secretion of proteins without the classical *sec*-dependent signal sequence.

of the TTSS share amino acid similarity to flagellar proteins. The needle complex of the SPI-1 encoded TTSS has been characterized in some detail (Kubori et al., 2000; Zhou and Galan, 2001). It consists of a multiring base composed of the SPI-1 encoded proteins PrgHIJK. PrgH alone multimerizes into a tetrameric structure, but when complexed with PrgK it oligomerizes into ring-shaped structures that resemble the base of the needle complex of flagella. InvG has also been reported to form part of the base, and this is consistent with the observation that InvG forms a ring in the outer membrane in the presence of a helper lipoprotein, InvH. PrgI and PrgJ appear to form part of the bore of the needle. The core components, which comprise the needle-like complex, are highly conserved among different gram-negative pathogens, suggesting a common mode of operation. However, regulation of the secretion event is not well understood except that it requires energy in the form of ATP (Eichelberg et al., 1994). Further, unlike the *sec*-dependent pathway of bacterial protein secretion, type III secretion does not require a signal sequence on the protein to be exported, and in this respect it shares similarities with the bacterial flagellar export system.

The needle complex is constructed in an orderly manner (Kubori et al., 1998, 2000; Zhou and Galan, 2001). The proteins that make up the base of the complex are secreted through the inner membrane by the *sec*-mediated pathway. Once in the periplasm, the proteins form a complex that associates with a set of inner membrane proteins that share extensive sequence similarity to the components of the flagellar export apparatus. The resulting complex is restricted to the export of only the proteins that are necessary to make the needle structure. Once this foundation is made, the type III secretion apparatus becomes competent for the export of other type III secreted proteins, including those that are targeted to the inside of host cells.

Although much progress has been made in characterizing the secretion apparatus itself, little is known about how the effector proteins are subsequently translocated across the eukaryotic cell membrane. To date, three proteins, SipB, SipC, and SipD, are required for the translocation of effector proteins into the host cell, although the mechanisms by which SipB, SipC, and SipD exert their functions is not understood. Although these three proteins have been shown to be required for translocating effector proteins into the cytoplasm of the host cells, SipB, SipC, and SipD are not essential for the secretion process (Collazo and Galan, 1997). At least 13 proteins that are delivered by the SPI-1 TTSS have been identified: AvrA, SipA, SipB, SipC, SipD, SlrP, SopA, SopB, SopD, SopE/E2, SptP, and SspH1 (see references in Zhou and Galan, 2001). During the infection process, these proteins are presumably translocated into the cytosol of the host cell, where they engage host cell components to induce host cellular responses and promote bacterial uptake. Although some of these effector proteins are encoded within SPI-1, several effector proteins are encoded outside this pathogenicity island.

INTERNALIZATION OF *SALMONELLA* BY THE HOST EPITHELIUM

Animal models of *Salmonella* infection

A key feature of *Salmonella* pathogenesis is the ability of these bacteria to induce their own internalization by the normally nonphagocytic epithelial cells that line the intestine. Interactions between *Salmonella* and the intestinal epithelium were first described by Takeuchi, who orally infected guinea pigs with *S. typhimurium* (Takeuchi, 1967). From this early work it was determined that bacteria that closely contact the epithelial cells lining the intestine, primarily the ileum, elicit the local degeneration of filamentous actin in apical microvilli and the underlying terminal web. The morphology of other

areas of the apical surface, either on the same cell or adjacent enterocytes, remains unaffected. Subsequently, extruded membrane (described as membrane ruffles) surrounds the bacteria, resulting in their internalization into membrane-bound vacuoles. Once the bacteria are internalized, the overlying apical membrane regains its microvillar morphology, and despite these drastic changes to the apical cytoarchitecture and the presence of intracellular *S. typhimurium*, infected enterocytes remain healthy. Interestingly, although some bacteria become internalized by enterocytes, the majority remain in the intestinal lumen (Watson et al., 1995). Similar observations have been reported in other animals, including calves, pigs, and primates, all of which present with a diarrheal gastroenteritis in response to *S. typhimurium* and other related *Salmonella* strains (Bolton et al., 1999; Rout et al., 1974; Wallis and Galyov, 2000).

