Abstract

A common theme in bacterial disease is the manipulation of the eukaryotic cytoskeleton by pathogenic factors. Understanding how virulence factors operate to achieve these alterations is a growing and important research effort. This review focuses on several virulence factors from the pathogen *Salmonella*, and considers the contributions of structural biology to our appreciation of bacterial modulation of cytoskeletal dynamics.

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1. Introduction

While both viruses and bacteria are now known to manipulate the host cell in highly specific manners, the differences between these types of infectious agents require very different strategies of infection. In particular, the size differences between the two dictate that the much larger bacteria will have to grapple with problems that the smaller viruses can ignore. A very common theme in bacterial pathogenesis is that of manipulation of the host cell structure, and in particular, by modulating the eukaryotic cytoskeleton [1–5]. By altering the structure of cells, bacteria are able to induce their uptake into normally non-phagocytic host cells, or prevent their uptake into professional phagocytes. In some cases, internalized bacteria remodel elements of the actin cytoskeleton and microtubule networks for purposes ranging from the creation and maintenance of a specialized intracellular vacuole to the propulsion within and between cells [4,6]. In the least sophisticated instances, bacteria can use toxins to irreversibly direct host cytoskeletal structure, often with rapid death of the eukaryotic cell [5].

*Salmonella* spp. are Gram-negative, facultative intracellular pathogens that depending on the serotype, can cause a broad range of illnesses and infect various organisms, from reptiles to mammals [4]. *Salmonella* spp. are highly successful as pathogens, and more than one billion human infections are reported annually [7]. Central to the virulence of *Salmonella* is a type III protein secretion system (TTSS) employed in the translocation of virulence factors across the bacterial envelope and eukaryotic cell membrane [8]. The TTSS is associated with a supramolecular organelle-sized structure variously termed the “needle complex,” “injectosome,” or the “molecular syringe” for its structure as revealed by electron microscopy and its function in transporting bacterial virulence factors into the host cell [9]. The apparatus spans the bacterial inner and outer membranes [10] and is constructed of a handful of protein oligomers that assemble in a highly regulated process [9,11].

Two well-studied virulence-associated type III secretion systems in *Salmonella* are encoded in genomic islands, called SPI-1 and SPI-2, on the bacterial chromosome [4,12]. The SPI-1-encoded type III secretion system is specialized for invasion of epithelial cells, which is achieved through the delivery of bacterial effector (virulence) proteins directly into the host cell cytoplasm, where they manipulate host biochemical functions such as signal transduction, cytokine production, and actin cytoskeletal structure. One of the most striking features of *Salmonella* is its ability to invade normally non-phagocytic cells of the intestinal epithelium. *Salmonella* achieves entry by forming actin-rich membrane ruffles that drive bacterial internalization, leading eventually to the formation of the *Salmonella*-containing vacuole (SCV) [4].
vacuole is determined, in part, by translocation of effector proteins encoded by SPI-2, which is required for the establishment of systemic infections [4]. Actin and tubulin dynamics are also linked to these late stages of Salmonella infection, and the formation of an F-actin meshwork around bacterial vacuoles has been observed [13].

The last several years have witnessed an increasing number of identified SPI-1- and SPI-2-encoded effector proteins, which have been found to be involved in cytoskeletal modulation. Although the molecular mechanisms underlying the activity of the many proteins have yet to be revealed, important progress has been made over the last few years with a subset of these molecules. In this review we examine from a structural perspective several important factors that contribute to cytoskeletal modulation of the host by Salmonella.

2. Salmonella and actin cytoskeletal changes

Upon contact with Salmonella, host cells acquire actin-rich structures resembling the effects of activated oncogene on their surface [4]. This membrane ruffle formation is essential for encapsulation of bacteria and subsequent internalization into the host cell. Inhibitors of actin polymerization, such as cytochalasins and latrunculin, can inhibit formation of membrane ruffles and bacterial entry, indicating a critical role of actin polymerization in the process of bacterial internalization [14].

The regulation of the actin cytoskeleton is very complex in eukaryotic cells and is managed by a large number of cellular factors. Some of these proteins are involved in F-actin bundling, such as profilin and gelsolin, while others are involved in F-actin severing, such as plastin and ADF-cofilin. The processes of actin nucleation and polymerization of actin monomers are strongly regulated by actin-binding proteins and signal transduction molecules such as Rac and Arp2/3 complex [15,16]. Exploiting these regulatory pathways, several effector proteins of Salmonella are able to activate Rac and indirectly, through downstream signaling pathways, promote extensive cytoskeletal rearrangements (Fig. 1). In addition to this, Salmonella also directly induces actin cytoskeletal changes by employing actin-binding proteins.

