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Hans Georg Mannherz *Editor*

The Actin Cytoskeleton and Bacterial Infection

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The Actin Cytoskeleton and Bacterial Infection

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Preface

Three types of cytoplasmic filaments form the cellular cytoskeleton. These filaments often extent from the cell nucleus to the plasma membrane. They stabilise cell shape and provide tensile strength. Different classes of proteins form the different types of filaments: A large number of tissue specific proteins (the intermediate filament proteins) form the intermediate filaments, proteins of the tubulin family build the microtubules, and the actins the microfilaments, which organize the actin cytoskeleton. The cytoskeletal filaments differ in structure and functions. The intermediate filaments have mainly shape stabilising functions. In addition to cell shape stabilising functions the microtubules provide the routes for the intracellular transport of vesicles and form the spindle apparatus during cell division, the actin cytoskeleton participates in a number of motile processes like cell locomotion and more specialized processes as endo- and exocytosis, vesicular transport, and cytokinesis. The supramolecular structure of the cytoskeleton is subjected to constant reorganization according to the cellular activities. In the past the different filament types have been regarded as separate functional entities, recent data, however, strongly indicate structural and functional linkages (as also demonstrated by the contribution by Schwan and Aktories).

Actin is the most abundant protein in many eukaryotic cells. In mammals six tissue-specific isoforms assemble to the actin containing microfilaments, which are often organized into bundles or higher ordered networks. The highly dynamic behaviour of the actin cytoskeleton is regulated by a large number of actin binding proteins (ABP). The ABPs can be grouped into different functional classes, which regulate the assembly or disassembly of actin filaments and are often targets of signalling pathways.

Bacteria have evolved a large arsenal of toxins and effectors, some of which are injected into host cells by type-3 secretion mechanisms to avoid phagocytosis by professional macrophages (see contribution by Aepfelbacher & Wolters). Alternatively, bacterial toxins or virulence factors induce special plasma membrane extensions of non-professional phagocytotic cells, which embrace bacteria leading to their uptake into host cells with the aim to secure their survival by gaining access to nutrients for proliferation, to achieve transport within the cytoplasm or from cell

to cell and finally their release into the environment for further dissemination (see contribution by Stradal & Costa). The actin cytoskeleton is essential for all these processes. Therefore it is not surprising that actin is a preferred target of many bacterial toxins and effector proteins also due to its highly conserved primary and ternary structure and its intracellular abundance.

With the 11 contributions this volume tries to introduce this fast progressing field of research without pretending completeness. Bacteria have evolved a vast number of tricks to secure their survival and proliferation. Therefore only a few bacteria and their toxins and effectors will be introduced with the intention to indicate common modes of action. The structure, properties and dynamic behaviour of actin cytoskeleton itself and the different classes of actin-binding proteins (ABP) will introduced with constant reference to bacterial toxins and effectors, which interfere their functions by modifying their normal behaviour (Kühn & Mannherz).

Bacterial toxins or effectors affect either the actin cytoskeleton by modifying actin directly or subverting certain ABPs or signalling pathways leading to the actin cytoskeleton by modifying small GTP-binding proteins terminating at regulatory ABPs (see contributions by Aepfelbacher & Wolters, Lemichez, and Stradal & Costa). Often a bacterium has developed toxins aiming on both mechanisms to influence the actin cytoskeleton like for instance *Photorhabdus luminescens* (contribution by Lang et al.), *Clostridia* (Schwan & Aktories), *Yersinia* (contribution by Aepfelbacher & Wolters).

A number of bacterial toxins modify actin directly by ADP-ribosylation or cross-linking. These modifications subvert the normal functioning of the actin cytoskeleton and thereby secure bacterial survival by inhibiting phagocytosis. This area will be covered by four contributions, which extend from their cellular effects to structural studies illuminating their selectivity. How phagocytosis is normally able to eliminate bacterial invasion is detailed for the uptake of Borrelia by macrophages in one contribution (Naj & Linder).

ADP-ribosyltransferases also modify small GTP-binding proteins of the Rho-family resulting in dramatic effects in their signalling capacity to the actin cytoskeleton (one special contribution and part of a number of other contributions). Alternatively, extracellular gastrointestinal pathogens manipulate the signalling pathways leading to the actin cytoskeleton by secreting virulence factors that modify the GTPase cycle of host Rho-GTPases thus securing their survival (This topic will be covered with two contributions (by Lemichez and Stradal & Costa).

Some intracellular bacteria hijack the actin cytoskeleton to their own needs by presenting on one pole bacterial proteins that lead to the assembly of a comet-like F-actin tail able by rapid actin subunit treadmilling to propel the pathogen within the cytoplasm of the host cell and for further dissemination from cell to cell (see contribution by Pillich et al.).

In many instances the analysis of the action of bacterial toxins and virulence factors provided important clues to the function of endogenous ABPs or even helped to identify ABPs, which were binding partners of certain bacterial effector proteins like the Arp2/3 complex as partner of ActA of Listeria. Future studies on

bacterial toxins will certainly uncover new insights into their functions and also unravel new aspects of the inherent regulatory mechanism of the actin cytoskeleton, which might also lead to the development of new antibiotic drugs. As a further outlook in this perspective this volume includes a description of the bacterial actins, which are different in primary but similar ternary structure to their eukaryotic counterparts, and their interactors (contribution by Gayathri).

Bochum, Germany Hans Georg Mannherz

Contents

Actin: Structure, Function, Dynamics, and Interactions with Bacterial Toxins

Sonja Kühn and Hans Georg Mannherz

Abstract Actin is one of the most abundant proteins in any eukaryotic cell and an indispensable component of the cytoskeleton. In mammalian organisms, six highly conserved actin isoforms can be distinguished, which differ by only a few amino acids. In non-muscle cells, actin polymerizes into actin filaments that form actin structures essential for cell shape stabilization, and participates in a number of motile activities like intracellular vesicle transport, cytokinesis, and also cell locomotion. Here, we describe the structure of monomeric and polymeric actin, the polymerization kinetics, and its regulation by actin-binding proteins. Probably due to its conserved nature and abundance, actin and its regulating factors have emerged as prefered targets of bacterial toxins and effectors, which subvert the host actin cytoskeleton to serve bacterial needs.

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1 Introduction

After host infection, bacteria invade non-phagocytic cells to secure their survival and multiplication. At the same time, they have to evade or block phagocytosis and destruction by professional phagocytes like polymorphonuclear cells (neutrophiles) and macrophages. Both, uptake by non-phagocytic host cells and phagocytosis by macrophages depend on plasma membrane extensions driven by rearrangements of the host actin cytoskeleton. Bacteria have devised a large arsenal of toxins to subvert cellular actin structures for their purposes by attacking actin directly or its manifold regulatory partners. Therefore, a more detailed knowledge of actin and its modes of regulation is essential to fully appreciate the many tricks bacteria have developed to hijack the actin cytoskeleton. Not surprisingly, the study of bacterial toxin actions was in many cases instrumental to deeper understand the fundamental, molecular mechanisms that drive the reorganization of dynamic cellular actin structures (see also Haglund and Welch 2011).

Actin is one of the most ubiquitous proteins in nature. Besides its abundant presence in all types of muscle cells and its participation in muscle contraction, it is present in almost any non-muscle eukaryotic cell (ranging from yeast to mammals) and in most cases in high concentration. It exists also in plant cells, where it fulfils similar functions like in metazoan cells. Furthermore, actin-analog proteins even exist in many bacteria where they may fulfil also cytoskeletal functions (see Gayathri 2016).