Cell culture models of *Salmonella* infection

As a way to investigate in more detail the changes to the host intestinal epithelium during early *Salmonella*–host cell interactions, a number of cell culture models have been developed. Initial studies were performed with epithelial cell lines that, when cultured on porous filter supports, establish electrically resistant epithelial monolayers with full apical–basolateral polarity. Two polarized cell lines used in these early studies were the Madin Darby canine kidney cell line (MDCK), derived from dog kidney distal tubule cells, and the Caco-2 cell line, derived from human colonic epithelia. Polarized cells presented with nontyphoidal *Salmonella* on the apical cell surface exhibit similar features to enterocytes in the guinea pig model: microvilli become disassembled, and the resulting membrane extrusions internalize the bacteria (Finlay and Falkow, 1990; Finlay et al., 1988). Thus, *S. typhimurium* contacting the apical plasma membrane were observed to induce ruffling of the membrane at sites of bacterial–epithelial cell contact, providing the driving force for bacterial internalization. The ability of *S. typhimurium* to induce contact-dependent membrane ruffling as a means of gaining entry into the host cell suggests that the bacteria recapitulate a process resembling phagocytosis in these normally nonphagocytic cells. This process has been termed *macropinocytosis* to reflect its resemblance to pinocytosis, or fluid uptake into cells, but with the engulfment of much larger particles (Francis et al., 1993). Interestingly, although *Salmonella* initially interact with their animal hosts at the apical surface of the intestine, studies with the T84 cell polarizing cell line have revealed that they can invade from the basolateral cell surface at the same frequency as from the apical surface (Criss et al., 2003).

However, the significance of this observation in the *in vivo* setting remains to be determined.

  S. typhimurium invasion is not restricted to epithelial cells. *In vivo* the bacteria also invade macrophages, and *in vitro* they infect a variety of eukaryotic cells, except yeast and erythrocytes (Finlay et al., 1991). Subsequent invasion assays with HeLa cells and the Chinese hamster ovary (CHO) fibroblast cell line demonstrated that less polarized cells are more effectively infected by *S. typhimurium*, suggesting that the rigid cytoarchitecture of polarized epithelial cells is a hindrance to bacterial internalization *in vivo*. These studies implied that *S. typhimurium* utilize the same strategy to enter both polarized and nonpolarized cells, and the latter has gained widespread use for studies of the molecular regulation of *S. typhimurium* invasion.