3. Indirect modulation of the actin cytoskeleton by Salmonella

The activation of the eukaryotic Rho-family of low-molecular-weight GTPases is one of the most important signaling pathways in cells. Structurally, Rho GTPases, such as Cdc42 and Rac1, have two peptide regions, called Switch I and Switch II that undergo conformational changes that are dependent upon the state of the nucleotide that is bound [17]. GTP-binding is associated with the active state of the protein in which the conformations of Switch I and Switch II allow signaling molecules to bind and thereby initiate signaling cascades that lead to cytoskeletal changes (among other effects). Hydrolysis of GTP to GDP is associated with an inactive signaling state of the GTPases due to the conformational changes in Switch regions, which occurs upon loss of the γ-phosphate.

Activation of Cdc42 and Rac1 is achieved by translocated virulence proteins of Salmonella: SopE, SopE2 and SopB/SipD [18–20]. SopE and SopE2 are guanine nucleotide exchange factors (GEFs) that catalyze exchange of GDP for GTP [18–20]. This activation contributes to the formation of membrane ruffles and bacterial internalization. SopE is a highly active GEF and increases guanine nucleotide exchange 105-fold above the intrinsic reaction rate [20,21]. Inactivation of Cdc42 and Rac1 is also achieved by translocated virulence proteins of Salmonella, of which SptP is a prime example (discussed below) [22].

3.1. SopE: a guanine nucleotide exchange factor

The crystal structure of SopE in complex with Cdc42 reveals that activation of Cdc42 is based on a unique insertion of a highly conserved loop into the nucleotide-binding site of Cdc42 that rearranges Switch I and Switch II regions and promotes nucleotide release [23]. SopE possesses a unique fold that is, in particular, unrelated to any known host GEF enzymes. The crystallized, catalytic domain of SopE (resi-
dues 78–240), consists of a pair of three helix bundles oriented such that each bundle contacts the other in a “V” shape (Fig. 1). A small, two-stranded β-sheet is located at the bottom of the molecule proximal to the end of one bundle, and a loop extending from one strand of this sheet crosses over to the other bundle. This loop, as discussed below, is presented outward to solvent and is the key element in the activity of SopE.

SopE binds primarily to the Switch regions of Cdc42, burying a relatively large surface area of 2800 Å², but leaves the nucleotide-binding pocket open to solvent (and thus to GTP). No GDP is bound in the complex. The interaction between SopE and Cdc42 induces dramatic conformational changes in Switch I and Switch II. The catalytic loop (with sequence GAGA) that connects the short β-strand with one of the three helix bundles inserts between the two Switch regions of Cdc42 in a manner to push aside Switch I and pull Switch II closer to the loop (Fig. 2). An important effect on the conformation of Switch II is the induction of a peptide flip involving Ala59 very near to the location of the magnesium-binding site. In Cdc42 (and all the small Rho GTPases) the magnesium ion plays a role in the coordination of the nucleotide. By reorienting Switch II to sterically prevent magnesium binding, SopE further contributes to the loss of contacts already achieved by the repositioning of nucleotide-binding regions in the Switch polypeptides.

3.2. SopE2 and SopB

Two other effector proteins from Salmonella, SopE2 and SopB, also contribute to the actin cytoskeletal changes and ruffle formation [18,19,24]. The crystal structures of these two proteins are not known but they have been characterized biochemically. SopE2 is 69% identical with SopE [18], contains the conserved GAGA insertion sequence, and threads very well against the SopE structure: it likely functions in a very similar fashion to SopE. One difference between SopE and SopE2 appears to be with regard to specificity: SopE2 is an efficient GEF like SopE, but with significant activity only toward Cdc42 and not Rac1 [25].

SopB (also called SigD) is the third effector protein that activates Cdc42, although it is not an exchange factor, but functions by inducing changes in phosphoinositide metabolism [26–28]. This protein is located at the host cell membrane and is ubiquitinated within the host cell [29]. SopB shows very limited sequence similarity to motifs found in phosphoinositid phosphatases [26] and a synaptojanin-homologous region [30], but other sequence similarities have not been uncovered, nor are there any structural data on this protein.

SopE/SopE2 and SopB show some redundancy in function, and disruption of any one of these three genes does not prevent induction of actin cytoskeleton modifications or Salmonella entry [24]. However, simultaneous inactivation of these three proteins severely inhibits bacterial entry.