The actin protein was first isolated by Bruno Straub in 1943 working in the Laboratory of Albert Szent-Györgyi of the Department of Biochemistry at the University of Szeged (Hungary). Straub was credited for the isolation of actin from skeletal muscle only after the end of the World War II, since his results were initially published in a largely unknown journal edited by the University of Szeged (Straub 1942, 1943; see also Schoenenberger et al. 2011). The existence of two components necessary for muscle contraction had been implicated a century earlier by the work of the physiologists Wilhelm Friedrich Kühne (1837–1900) (University of Heidelberg, Germany), who first isolated a contractile extract from frog muscle—most probably actomyosin (Kühne 1859), and by William Dobson Halliburton (1860–1931) (Kings College, University of London, UK) (Halliburton 1887).

Today, we know much more about actin. In muscle tissues, actin containing thin filaments interdigitate with myosin containing thick filaments. Both types of filaments slide past each other during muscle contraction (Huxley and Niedergerke 1954; Huxley and Hanson 1954). The power for the sliding movement is generated by the head regions of the myosin motor molecule, which possess ATPase activity and the ability to cyclically interact with actin molecules of the thin filaments. The cyclical interaction of myosin motor domains with actin is linked to different steps of ATP binding and ATP hydrolysis. Thus, the chemical energy stored in the β-γ-phosphoanhydride bond of ATP is transformed into mechanical work by the interaction of the motor protein myosin with actin. Similar to muscle contractility, actin in non-muscle cells participates in many motile events like cell locomotion, intracellular transport processes like vesicular movements during exo- or endocytosis, phagocytosis, and cytokinesis, the final stage of mitosis. These motile processes often depend on the interaction of actin with specific myosin variants, but a number of essential motile events are executed also by mere polymerization and depolymerization of actin itself.

In addition, actin-containing filaments are essential for the structural and functional integrity of cells. Maintenance of cell polarity and the formation and stability of surface extensions like lamellipodia, microvilli, or filopodia critically depend on the local architectural stability of networks or bundles of actin filaments.

2 Actin

The actin protein is composed of a single polypeptide chain of 375 amino acid residues (skeletal muscle actin) with a molecular mass of 42 kDa, whose sequence was determined by Elzinga and coworkers (1973). Its amino acid sequence is highly conserved between different organisms, and the actin protein occurs abundantly in eukaryotic cells. Mammals express 6 different actin isoforms encoded by different genes. The actin isoforms are distributed in a tissue-specific manner and classified according to their isoelectric points: the most acidic isoforms being the three α-actins (one specific isoform expressed in skeletal, cardiac, and vascular smooth muscle), the β-actin in contractile structures like the so-called stress fibres (also termed cytoplasmic actin in non-muscle cells), and two γ-actins (one cytoplasmic actin in non-muscle cells and one enteric smooth muscle form) (Rubenstein 1990). Both cytoplasmic actin isoforms are ubiquitously expressed. These different mammalian actin isoforms vary only slightly in their amino acid sequences (Vandekerckhove and Weber 1978). The main differences were observed at their negatively charged N-terminus, whose composition and length vary in an isoform-specific manner.

All mammalian actins exist intracellularly in two main states of organization: the monomeric, globular G-actin or the polymerized, filamentous form (F-actin).

F-actin is physiologically the more relevant form, since it is only F-actin that is able to stimulate the myosin-ATPase activity, which provides the energy necessary for the performance of the cyclical force-producing interactions with myosin motor domains during muscle contraction or other cellular motile events. In addition, non-muscle F-actins often organize into higher-ordered supramolecular structures like stress fibres or bundles present in plasma membrane extension like microvilli or filopodia. Thus, besides its participation in motile events, actin filaments fulfil cytoskeletal functions like stabilizing cell form or specialized membrane extensions.

For these many diverse functions, actin has to be able to specifically interact with a large number of actin-binding proteins. Actin is one of the evolutionarily most conserved proteins, most probably due to its "promiscuous" nature. Actin is designed to interact with a large number (more than 160) of different actin-binding proteins, which regulate its spatial and temporal polymerization to actin filaments and their supramolecular organizations into bundles or networks.

2.1 Actin Structure

The three-dimensional (3D) structure of actin was solved to high-resolution by X-ray crystallography (Kabsch et al. 1985, 1990). Because increasing the ionic strength induces actin polymerization, it was found impossible to obtain crystals of monomeric actin suitable for X-ray analysis. Therefore, binary complexes of actin with an actin-binding protein (ABP) stabilizing it in the monomeric form were employed for crystallization. The 1:1 complex of skeletal muscle α-actin with deoxyribonuclease I (DNase I) was the first complex whose 3D structure was solved (Kabsch et al. 1985, 1990). Subsequently, complexes of skeletal muscle α-actin with gelsolin G1 (McLaughlin et al. 1993) and profilin in complex with cytoplasmic β-actin (Schutt et al. 1993) were determined. Meanwhile, about 80 3D structures of actin in complex with a number of different ABPs or small molecules have been determined, which all confirmed its basic 3D structure (see Dominguez and Holmes 2011).

Actin is a rather flat molecule with dimensions of about 5.5 × 5.5 × 3.5 nm (Fig. 1a–c). The molecule is divided into two main lobes of about equal sizes separated by a deep upper cleft whose bottom contains the nucleotide (ATP or ADP) and divalent cation-binding sites (Fig. 1a). A smaller incision is seen at its lower side, which represents the main target area for binding of many ABPs (see later). The two main lobes are connected by a small bridge with the peptide chain crossing twice between the two main lobes. This connection may function as a hinge allowing rotations of the two main lobes relative to each other during G- to F-actin transitions (Oda et al. 2009). Each main lobe is subdivided into two clearly discernible subdomains (SD1–4, see Fig. 1a), which are composed of a central ß-pleated sheet and surrounded by α-helices linked by loops of varying lengths. SD1 and 3 have a similar architecture built from a five-stranded ß-pleated sheet, whereas