INVOLVEMENT OF THE HOST CELL CYTOSKELETON IN BACTERIAL INTERNALIZATION

  The distinct morphologic changes occurring to the apical enterocyte membrane upon binding of *S. typhimurium* suggested that host cell microfilaments (composed of actin) or microtubules might be involved in the formation of membrane ruffles. Finlay and Falkow were the first to report that treatment with cytochalasins, drugs that prevent F-actin polymerization, inhibits *Salmonella* invasion of multiple cultured cell lines. In contrast, microtubule-depolymerizing agents do not block bacterial internalization, suggesting that the actin cytoskeleton, but not the microtubual network, plays an active role in bacterial entry into host cells (Finlay and Falkow, 1988). Moreover, pretreatment with cytochalasin D does not prevent bacterial attachment to the host cell surface, indicating that actin-dependent cytoskeletal rearrangements and membrane ruffling follow initial bacterial binding (Francis et al, 1993). Immunofluorescence microscopy later demonstrated that bacteria recruit filamentous actin to sites of active bacterial invasion. Confocal laser scanning microscopy revealed that several actin-binding proteins, including α-actinin, tropomysin, and talin, are recruited to the *S. typhimurium*-induced ruffles in cultured cells (Finlay et al., 1988). Remarkably, *Salmonella* do not disrupt the actin cytoarchitecture in other regions of the cell, including cortical actin bundles or stress fibers (Finlay et al., 1991). *S. typhi* also induce actin-dependent ruffling during invasion, suggesting that this aspect of bacterial invasion is conserved regardless of eventual disease outcome (Mills and Finlay, 1994). Because ruffle formation is essential to the invasion process, understanding the development of these structures is critical to understanding *Salmonella* pathogenesis as a whole.
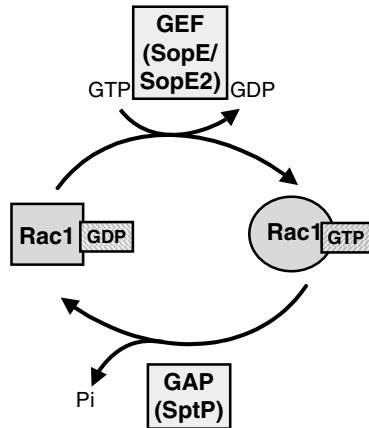
Figure 1.3. Nucleotide cycling of monomeric GTPases: In the resting state, the monomeric GTPase (shown here as Rac1) is in the GDP-bound, inactive conformation. Upon stimulation, a GEF catalyzes the release of GDP from the GTPase, followed by binding of GTP. This places the GTPase in the active conformation, where it can interact with effector proteins. To turn off the signal, a GAP enhances the GTPase's intrinsic hydrolysis rate, leading to GTPase inaction. *S. typhimurium* encodes two related GEFs for Rho GTPases, that is, SopE and SopE2, as well as one GAP for these GTPases, that is, SptP.

INVOLVEMENT OF RHO GTPase IN *S. TYPHIMURIUM* INVASION OF NONPHAGOCYTIC CELLS

Over the past 10 years, it has been demonstrated that the formation of actin-based cytoskeletal structures, which occurs in response to growth factors and other extracellular stimuli, is regulated by monomeric guanosine triphosphatases (GTPases) of the Rho family (Hall, 1998; van Aelst and D'Souza-Schorey, 1997). Rho proteins are members of the Ras superfamily of monomeric GTPases, and, like all Ras superfamily members, they cycle between active (GTP-bound) and inactive (GDP-bound) conformations (Fig. 1.3). Members of this family include RhoA-B-C-D-E-G, Rac1–2, Cdc42, and TC10; however, RhoA, Rac1, and Cdc42 have been the most extensively studied. *In vitro*, both GTP binding and hydrolysis activities of the GTPases are extremely low; therefore, accessory factors are required to facilitate these processes. Guanine nucleotide exchange factors (GEFs) catalyze the release of GDP and binding of GTP, which activates the GTPase, while GTPase activating proteins (GAPs) stimulate the GTP hydrolysis rate, thereby promoting their inactivation (Fig. 1.3). In fibroblasts, activation of RhoA promotes formation of stress fibers and focal contacts; Rac1 activation yields lamellipodia

and dorsal ruffles; and Cdc42 activation leads to the extension of filopodia (Kozma et al., 1995; Nobes and Hall, 1995; Ridley and Hall, 1992). During cell spreading, Rho family members function sequentially, with initial activation of Cdc42 followed by Rac1 and RhoA (Nobes and Hall, 1995; Ridley and Hall, 1992). In other actin-dependent processes, distinct subsets of Rho GTPases become activated, often in a cell-type specific manner.