3.3. SptP: a GTPase activating protein

The activity of Rho GTPases, and subsequent membrane ruffle formation, is suppressed by GTPase activating proteins (GAPs). SptP is an effector protein of Salmonella that abolishes Rho GTPase activity induced by SopE, and thereby has a reversible effect on cytoskeletal changes induced during Salmonella entry [22]. Mutagenesis has shown that the GAP activity is essential for the regaining of normal cytoskeletal structure and cell recovery after bacterial internalization [22,31]. In a fascinating recent publication, it was shown that host cell proteasome-dependent degradation temporally regulates the quantity, and thereby the activity, of SopE and SptP, resolving a conundrum of how these two molecules with opposing activities could be used so effectively in the same host cell by the bacterium [32].

The carboxy-terminal domain of SptP has tyrosine phosphatase activity and shows similarity to the Yersinia tyrosine phosphatase, YopH, and other eukaryotic tyrosine phosphatases. Adjacent to the phosphatase domain, and distinct from a domain involved in the secretion and translocation process, is a region of SptP that demonstrates GAP activity for Rho GTPases, Cdc42 and Rac1 [22,31,33–36]. The crystal structures of the GAP and tyrosine phosphatase domains of SptP, as well as both in complex with Rac1, have been determined [34].

The GAP domain of SptP is a four-helix bundle with an up–down–up–down topology including supercoiling in heli-
ces and heptad repeats (Fig. 1). A deviation from the standard bundle structure creates a bulge at one end of the domain. Some segments of the molecule fold out and make interactions with the nucleotide GDP and Rac1 elements in the Switch I and Switch II regions (Fig. 3). The catalytically essential residue for the GAP activity, Arg-209 in SptP, extends from one helix and into the active site of Rac1.

Residues from SptP contact important elements in Rac1 associated with the GTP hydrolysis reaction, including the catalytically crucial Gln-61 of Switch II, the γ-phosphate mimic aluminum fluoride (used to trap the complex a transition-like state), the phosphate-binding region (P-loop), and the nucleotide itself. Arg-209 of SptP inserts into the active site, resulting in strong hydrogen bonds of its guanidinium group to the (Pγ-bridging) β-phosphate oxygen of GDP and a γ-phosphate mimic fluoride atom of AlF₃, an interaction thought to stabilize the negative charge that develops on the leaving group during the phosphoryl transfer reaction. An attacking nucleophilic water molecule is effectively positioned for the phosphoryl transfer reaction through a strong hydrogen bond to the residue Gln-61: SptP devotes several residues to the positioning of Switch II, including this catalytic glutamine. SptP also makes significant contacts to the ribose and guanine groups of GDP (Fig. 3). Overall, SptP is found to recapitulate from a unique scaffold nearly all the important contacts to Rac1 found spread throughout several host Rho-family GAP enzymes [34,37].

4. Direct control of actin cytoskeletal structure

In addition to the indirect influence of effector proteins on actin cytoskeletal rearrangements, Salmonella also engages cytoskeletal proteins directly, and thereby modulates actin dynamics. Two important factors involved in such activities are the SipC and SipA proteins (also called SspC and SspA), which function cooperatively to manipulate processes of actin nucleation, polymerization, and interaction with proteins [38–40]. In addition, several strains of Salmonella harbor the SPV virulence plasmid, on which lies the SpvB gene. SpvB functions as an ADP-ribosyltransferase, covalently modifying actin and preventing its polymerization [41,42].

4.1. SipC: an actin nucleation, polymerization, and bundling factor

SipC is a protein crucial for early bacterial entry. SipC is inserted into the host epithelial plasma membrane [43], and it is essential for the translocation of effectors such as SopE, SopB, and SptP [31,44,45]. SipC can be divided into three domains: an N-terminal domain (1–120) involved in F-actin bundling, a C-terminal domain (200–409) that directly nucleates actin in vitro, and a middle, transmembrane domain that is thought to form a component of the translocon pore [46]. SipC does not share any sequence similarity to any of the known eukaryotic actin-binding proteins, and no structural data is currently available.

4.2. SipA: an actin polymerization factor

Mutants of Salmonella typhimurium with a disruption of the Salmonella invasion protein A, or sipA, gene show an attenuated virulence in bovine intestinal models [28], impaired ability to invade cells [38,47], and less robust and localized membrane ruffling as compared with the wild-type strain [38,48]. The carboxy-terminal domain of SipA is involved in binding to F-actin [38,39], polymerization of G-actin in low salt [49], and reduction of the critical G-actin concentration required for polymerization [38]. The activity of SipA enhances F-actin bundling by host fimbrin (plastin) [50] and SipC-induced actin nucleation and bundling both in vitro and in cultured cells [39]. Moreover, SipA prevents F-actin filament depolymerization in vitro [38,39], and it locally inhibits ADF/cofilin- and gelsolin-directed actin disassembly [40,51].
The crystal structure of the carboxy-terminal domain of *S. typhimurium* SipA (residues 497–669) has been determined [52], revealing this molecule to be a compact, heart-shaped fold dominated by helical secondary structure measuring roughly 30 x 40 x 40 Å. SipA\(^{497–669}\) is active in actin-binding and polymerization, and has a novel three-dimensional structure. In the crystal, the protein is ordered only between residues 513 and 657, and the N- and C-termini are positioned at opposite sides of the molecule (Fig. 1).