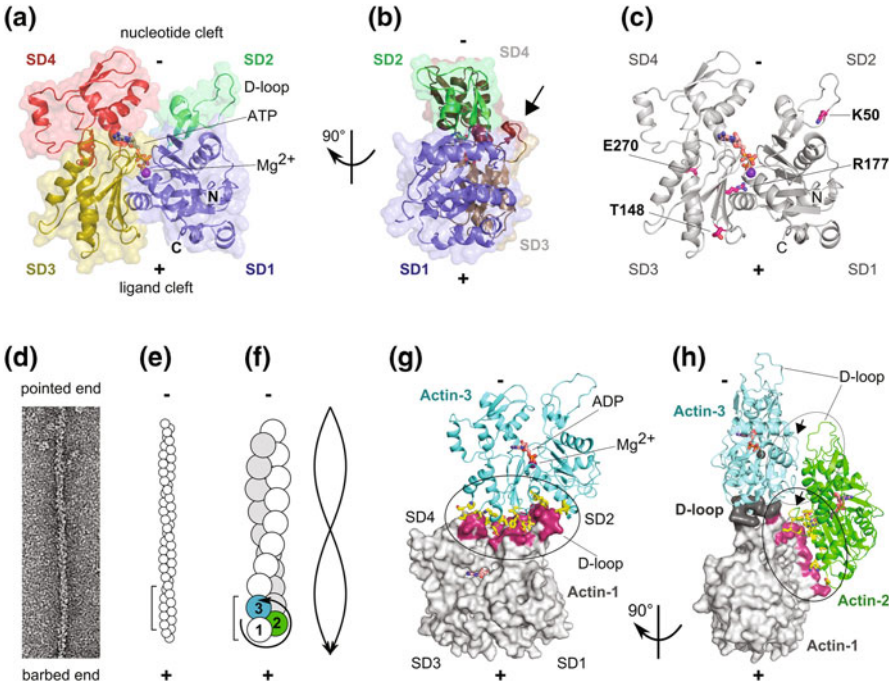

Fig. 1 Structure of G- and F-actin. (**a**–**c**) G-actin structure shown as ribbon with semitransparent surface (**a**–**b**), the ATP nucleotide as stick representation and bound Mg^{2+} cation as sphere. The actin subdomains (SD), pointed (−) and barbed (+) faces are indicated. Front (**a**) and side (**b**) view of G-actin with its different coloured four subdomains (SD1–SD4). Note the two large domains (*blue* SD1 and *red* SD4) and bound ATP at the bottom of the deep nucleotide binding cleft between SD2 and SD4. The hydrophobic ligand-binding cleft that enables interaction of most actin-binding proteins (ABPs) with actin is located on the opposite between SD1 and SD3. The DNase I-binding loop (D-loop) is mainly involved in maintaining important intrastrand F-actin contacts (see **g**–**h**). The *arrow* indicates the hydrophobic plug that forms interstrand contacts (see **h**) (PDB: 1ATN). **c** Sites of direct actin modifications of bacterial toxins. Modified amino acids are indicated in *pink stick* presentation. Arg177 of actin (R177) is ADP-ribosylated by binary toxins like C2 or Iota, while Thr148 (T148) is ADP-ribosylated by the TccC3 toxin. Cross-linking of two actin molecules by bacterial toxins of the MARTX family occurs between Lys50 (K50) and Glu270 (E270). **d**–**h** F-actin subunit organization. **d** shows an electron microscopic image of a single actin filament with depicted pointed (−) and barbed (+) ends, (**e**) the arrangement of the actin subunits within the filament and (**f**) their helical organization (see text). The *bracket* in (**e**) corresponds to the displayed detail in (**f**), while the *bracket* and F-actin protomer numbering at the filament barbed end in (**f**) belongs to the actin dimer and trimer in (**g**–**h**). **g**–**h** Electrostatic and hydrophobic interactions of actin protomers within the actin filament. The contacts are formed between three surfaces: site (I) (actin-1:actin-3), (II) (actin-1:actin-2), and (III) (actin-2:actin-3). Binding sites (II) and (III) are identical. **g** Intrastrand contacts of site I (*circle*) between actin molecules 1 (actin-1, *grey*, as surface) and 3 (actin-3, *blue*, as ribbon) of the long-pitch dimer. Resides of actin-3 (SD1 and SD3) involved in the interaction with actin-1 (SD2 and SD4) are presented as sticks (*yellow*), while the actin-3-binding surface on actin-1 is coloured in *pink*. **h** Interstrand contacts of site II (*black circle*) between actin-1 (*grey*, surface) and actin-2 (*green*, ribbon) of the lateral dimer and of site III (*grey circle*). The hydrophobic plug (see **b**) connects all three actin protomers at the interior of the actin filament and is highlighted (*arrow*). The D-loop of actin-1 (*dark grey*) forms hydrophobic and electrostatic contacts with actin-3 (site I) , while adjacent residues in SD2 of actin-1 are involved in interstrand contacts with actin-2 (site II). Residues of actin-2 involved in the interaction with actin-1 are presented as sticks (yellow), while the actin-2-binding surface on actin-1 is colored in pink

SD2 and SD4 differ in their size and 3D structure. The N- and C-termini are located in SD1. Therefore, SD1 is built from residues 1–32, 70–144, and 338–375, SD2 from residues 33–69, SD3 from residues 145–180 and 270–337, and SD4 from residues 181–269 (Fig. 1a–b).

This basic actin fold was also found in the so-called actin-related proteins (the Arp proteins), which considerably differ in their sequence but are specifically enriched in cell nuclei or present in the cytoplasm of many eukaryotic cells, like within the Arp2/3 complex. In addition, many of the prokaryotic actin-like proteins like MreB and ParM share a high structural homology to actin in spite of high sequence divergences (see Gayathri 2016). Surprisingly, a number of proteins with completely different sequences and functions like hexokinase and the heat-shock protein HSP70 possess also a high structural similarity to actin probably due to a common architecture of their ATP-binding sites (Flaherty et al. 1991).

2.2 Binding Sites on Actin for Actin-Binding Proteins

Subdomain 1 appears to be the main binding site for myosin motor heads (see Geeves and Holmes 1999; Behrmann et al. 2012). From SD2 extents a loop that in many solved structures appeared unstructured, but forms the main binding site for DNase I and therefore was named the DNase-binding (or D-) loop (Kabsch et al. 1990). The D-loop is also involved in actin-actin contacts along the long-pitch strand (see below). The small incision at the base between SD1 and SD3 forms an important target area for a large number of actin-binding proteins like gelsolin segment 1 (G1), profilin, cofilin, and thymosin beta 4, which binds to actin with its so-called WH2 domain. WH2 domains are present in a large number of other actin-binding proteins enabling similar interactions with this region of actin (see Dominguez and Holmes 2011).

2.3 Filamentous (F-) Actin

The physiologically active form of actin is F-actin. In the test tube, actin can be maintained in monomeric (G-) state only at low-salt conditions of mono- and divalent cations. When raising the ionic strength by addition of cations (KCl to 100 mM and/or to 2 mM $MgCl_2$, in other words to about the intracellular ionic concentrations), actin polymerizes to form filamentous (F-) actin.

Normally, monomeric actin contains firmly bound one molecule of ATP, which is essential for the maintenance of its native configuration. Nucleotide-free actin denaturates rapidly and irreversibly. After incorporation into a growing filament,

the actin-bound ATP is quickly hydrolysed into ADP and inorganic phosphate (Pi). While the ADP nucleotide remains bound to the actin molecule, the Pi is slowly released ($t_{1/2}$ = 6 min).

The actin filament (Fig. 1d–f) has a diameter of about 8.0 to 10.0 nm with the larger domain being in the centre of the filament axis. Cellular actin filaments can be composed of about 1000 actin monomers and attain a length of 1 µm like the thin filaments of skeletal muscles. F-actin can be described as a left-handed two-start long-pitch helix (half pitch rise 380 nm) or as a right-handed generic helix with an inter-subunit raise of 27.5 nm and a 166° rotation angle (Fig. 1f). Since the actins in both strands have the same orientation, the filament ends expose different surfaces of the actin molecule. The contact sites of a single actin subunit with its neighbouring subunits are shown in Fig. 1g–h.

Because F-actin cannot be crystallized, numerous attempts using fibre diffraction procedures or electron microscopy of parallel aligned F-actin (F-actin paracrystals) have been undertaken to elucidate the atomic structure of F-actin at high resolution. Recently, the advancement of cryoelectron microscopy has provided the necessary resolution to define the conformational changes G-actin undergoes when incorporated into F-actin. This structural transition is characterized by a 20° tilt of the two main domains relative to each other resulting in a flatter actin conformation, which appears to be essential for filament incorporation (Oda et al. 2009). Recent data of about 3.7 Å resolution show more clearly the interfilament actin-actin contacts (von der Ecken et al. 2014).

Within the filament, each actin subunit contacts four neighbouring actins: two longitudinally related actins along the long-pitch strand and two lateral subunits of the neighbouring strand. The contacts between actin subunits along the long-pitch strands are more numerous and apparently stronger than the interstrand contacts. The longitudinal contacts are formed between subdomains 2 and 4 of each subunit with subdomain 3 of the respective upper subunit (Fig. 1g, h). This contact area contains a number of electrostatic and hydrophobic interactions (highlighted in Fig. 1g, h). The residues involved are 166–169 (SD 3) to 41–45 (SD2), residues 286–289 (SD3) to 202–204 (SD4), and residues to 322–325 (SD3) to 243–245 (SD4) of the upper to the lower subunit (see Fig. 1g, h; for review see Mannherz 1992). A major hydrophobic contribution is provided by the D-loop (SD2) with the lower surface of the ß-pleated sheet of SD3 of the actin subunit above, where it contacts residues around Tyr169 (von der Ecken et al. 2014).