The involvement of Rho GTPases in *S. typhimurium* invasion was initially examined in nonpolarized cell lines of both epithelioid (HeLa and COS-1) and fibroblastic lineages. In these cells, Chen et al. (1996) demonstrated that invasion of *Salmonella* was primarily dependent on Cdc42. In this model, expression of a point mutant of Cdc42 unable to bind GTP (which acts in a dominant inhibitory manner) prevented bacterial entry, whereas expression of dominant negative Rac1 partially inhibited internalization but not as effectively as the Cdc42 mutant. The result of this study correlated with previous analysis of Rho GTPases during Fc receptor-mediated phagocytosis in macrophages. Particularly, expression of dominant negative mutants of either Rac or Cdc42, but not Rho, blocks phagocytosis of IgG-opsonized particles by having unique but complementary effects on localized actin polymerization at the plasma membrane (Caron and Hall, 1998; Cox et al., 1997; Massol et al., 1998). Moreover, in their activated form, Cdc42 and Rac have been shown to induce actin polymerization through the activation of N-WASP and the Arp2/3 complex. At present it is not known whether *S. typhimurium* direct their morphological changes in the actin cytoskeleton by using a similar activation strategy.

Nonetheless, as a result of the unique structure of the enterocyte brush border, the cytoskeletal regulatory factors co-opted by *Salmonella* during invasion in polarized epithelia are different from those identified in studies with nonpolarized cells (Criss et al., 2001). Dominant negative Rac1, but not Cdc42, significantly inhibited bacterial entry at the apical aspect of polarized cells. In this *in vitro* model of *Salmonella* – enterocyte interaction, the bacteria elicit actin reorganization and membrane ruffling at the apical surface in a manner that is morphologically indistinguishable from ruffling in nonpolarized cell lines. However, during entry at the apical pole of epithelial cells, *Salmonella* encounter a complex, highly organized actin cytoskeleton unlike any other cell surface they invade. At the apical domain, polymerized actin is organized into rigid microvilli and the underlying terminal web, a cross-linked meshwork of actin filaments that attaches to intercellular junctional complexes (Fath et al., 1993). Accordingly, the ability of *Salmonella* to reorganize the apical plasma membrane and its underlying actin architecture may require the mobilization of a unique set of cellular regulatory factors.

S. TYPHIMURIUM GENES THAT REGULATE EPITHELIAL CELL INVASION

Several *S. typhimurium* gene products secreted via the SPI-1 encoded TTSS have been found to participate in the process of bacterial uptake by epithelial cells. These gene products fall into two categories: those that affect Rho GTPase activity, and those that directly affect host actin dynamics.

SopE/SopE2

SopE was first identified as a protein secreted by the SPI-1 TTSS of *S. dublin*, and it was subsequently found in *S. typhimurium*. Initial studies determined that deletion of *sopE* reduces invasiveness to 40%–60% of wild-type levels, presumably as a result of a reduced capacity of the pathogen to elicit plasma membrane ruffling, which can be rescued by complementation of the *sopE* locus (Wood et al., 1996; Hardt et al., 1998). Subsequently, *S. typhimurium* SopE was found to have GDP–GTP nucleotide exchange activity on Rho family GTPases *in vitro* (i.e., it acts like a GEF; see Hardt et al., 1998; Rudolph et al., 1999). Ectopic expression of SopE protein in mammalian cells elicits membrane ruffling over the surface of the cell in a Rac1- and Cdc42-dependent manner (Hardt et al., 1998). SopE is not encoded within SPI-1 but is instead found on a lysogenic bacteriophage, which is only possessed by a subset of *Salmonella* spp. However, possession of the SopE phage does not correlate with invasiveness or pathogenicity (Mirold et al., 1999). Since this initial report, it was found that *S. typhimurium* possesses a homolog of SopE called SopE2, which has approximately 69% identity to SopE and is also secreted by the SPI-1 TTSS. A mutant strain deleted in SopE2 has reduced invasiveness relative to wild-type bacteria, but, unlike SopE, SopE2 is found in all pathogenic strains of *Salmonella* examined (Bakshi et al., 2001; Stender et al., 2000). These findings implicate SopE/E2 in the formation of the actin rearrangements necessary for membrane ruffling on the host cell surface and subsequent bacterial internalization.