Electron microscopic studies of actin-SipA\(^{497–669}\) complexes show that SipA interacts with actin as a globular structure with two non-globular extensions connecting actin protomers on opposite strands (Fig. 4). These arms are located at opposite ends of the globular density and link subdomain four of the *n*th actin subunit in the filament to subdomain one of subunit *n* + 3. Previous studies with a longer construct (SipA\(^{446–684}\)) revealed that SipA is an extended and tubular molecule on the order of nearly 100 Å long [49,53].

Electron density maps obtained from electron micrographs of filaments with SipA\(^{497–669}\) and SipA\(^{446–684}\) were compared, and it was found that in both cases there is a central density into which the globular crystal structure can be positioned. What differs significantly is the length of the arms. Thus, the previously observed extended and tubular appearance of longer SipA constructs is due to the presence of additional polypeptide in non-globular extensions, or “arms.”

The combination of the SipA crystal structure and EM data has led to a model for SipA function termed the “molecular staple.” In this model, SipA possesses a globular domain for actin-binding and positioning the arms, and non-globular extensions to reach out and tether actin molecules on opposing strands of the filament [52]. Numerous deletion mutants in which the arms were truncated have supported this model, although difficulties in controlling actin polymerization in the presence of SipA have prevented high resolution crystallographic confirmation of this model.

### 5. The Cytoskeleton and SPI-2

The formation of membrane ruffles and dynamic actin cytoskeletal reorganization during *Salmonella* infection is essential for bacterial internalization into intestinal epithelial cells. These cytoskeletal changes are relatively short-lived, however, and the host cell membrane regains a normal cytoskeletal organization shortly after bacterial uptake. However, the role of the host cytoskeleton is also important in later stages of *Salmonella* infection, during replication and survival inside the SCV.

SPI-2-encoded proteins are required for replication and survival inside the SCV, and their activity is associated with actin reorganization, maintenance of the vacuolar membrane [54], and the formation of microtubule-associated *Salmonella*-induced filaments (Sifs) that extend dynamically from the SCV [55]. Actin assembly around the vacuole is pronounced, and significant amounts of F-actin are found associated with the vacuolar membrane that surrounds intracellular bacteria [13]. Several effector proteins, such as SifA, SifB, SseJ, co-localize with the SCV and the Sifs [56,57]. Two other proteins, SspH2 and SseI, are also found co-localized with the polymerizing actin cytoskeleton [58].

In addition to the formation of an F-actin meshwork, intracellular bacteria also cause a dramatic accumulation of microtubules around microcolonies, and they play an important role in vacuolar membrane dynamics [59]. SifA is an effector protein that associates with microtubules, and Sif formation is strongly dependent on the microtubular organization [56].

Despite the cumulative research of SPI-2 effector proteins involved in cytoskeletal modulation, *Salmonella* virulence factors involved in actin and microtubular dynamics around SCV are poorly characterized at present, and no structural data is available.

### 6. Conclusion and future directions

Virulence factors from *Salmonella* have been shown to induce alterations in the host cytoskeleton, both indirectly by modulating signal transduction, and directly, through interactions with cytoskeletal proteins. Initially, the stimulation of actin cytoskeletal rearrangements is essential for internalization and replication of *Salmonella*, while subsequent down-regulation enables survival of both the bacteria and the host cell. In addition to revealing the molecular mechanisms of this remarkable machinery utilized by the *Salmonella*, high-resolution structures are revealing fascinating themes in the mechanisms of host manipulation. As an example, such stud-
ies have shown that many bacterial pathogens mimic the function of host proteins in order to achieve benefits to the microorganism. This contrasts with the strategies used by some pathogens that involve microbial products with activities lacking clear counterparts in eukaryotic cells [37].

Detailed structural analysis of type III secretion virulence systems are only in their infancy. In particular, the available structural data regarding virulence factors involved in direct cytoskeletal modulation is limited by difficulties of working with factors that promote actin nucleation and polymerization. Nonetheless, the first structural information from these systems has been immensely informative, and very exciting from a biological point of view. Over millions of years, the co-evolution of host and pathogen has engendered highly complex and sophisticated biochemical exchange between different species. The coming years will surely only deepen this appreciation with additional examples for which structural insight will often play a key role.

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References