The main interstrand contact is formed by a loop (the so-called hydrophobic plug, Holmes et al. 1990; see also Dominguez and Holmes 2011) that extends into the middle of the filament from SD3 to SD4 of each actin subunit and contacts a pocket formed by the interface of two adjacent actin subunits of the opposing strand (arrows in Fig. 1b, h). This loop is formed by residues extending from Pro264 to Ser271, but contrary to its original denomination forms salt bridges between the three actins (Fujii et al. 2010). It contacts residues 40–45 and 63–65 (SD2 of the lower opposing subunit) and residues from 166 to 171 and 285 to 289 (SD3 of the opposing upper subunit) (reviewed in Mannherz 1992; von der Ecken et al. 2014).

2.4 Actin Dynamics: Polymerization Behaviour

Actin proteins in living cells are in a tightly regulated, dynamic equilibrium between the G- and F-actin states. The spontaneous assembly of monomeric to filamentous actin is a two-step polarized condensation process, which depends on an initial nucleation phase followed by a rapid elongation process until the steady state is reached. During the nucleation phase, an actin oligomer consisting of three or four G-actin subunits has to be assembled for elongation to occur (Fig. 2). The formation of actin nuclei represents the rate-limiting step. It is kinetically highly unfavoured, since the net negative charge at physiological pH hinders the formation of actin dimers ($k_+ = 10\ \mu M^{-1}\ s^{-1}$, $k_- = 10^6\ s^{-1}$ giving a K_d of 100 mM). A more stable nucleus is formed only after binding of a third actin protomer ($k_+ = 10\ \mu M^{-1}\ s^{-1}$, $k_- = 10^3\ s^{-1}$ giving a K_d of 0.1 mM), or requires even the stabilizing effect of binding to a fourth one. Further actin monomers assemble at both sides of this actin nucleus during early filament elongation (Wegner and Engel 1975; Gilbert and Frieden 1983; Tobacman and Korn 1983; Sept and McCammon 2001).

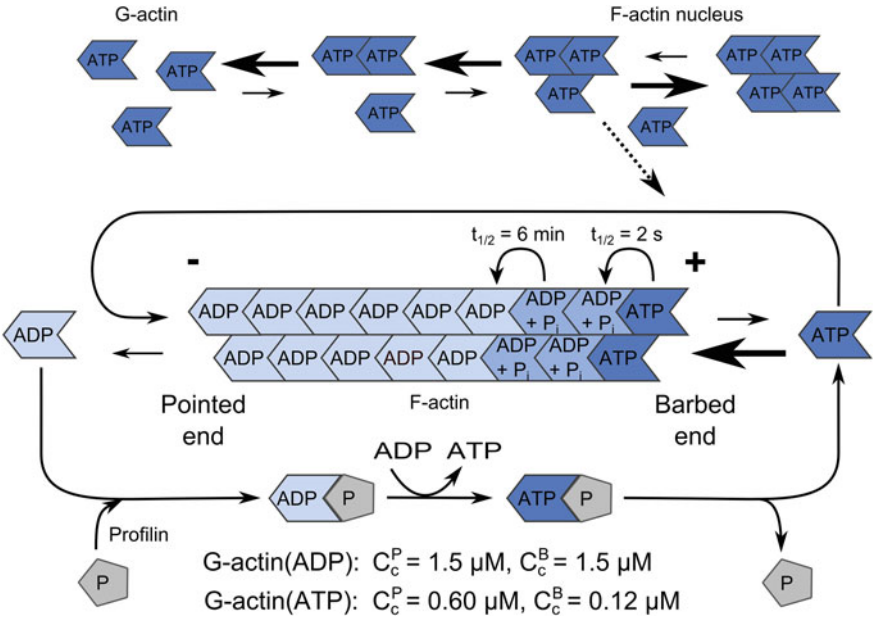

Fig. 2 The nucleation and polymerization (treadmilling) process of the G- to F-actin transition. The *upper row* illustrates the formation of nuclei (dimer to tetramer) from ATP-containing actin molecules and the cyclic scheme the treadmilling process of F-actin with barbed end (+) ATP-containing actin subunits and pointed end (−) ADP-containing subunits. The critical concentrations for ATP- or ADP-actin addition to the pointed (P) or barbed (B) end are shown (for detail, see text). $t_{1/2}$ describes the half time for ATP hydrolysis or for P_i dissociation from the actin protomers after ATP hydrolysis

Due to the structural polarity of F-actin, two filament ends with different exposed surfaces and with distinguishable elongation rates are generated: the fast-growing plus or barbed end (+) and the slow-growing minus or pointed end (−) (Fig. 2). With the different rates of actin monomer addition and dissociation, the barbed end of F-actin elongates up to ten times faster than the pointed end (rate constants (+)-end: $k_+ = 12\ \mu M^{-1}\ s^{-1}$, $k_- = 1.4\ s^{-1}$; (−)-end: $k_+ = 1.3\ \mu M^{-1}\ s^{-1}$, $k_- = 0.3\ s^{-1}$) (Pollard et al. 2000). During F-actin growth, the concentration of soluble actin monomers decreases, until at steady state G-actin molecules exchange with the filament ends without increasing the total F-actin amount. The equilibrium concentration of the monomeric G-actin pool at steady state is termed the critical concentration C_c. Monomeric actin polymerizes at G-actin concentrations above C_c, while lower values lead to F-actin depolymerization. The C_c values for ATP-G-actin differ at both filament ends, and the C_c is much lower at the barbed end ($C_c^+ = 0.12\ \mu M$ vs. $C_c^- = 0.60\ \mu M$). Monomeric actin polymerizes at the barbed end at G-actin concentrations above C_c^+ and below C_c^-, while the pointed end depolymerizes. This process is termed treadmilling and describes the presence of equal net rates of F-actin barbed end assembly and pointed end disassembly after reaching the steady-state G-actin concentration of 0.12 μM. During the treadmilling cycle, the overall length of the actin filament remains constant (Kirschner 1980; Wegner 1976, 1982; Bonder et al. 1983; Bugyi and Carlier 2010).

In filamentous actin, the actin-bound ATP nucleotide is irreversibly hydrolysed to ADP and inorganic phosphate ($0.3\ s^{-1}$) followed by the slow release of the phosphate ($0.002\ s^{-1}$) (Pollard and Weeds 1984; Carlier et al. 1984; Korn et al. 1987; Pollard et al. 2000). The ATP hydrolysis rate of actin is thereby slower compared to the assembly kinetics at the barbed end (see Fig. 2). This leads to an F-actin barbed end with terminal actin protomers enriched in ATP or ADP + Pi, while the remaining filament is in the ADP-bound state. Thus, actin polymerization is an energy-consuming process that results in two ends that differ with regard to their structure and kinetics, but also with their energy charge.

Notably, the intrinsic treadmilling rate of actin and thereby the net flux of actin protomers from the filament's barbed end to the pointed end is very slow. The fast, polarized assembly of actin filaments found in living cells is solely achieved by the interaction of actin with actin-binding proteins (ABPs) which tightly regulate the treadmilling process (see Sect. 3).