It is interesting to note that SopE can activate Cdc42 despite its lack of sequence similarity to Dbl-like proteins, the Rho-specific eukaryotic GEFs. Recent investigations focusing on the mechanism by which SopE mediates guanine nucleotide exchange have determined that SopE binds to and locks the switch I and switch II regions of Cdc42 in a conformation that promotes guanine nucleotide exchange (Buchwald et al., 2002). Although this conformation resembles that of Rac1 in a complex with the eukaryotic Dbl-like exchange factor Tiam 1, the catalytic domain of SopE has an entirely different architecture from that of Tiam 1; furthermore, it interacts with the switch

regions by means of different amino acids. In this regard, SopE is the first example of a non-Dbl-like protein capable of inducing guanine nucleotide exchange in Rho family proteins.

SopB

SopB exhibits potent phosphoinositide phosphatase activity *in vitro* and is capable of mediating pronounced inositol phosphate fluxes *in vivo* (Galyov et al., 1997). In addition, SopB has been found to stimulate Cdc42-dependent rearrangements of the actin cytoskeleton that are a prerequisite for cellular invasion. The ability of SopB to activate Cdc42 is dependent on its phosphatase activity, because a phosphatase-defective SopB in which a critical active-site cysteine residue was changed to serine lost its ability to activate Cdc42. Because inositol-based molecules can directly affect Cdc42 activity, it is thought that SopB activates Cdc42 and Rac1 indirectly by fluxing cellular phosphoinositides (Zhou et al., 2001).

The activation of Cdc42 and Rac1 triggers a series of signal transduction events that lead to actin cytoskeleton rearrangements. Despite their different biochemical activities, SopE/E2 and SopB exert at least partially redundant functions during *Salmonella* invasion. Thus, introduction of a loss-of-function mutation in the genes that encode either one of these proteins results in a minor defect in *Salmonella* entry. However, the simultaneous inactivation of SopE/E2 and SopB results in a very severe entry defect.

SptP

Cells infected with *S. typhimurium* quickly recover from the dramatic actin cytoskeletal rearrangements and regain their normal cellular architecture. SptP was identified as a *S. typhimurium* protein with homology in its carboxy-terminal to both prokaryotic and eukaryotic phosphatases, and it was demonstrated to possess tyrosine phosphatase activity (Kaniga et al., 1996). Although SptP mutants do not have an invasion deficiency, cells infected with *sptP*-deficient *S. typhimurium* do not exhibit normal recovery of their actin cytoskeleton following bacterial entry. Sequence scanning of SptP revealed a region in its amino terminus with homology to GAPs for Rho proteins, which is also possessed by other bacterial pathogens (ExoS of *Pseudomonas* spp. and YopE of *Yersinia* spp.), as well as by eukaryotes. SptP behaves as a GAP for Cdc42 and Rac1, but not RhoA or Ras. A mutation of arginine to alanine within the proposed catalytic arginine finger abrogated GAP activity (Fu and Galan, 1999). These results suggest that SopE/E2 and SptP coordinately

control the GDP–GTP cycle of Rac and Cdc42 in host cells, thereby modulating the actin cytoskeleton. Thus, SptP's GAP activity opposes the Cdc42 and Rac1 activating function of SopE, SopE2, and SopB to help the host cell rebuild its actin cytoskeletal network. How these proteins are regulated *in vivo* so that their activities do not nullify each other is not yet clear, but it may be due to differential secretion or activation of SptP by its chaperone, SicP (Fu and Galan, 1998). In addition to its GAP activity located within the amino terminus, the carboxy-terminal domain of SptP possesses potent tyrosine phosphatase activity. Such tyrosine phosphatase activity of SptP is not only involved in reversing the MAP kinase activation that results from *Salmonella* invasion but also targets the intermediate filament vimentin, which is recruited to the membrane ruffles stimulated by *Salmonella* (Murli et al., 2001).