3 Interactions with Actin-Binding Proteins (ABPs)

A large number of about 160 different actin-binding proteins (ABPs) have been identified so far. Almost all actin-binding proteins either control cellular actin assembly dynamics or maintain the supramolecular F-actin organization and its connection to other cellular components (see Fig. 3). There are only few examples known where G-actin influences the enzymatic activity of another protein: first, the inhibition of DNase I activity (Mannherz et al. 1980). DNase I is an extracellular or serum protein, and its chromatin-degrading activity will harm any cell when gaining

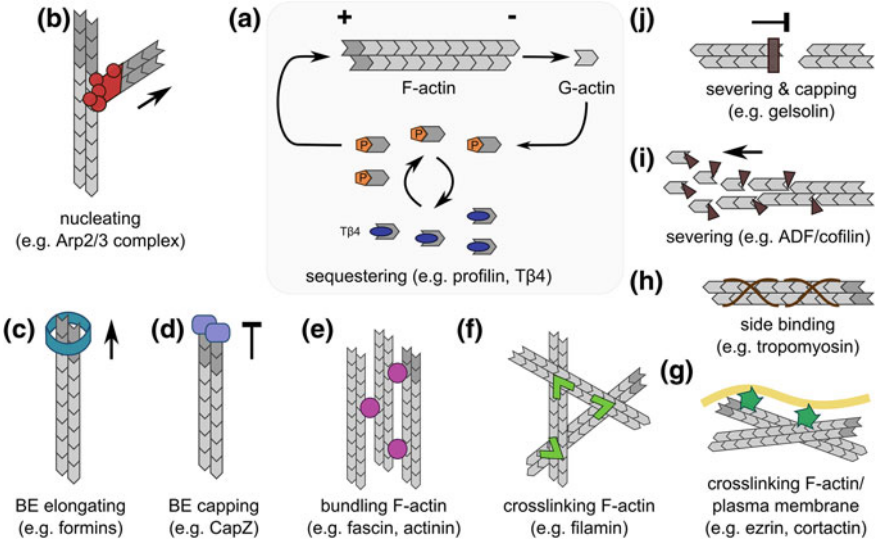

Fig. 3 The effect of the diverse classes of actin-binding proteins (ABPs) on actin dynamics. **a** F-actin in equilibrium with G-actin that can be sequestered by binding to profilin (P) or thymosin beta 4 (Tß4). **b–j** The effects of different ABP classes on F-actin are illustrated (for details see text)

access to the nucleus. DNase I inhibition by G-actin appears to be a safeguard mechanism to prevent chromatin degradation when DNase I inadvertently diffuses into cells with damaged plasma membranes (Eulitz and Mannherz 2007). Secondly, the kinase activity of a bacterial toxin (Yersinia YopO protein; see later) is stimulated by G-actin binding (Trasak et al. 2007; Lee et al. 2015; see also Aepfelbacher and Wolters 2016). Furthermore, binding of G-actin within the cytoplasm to MAL (or MRTF: myocardin-related transcription factor) negatively regulates the transcriptional activity of the serum response factor (SRF) by preventing the translocation of MAL into the nucleus. Only dissociation of the G-actin-MAL complex liberates MAL to diffuse into the cell nucleus and to stimulate SRF transcriptional activity leading to the expression of SRF-dependent genes (Olson and Nordheim 2010).

Apart from these examples, most ABPs that influence cellular actin dynamics either induce the formation of F-actin nuclei to overcome the energetic barrier of F-actin nuclei formation, or regulate the polymerization behaviour of F-actin. The latter is achieved either by increasing the rate-limiting step of the treadmilling cycle (e.g. severing and pointed end depolymerization), by manipulating the F-actin assembly dynamics by increasing the barbed end elongation rate or preventing the assembly of additional G-actin protomers, or by destabilizing F-actin by side binding and severing. In cells, the supramolecular organization of actin is maintained by cross-linking and bundling actin filaments and by connecting actin filaments with other cytoskeletal filaments or cellular membranes (Fig. 3). Most actin regulators share redundant functions with others and can be grouped into different classes according to their general activity (Fig. 3). The main groups are briefly

introduced below. Of note, many of these proteins are hijacked by bacterial pathogens to induce the pathogen-required temporal and spatial regulation of the host actin regulatory machinery.

3.1 G-actin-Sequestering Proteins

The maintenance of a cellular, monomeric actin pool is of importance, because the elongation rate of barbed ends depends on the availability of ATP-G-actin. This cellular G-actin pool is bound in 1:1 complexes to sequestering proteins like the abundant G-actin-binding proteins profilin and β-thymosins (main component: thymosin beta 4 or Tβ4) that prevent spontaneous F-actin nucleation (Rosenblatt et al. 1995; Fig. 3a). This sequestered G-actin pool is in rapid equilibrium with monomeric, unsequestered actin but not with the filamentous one. β-Thymosins form 1:1 complexes with preferably ATP-G-actin, which are by themselves polymerization resistant and thereby responsible for the maintenance of the high intracellular pool of unpolymerized actin. Since the affinity of β-thymosins to actin is only in the micromolar range (K_d = 1–5 μM), the actin in this complex is in rapid equilibrium with free and assembly-competent G-actin. Only the often high intracellular concentration of β-thymosins (up to 500 μM in non-muscle cells) can establish an appreciable amount of sequestered actin (see Fechheimer and Zigmond 1993; Mannherz and Hannappel 2009).

Profilin enhances the ADP exchange rate of G-actin leading to sequestered profilin:ATP-G-actin complexes. Like many other actin-binding proteins, profilin interacts with the ligand-binding cleft of actin at the edge of subdomains I and III (for review, see Dominguez and Holmes 2011). As a consequence, profilin does not participate in pointed end assembly and enhances thereby the processivity of treadmilling. Additionally, profilin effectively competes with β-thymosins for ATP-G-actin binding due to its higher binding affinity for actin. In contrast to Tβ4, profilin-actin is able to associate specifically with the barbed end of F-actin to participate in its elongation. Profilin-bound actin therefore represents the main cellular source of actin for polymerization and serves as a kind of carrier between the non-available Tβ4-actin pool and free F-actin barbed ends. Interestingly, some bacterial actin modifications specifically interfere with actin binding to Tβ4, but not to profilin (see Sect. 4.1).

3.2 F-actin-Nucleating Proteins and Their Nucleation-Promoting Factors (NPFs)

Proteins that initiate the polymerization of actin filaments by stabilizing actin nuclei involve the Arp2/3 complex and its nucleation-promoting factors (NPFs), multiple WH2 domain-containing proteins, and some formin family members (Fig. 3b, c).

The main cellular F-actin-nucleating machinery represents the Arp2/3 complex that initiates branched actin filaments at the sides of pre-existing mother filaments (Fig. 3c) and that is involved in the formation of the branched actin meshwork in membrane ruffles and lamellipodia (Mullins et al. 1998a, b; Amann and Pollard 2001; Lai et al. 2008). Nevertheless, the activation of the Arp2/3 complex by additional nucleation-promoting factors (NPFs) is necessary for efficient F-actin nucleation activity. NPFs are WH2 domain-containing proteins and provide an actin monomer for the formation of an actin trimer with the two actin-related proteins of the Arp2/3 complex (Higgs and Pollard 1999; und 2001; Robinson et al. 2001; Goley and Welch 2006). The NPFs WASP, N-WASP, and the WAVE complex are mainly involved in actin remodelling at the plasma membrane and often manipulated by invading pathogens (Pollard and Borisy 2003; Veltman and Insall 2010). Some formins have been described to possess F-actin nucleation activities. This class of autoinhibited nucleators is activated by GTP-binding proteins of the Rho family, which thereby links the organization of the intracellular actin cytoskeleton to extracellular signals.

3.3 F-actin-Elongating Proteins

This group of ABPs comprises Ena/VASP proteins and formins that both processively elongate F-actin barbed ends and promote the dissociation of barbed end assembly antagonists (Fig. 3c). Besides their nucleation activity, dimeric formins are prominent barbed end trackers that support the fast formation of long straight filaments and compete with capping proteins for barbed end binding (Pruyne et al. 2002; Paul and Pollard 2009; Shekhar et al. 2015; Bombardier et al. 2015). Formins bind profilin-G-actin with their FH1 domain and F-actin barbed ends with their ring-shaped FH2 domain that processively incorporates new actin monomers into the filament (Goode and Eck 2007; Kühn and Geyer 2014). Tetrameric Ena/VASP proteins contain WH2 domains and interact with G- and F-actin to add profilin-bound actin monomers to the bound filament barbed end (Bachmann et al. 1999; Applewhite et al. 2007; Breitsprecher et al. 2008; Ferron et al. 2007; Bear and Gertler 2009).

3.4 F-actin-Capping Proteins

The large and diverse group of capping proteins bind with high affinity to the ends of actin filaments and prevent further subunit association or dissociation (Fig. 3d). They vary in their abundance, domain composition, and F-actin-binding affinity. Most capping proteins interact with F-actin barbed ends to block filament growth, like gelsolin or capping protein (CP) (Cooper and Sept 2008; Silacci et al. 2004). There are only a few pointed end capping proteins, like tropomodulin in muscle tissue.

3.5 F-actin-Bundling and Cross-linking Proteins

Existing actin filaments can form long straight bundles with the help of bundling proteins, whose length determines the distance between individual filaments inside an actin bundle (Fig. 3e). Villin and fimbrin fulfil this function in bundles within microvilli in epithelial cells, while fascin is involved in F-actin bundling in filopodia (Bretscher and Weber 1979; Edwards and Bryan 1995; Khurana and George 2008). Besides bundling, F-actin can be organized into large, cross-linked, and netlike actin webs by proteins of the filamin family (Fig. 3f; Razinia et al. 2012). Cross-linking does also occur to mediate the interaction of F-actin with cellular components like the plasma membrane as it has been described for members of the ezrin/radixin/moesin (ERM family; Niggli and Rossy 2008) and spectrin protein families (Broderick and Winder 2005) (Fig. 3g). The ERM proteins attach F-actin network to the cytoplasmic face of the plasma membrane by binding with their N-terminal FERM domain to integral membrane proteins. By their preferred binding to F-actin, these proteins stabilize also the filamentous form of actin. Spectrin molecules form with short actin filaments a network within the so-called terminal web of microvilli bearing epithelial cells or the membrane cytoskeleton of red blood cells.

3.6 F-actin-Stabilizing Proteins

The large group of tropomyosins encompasses elongated proteins built from an α-helical coiled-coil, which stabilize existing actin filaments by side binding along both long-pitch strands of F-actin (Fig. 3h; von der Ecken et al. 2014; Pittenger et al. 1994; Wang and Coluccio 2010).

3.7 F-actin-Severing Proteins

The diverse group of severing proteins consists of two main families, ADF/cofilin and gelsolin-like proteins, that are responsible for the sudden, rapid breakdown of straight or branched actin filaments (Fig. 3i; Andrianantoandro and Pollard 2006; Nag et al. 2013). They bind at least transiently to G-actin forming either 1:1 or 1:2 complexes and fragment F-actin in a Ca^{2+}-dependent (gelsolin) or Ca^{2+}-independent (ADF/cofilin) manner.

After severing actin filaments, proteins of the gelsolin family remain associated with the newly formed barbed end and prevent further monomer addition. Gelsolin proteins thereby act as capping proteins, while the uncapped pointed end rapidly disassembles (Fig. 3j; Silacci et al. 2004). Severing proteins also promote filament polymerization depending on the physiological status of the cell. Members of the

ADF (actin-depolymerizing factor)/cofilin protein family bind specifically to ADP-bound G- and F-actin and facilitate indirectly barbed end assembly. They act in synergy with barbed end capping proteins to cause an increase in the critical concentration of the pointed end (C_c^-) by enhancing its disassembly rate, which increases the monomeric ATP-actin concentration and stimulates spontaneous nucleation and faster barbed end polymerization of new actin filaments (Carlier et al. 1999; Tania et al. 2013). Thus, severing contributes to the turnover of actin filaments and may result in a net increase in the F-actin amount.

3.8 Regulation of the Activity and Localization of ABPs

Many ABPs are directly regulated downstream of key extra- and intracellular signalling cascades that control actin dynamics during cell migration, cytokinesis, exocytosis, and endocytosis (Pollard and Cooper 2009). The activity and spatial localization of these actin regulators depend on Ca^{2+} ion concentrations, phosphatidylinositol phosphate interactions, active Rho GTPases, phosphorylation by kinases, and their recruitment by membrane-bound scaffold proteins. As an example, the nucleation-promoting factors N-WASP and WAVE complex of the F-actin-nucleating Arp2/3 complex are effectors of the Rho GTPases Cdc42 and Rac1, respectively. In addition, they become activated and recruited by kinase phosphorylation (e.g. Abl and ERK2), phospholipid binding at the plasma membrane ($PI(4,5)P_2$ and PIP_3, respectively), and interaction with membrane-bound adapter proteins like IRSp53 and Nck. Rho GTPases themselves are activated by extracellular stimuli and intracellular signal cascades that involve tyrosine phosphorylation, changes in the lipid composition of membranes, and scaffold proteins. Of note, Rho proteins themselves represent targets of bacterial toxins (see Lemichez 2016). Severing proteins of the gelsolin family are regulated by Ca^{2+} ion levels and phosphorylation, while their activity is inhibited by the phospholipid $PI(4,5)P_2$.

4 Examples of Bacterial Proteins that Subvert the Host Actin Cytoskeleton

In contrast to the above described eukaryotic actin-binding proteins, bacteria have developed actin cytoskeleton regulators, which either directly mimic and functionally override some of the ABP functions, or covalently modify actin in order to functionally disturb the actin cytoskeleton. The following paragraphs try to give a brief introduction into the considerable diversity of the strategies that bacterial pathogens have developed to attack or to survive in host eukaryotic cells (as summarized also in Tables 1, 2 and 3).

Table 1 G-actin modifying bacterial toxins

Bacterium	Toxin	Actin modification	Effect on F-actin cytoskeleton	Reference
Clostridium botulinum	C2	R177 ADP-ribosylation	Depolymerization	Aktories et al. (1986)
Clostridium perfringens	Iota	R177 ADP-ribosylation	Depolymerization	Stiles and Wilkins (1986), Simpson et al. (1987)
Clostridium difficile	CDT	R177 ADP-ribosylation	Depolymerization	Perelle et al. (1997)
Clostridium spiroforme	CST	R177 ADP-ribosylation	Depolymerization	Popoff and Boquet (1988), Simpson et al. (1989)
Bacillus cereus	VIP	R177 ADP-ribosylation	Depolymerization	Han et al. (1999)
Salmonella enterica	SpvB	R177 ADP-ribosylation	Depolymerization	Otto et al. (2000), Tezcan-Merdol et al. (2001), Hochmann et al. (2006)
Aeromonas salmonicida	Aext	R177 ADP-ribosylation, Rho GAP activity	Depolymerization	Braun et al. (2002), Fehr et al. (2007), Litvak and Selinger (2007)
Photorhabdus luminescens	Photox	R177 ADP-ribosylation	Depolymerization	Visschedyk et al. (2010)
Aeromonas hydrophila	VahC	R177 ADP-ribosylation	Depolymerization	Shniffer et al. (2012)
Streptococcus pyogenes	SpyA	ADP-ribosylation of actin and vimentin	Depolymerization	Coye and Collins (2004), Icenogle et al. (2012)
Photorhabdus luminesens	Tc toxins (e.g. TccC3)	T148 ADP-ribosylation	Actin clustering	Lang et al. (2010)
Vibrio cholerae	RtxA (MARTX family)	K50-E270 cross-linking of G-actin	Depolymerization	Fullner and Mekalanos (2000), Satchell (2011), Sheahan et al. (2004)
Vibrio cholerae	VgrG1	K50-E270 cross-linking of G-actin, ADP-ribosylation	Depolymerization	Durand et al. (2012), Suarez et al. (2010)