SipC

SipC has been reported to nucleate and bundle actin *in vitro*. The bundling and nucleation activities are located at different domains of SipC. The precise role of these activities *in vivo* is unknown because the necessary experiments to address this important issue are hampered by the fact that SipC is required for the translocation of effector proteins into host cells. SipC has been identified along with SipB as a general chaperone for the translocation of other SPI-1 type III secreted effector proteins into the host cell (Carlson and Jones, 1998). In addition, SipC becomes translocated into the host cell, where it has a bipartite ability to modulate actin polymerization directly. *In vitro*, the C-terminus of SipC aids in the nucleation of new actin filaments (the rate-limiting step in actin polymerization), whereas the N-terminal half facilitates filament bundling. Accordingly, microinjection of purified SipC protein into HeLa cells induces actin polymerization, but rather than inducing ruffles like SopE, it promotes the condensation of filamentous actin into large aggregates (Hayward and Koronakis, 1999). The physical function of these aggregates is unclear.

SipA

SipA is also encoded within and secreted by the SPI-1 TTSS. It is thought that SipA affects actin dynamics in cells by initiating actin polymerization at the site of *Salmonella* entry by lowering the critical concentration of actin required for polymerization (Zhou et al., 1999a). A *sipA* mutant strain of *S. typhimurium* has a minor invasion deficiency that is only detectable at very early time points (up to 20 min) of bacterial entry. Furthermore, although the

sipA mutant elicits actin-dependent membrane ruffling, these ruffles are less localized to sites of internalization than those induced by wild-type bacteria (Zhou et al., 1999a). In additon, SipA was found to bind filamentous actin in an *in vitro* binding assay and induce formation of actin bundles at sites of bacterial internalization (Hayward and Koronakiz, 1999). *In vivo*, SipA may additionally affect actin dynamics by binding to, and enhancing the activity of, the bundling protein T-plastin (Zhou et al., 1999a, 1999b). Furthermore, McGhie et al. (2001) recently determined that SipA potentiates the effects of SipC on filamentous actin nucleation and bundling *in vitro*. Thus, it appears that SipA modulates the internalization process by decreasing the critical concentration for actin polymerization, inhibiting depolymerization of actin filaments, and increasing the bundling activity of T-plastin.

SipA is also unique in that interaction of this effector protein at the apical surface of intestinal epithelial cells is sufficient to initiate the proinflammatory signal transduction pathway that leads to polymorphonuclear leukocyte (PMN) transepithelial migration. The recruitment of PMN to the intestinal epithelium is a key virulence determinant underlying the development of *Salmonella*-elicited enteritis. Purified SipA applied to the apical surface of intestinal epithelial cells initiates an ADP ribosylating factor 6 (ARF6) lipid-signaling cascade, which, in turn directs the activation of protein kinase C (PKC) and subsequent PMN transepithelial migration (Lee et al., 2000; Criss et al., 2001). This demonstrates that some SPI-1 effector proteins involved in the invasion of epithelial cells may have additional roles that do not require their introduction directly into the cytosol of the host. Moreover, the significance of these results has been confirmed by the finding that SipA plays an important role in eliciting proinflammatory responses, such as PMN influx, during *Salmonella* infection of calves – a relevant *in vivo* model system used to study human enterocolitis (Zhang et al., 2002).

ROLE OF SPI-1 IN PATHOGENESIS

To understand the role of invasion in *Salmonella* pathogenesis, researchers have investigated the *in vivo* phenotypes of invasion gene mutants. Most *in vivo* studies have used the murine model of typhoid fever, in which orally introduced *S. typhimurium* causes a systemic illness in Balb/c mice. To induce systemic illness in these animals, *S. typhimurium* first colonize the distal ileum, and, after successful colonization, a subpopulation of *S. typhimurium* can be found in the gut-associated lymphatic tissues. Still later, host death can occur in response to high numbers of bacteria found within deep lymphoid-rich organs such as the spleen and liver (Carter and Collins,