Table 2 Direct manipulation of actin dynamics by bacterial effectors

Protein	Bacterium	Strategy (details and involved host proteins)	Effect on actin cytoskeleton	References
TARP	*Chlamydia trachomatis, C. pneumoniae*, *C. muridarum*, *C. caviae*	Mimicking host nucleators, indirect Arp2/3 complex regulation, recruitment of membrane-actin crosslinking proteins (actin binding domains: WH2, poly-Pro, FAB1/2; PI3K, FAK, vinculin recruitment; Rac1 GEF activation)	F-actin nucleation at bacterial entry site via Arp2/3 complex-dependent and independent mechanisms F-actin bundling	Jewett et al. (2006). Lane et al. (2008), Jiwani et al. (2013)
BimA	*Burkholderia pseudomallei, B. mallei*	Mimicking host Ena/VASP F-actin-tracking proteins (Actin-binding domain: WH2)	F-actin nucleation, barbed end elongation, bundling, uncapping	Sitthidet et al. (2010), Benanti et al. (2015)
VopL	*Vibrio parahaemolyticus*	Mimicking host Ena/VASP F-actin-tracking proteins (Actin-binding domain: WH2, FH1-like)	F-actin nucleation Stress fibres formation	Yu et al. (2011), Liverman et al. (2007)
VopF	*Vibrio cholerae*	Mimicking host Ena/VASP F-actin-tracking proteins (Actin-binding domain: WH2, FH1-like)	F-actin nucleation, elongation, uncapping, actin sequestering, filopodia formation, tight junction alterations	Dziejman et al. (2005), Pernier et al. (2013)
Sca2	*Rickettsia conorii*	Mimicking host formin F-actin assembly proteins (Actin-binding domain: WH2, FH1-like, FH2-like)	F-actin nucleation, profilin-dependent processive elongation, uncapping	Haglund et al. (2010), Madasu et al. (2013)
YopO	*Yersinia enterocolitica*	Actin-induced kinase activity for ABPs (inactivation of gelsolin, cofilin, VASP, EVL, Dia1, INF2, WASP), Rho GDI	F-actin disruption, impaired phagocytosis by misregulation of host ABPs	Trasak et al. (2007), Lee et al. (2015)

Table 3 Examples for indirect strategies of actin cytoskeleton manipulation by bacterial effectors

Protein	Bacterium	Strategy (details and involved host proteins)	Effect on actin cytoskeleton	References
ActA	*Listeria monocytogenes*	Direct Arp2/3 complex activation (mimics NPF N-WASP; actin binding domains: VCA, poly-Pro)	F-actin assembly into tail-like structures for bacterial motility	Domann et al. (1992), Kocks et al. (1992), Gouin et al. (2005)
RickA	*Rickettsia conorii*	Direct Arp2/3 complex activation (mimics NPFs for recruitment and activation)	F-actin nucleation	Gouin et al. (2004, 2005)
BimA	*Burkholderia thailandensis*	Direct Arp2/3 complex activation (mimics NPFs for binding and activation; actin binding domains: VCA, poly-Pro motif)	F-actin nucleation	Sitthidet et al. (2010), Welch and Way (2013), Benanti et al. (2015)
IcsA (VirG)	*Shigella flexneri*	Indirect Arp2/3 complex activation (recruits and activates N-WASP; actin binding domains: VCA, WH2, poly-Pro motif)	F-actin nucleation	Goldberg et al. (1993), Suzuki et al. (2002), Cossart (2000)
TccP (EspFu)	*EHEC/EPEC*	Indirect Arp2/3 complex activation (activates NPFs N-WASP and WASP by release from autoinhibition)	F-actin nucleation	Campellone et al. (2004), Cheng et al. (2008)
EspF	*EHEC/EPEC*	Indirect Arp2/3 complex activation (activates NPF N-WASP; actin, profilin, Abcf2, SNX9 binding)	F-actin nucleation, tight junction disruption, antiphagocytosis	Alto et al. (2007), Peralta-Ramírez et al. (2008)
Tir	*EHEC/EPEC*	Recruitment of membrane-bound scaffold proteins and ABPs (phosphorylation by host kinases leads to recruitment of Nck, IQGAP1, IRSp53, IRTKS)	F-actin nucleation and polymerization; actin pedestal formation	Brown et al. (2008), Campellone et al. (2002), Weiss et al. (2009), Campellone (2010), Gruenheid et al. (2007), de Groot et al. (2011)
IpaA	*Shigella flexneri*	Recruitment of membrane-actin crosslinking proteins (Vinculin)	Depolymerization of F-actin, reduction of adhesion	Bourdet-Sicard et al. (1999), Ramarao et al. (2007), Tran Van Nhieu (1997)
SptP	*Salmonella typhimurium*	Regulation of F-actin bundling and crosslinking proteins (phosphatase for villin, vimentin), regulation of Rho GTPases (GAP for Rac1, Cdc42)	Downregulation of actin cytoskeleton remodelling after bacterial entry	Fu and Galán (1999); Murli et al. (2001); Lhocine et al. (2008)

(continued)

Table 3 (continued)

Protein	Bacterium	Strategy (details and involved host proteins)	Effect on actin cytoskeleton	References
EspL	*EHEC/EPEC*	Regulation of membrane-actin crosslinking proteins (annexin 2)	F-actin accumulation, formation of pseudopod-like structures	Miyahara et al. (2009)
CagA	*Heliobacter pylori*	Phosphorylated by host kinases (effects on cortactin, ezrin, vinculin)	Actin cytoskeleton rearrangements that promote cell motility, cell scattering, and elongation	Tegtmeyer and Backert (2011), Rieder et al. (2005), Stein et al. (2002)
SipA	*Salmonella typhimurium*	Antagonizes with severing proteins (displaces ADF/cofilin, villin and gelsolin from F-actin, barbed end protection from severing and depolymerization)	Decreases critical concentration, F-actin stabilization, membrane ruffling for bacterial internalization	Zhou et al. (1999a, b), McGhie et al. (2001), Lhocine et al. (2008)
Ceg14	*Legionella pneumophilia*	Competition with sequestering proteins (profilin)?	Direct F-actin binding, inhibition of actin polymerization	Guo et al. (2014)
C3 toxins	e.g. *Clostridium botulinu,* C. limosum, *Staphylococcus aureus*	Direct modification of Rho GTPases (ADP-ribosylation prevents Rho GEF activation)	Inhibition of Rho interaction with its effectors, stress fibre depolymerization	Genth et al. (2003), Chardin et al. (1989)
SopE/E2	*Salmonella typhimurium*	Regulation of Rho GTPases (GEF for Cdc42, Rac1)	F-actin polymerization, membrane ruffling	Friebel et al. (2001), Hardt et al. (1998)
CadF (FlaA, FlaB)	*Campylobacter jejuni*	Modulation of Rho GTPase signaling (fibronectin binding, Cdc42 and Rac1 activation via integrin β1-FAK-Src-PI3K-Vav2 and FAK-DOCK180/Tiam-1)	Actin cytoskeleton rearrangements, membrane ruffling	Krause-Gruszczynska et al. (2011), Boehm et al. (2011), Eucker and Konkel (2012)

(continued)

Table 3 (continued)

Protein	Bacterium	Strategy (details and involved host proteins)	Effect on actin cytoskeleton	References
SteC	*Salmonella typhimurium*	Activation of kinase signaling pathways	F-actin remodelling around SCV	Poh et al. (2008), Odendall et al. (2012)
IpaC	*Shigella flexneri*	Recruitment of kinases (c-Src activates signal cascades for Rho GTPase activation, regulation of ABPs)	Actin reorganization, formation of filopodia and lamellipodia	Tran Van Nhieu et al. (1999), Mounier et al. (2009)
IpgD	*Shigella flexneri*	Modification of membrane lipid composition (dephosphorylates PIP_2 to PI(5)P)	Dissociation of actin cytoskeleton from plasma membrane, F-actin remodelling	Niebuhr et al. (2002), Mellouk et al. (2014)
SopB (SigD)	*Salmonella typhimurium*	Modification of membrane lipid composition (phosphatidylinositol 4- and 5-phosphatase)	Actin cytoskeleton rearrangements, membrane ruffling	Zhou et al. (2001), Terebiznik et al. (2002), Mallo et al. (2008)

Bacterial pathogens produce a huge variety of virulence factors that are able to target and to modify host proteins to interfere with or to inhibit cellular functions. Pathogens actively hijack the actin cytoskeleton to invade host cells, to move inside the host cytosol, to facilitate cell-to-cell spread, and to secure their survival by blocking phagocytosis. All these purposes require the controlled rearrangement of cellular actin structures. First, bacteria need to disrupt the membrane-associated, cortical actin cytoskeleton prior to cell invasion to access the host cytosol. For intracellular motility and cell-to-cell spread, pathogens secondly initiate actin filament nucleation and polymerization. Newly formed, free actin barbed ends (e.g. via the Arp2/3 complex) finally need to be elongated and bundled to generate the required pushing forces for the engulfment of pathogens and for intracellular pathogen motility. In order to secure their survival, bacteria often impede engulfment and destruction by professional phagocytotic host cells. Many bacteria have developed mechanism to block their engulfment by inhibiting actin reorganizations necessary for phagocytosis. Interestingly, most bacterial effectors do not directly interact with monomeric or filamentous actin but modulate host actin ABPs that regulate actin dynamics. A number of effects of bacterial virulence factors will be discussed in to more detail in later chapters.

4.1 Direct Interactions of Bacterial Effectors with Actin

4.1.1 Direct Modifications of G-actin

The intracellular abundance of actin and its essential role for many cellular functions have made it a preferred target for infectious agents, a fact that appears to be supported by its highly conserved sequence and structure. A number of bacteria have developed toxins that directly target actin (for review, see Aktories et al. 2011; see also Table 1 and Lang et al. 2016). Among these, two groups of toxins can be differentiated, which covalently modify actin. ADP-ribosyltransferases ADP-ribosylate actin at Arg177 (e.g. C2 toxin of *Clostridium botulinum*; Aktories et al. 1986) or Thr148 (e.g. TccC3 toxin of *Photorhabdus luminescens*; Lang et al. 2010) to induce the inhibition or promotion of actin polymerization, respectively, with the aim to secure their survival by inhibiting phagocytosis (see Lang et al. 2016).

A number of *clostridial bacteria* inject ADP-ribosyltransferases into host cells, which at least transiently form direct complexes with actin. The structure of the complex of G-actin with the ADP-ribosyltransferases from *Clostridium perfringens* (iota toxin) is shown in Fig. 4a (Tsuge et al. 2008; for further detail, see Tsuge et al. 2016). Actin Arg177-ADP-ribosylation introduces a bulgy side group in close proximity to the interstrand actin-actin interface and thereby interferes with the addition of a further actin subunit to the plus end (for further detail, see Schwan and

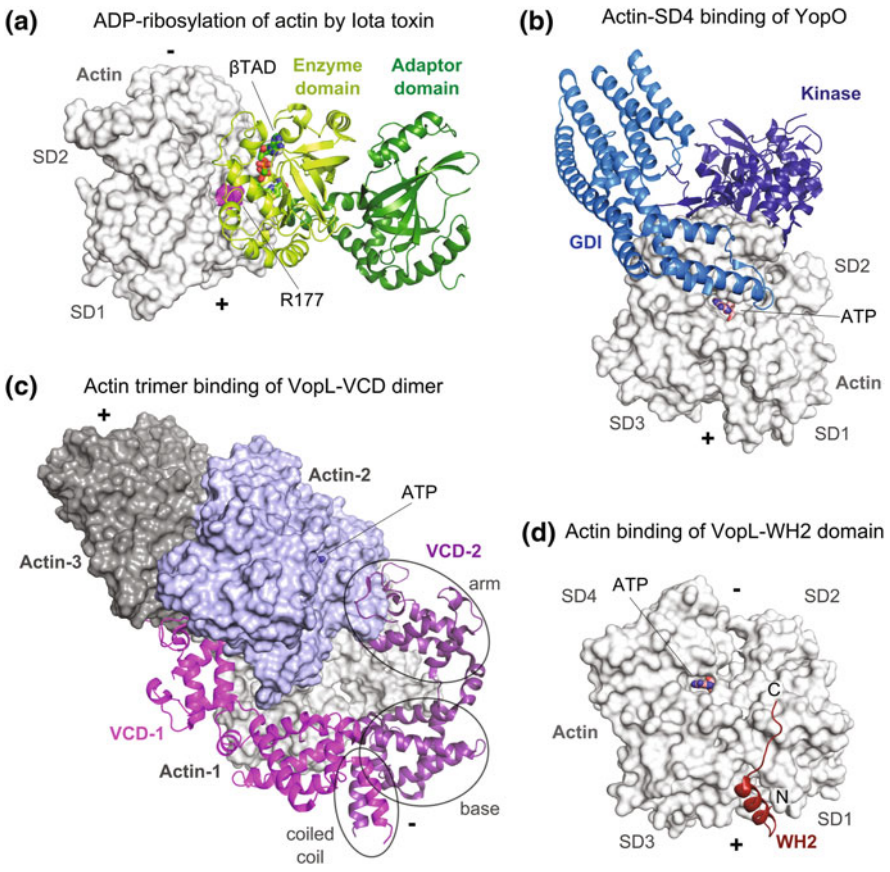

Fig. 4 Complex structures of actin with directly actin-binding bacterial proteins. Actin molecules are depicted as surface representation and bacterial proteins as ribbons, the actin subdomains (SD), pointed (−) and barbed (+) ends are indicated. **a** The ADP-ribosylating iota toxin consists of an N-terminal adaptor domain (*dark green*) with a C-terminal enzymatic domain (*light green*). The Arg177 residue of actin (R177) is highlighted in pink and in close proximity to βTAD (non-hydrolysable NAD analog) bound to the enzymatic iota domain (PDB: 3BUZ; Tsuge et al. 2008). The adaptor domain interacts with the binding domain (not shown) of the binary iota toxin. **b** Structure of the ABP-phosphorylating YopO effector in complex with G-actin. The front view on actin is shown with the YopO GDI domain (*GDI* guanine-nucleotide dissociation inhibitor) in *light blue* and the kinase domain in *dark blue* (PDB: 4CI6; Lee et al. 2015). Both, the GDI and the kinase domain interact with the pointed face of actin-encircling subdomain 4 (SD4). **c**–**d** The bacterial effector VopL comprises two different actin-binding domains: the VCD (VopL C-terminal domain; **c**), and three WH2 domains (WASP homology 2; **d**). VopL dimerizes with the coiled coil in the VCD (VCD-1: chain A, *pink*, VCD-2: chain B, *purple*). In addition, each VCD consists of arm and base regions, which interact with actin. The VCD-actin complex has been crystallized with three actin protomers in F-actin-similar conformation [strand 1: actin-1 and actin-3, strand 2: actin-2 (*light blue*)]. Note that the WH2 domain-binding sites at the barbed faces of actin protomers 2 and 3 are not occupied by the VCD dimer and would allow WH2 domain binding (PDB: 4M63; Yu et al. 2011; Zahm et al. 2013). **d** The cross-linked VopL-WH2-actin complex was crystallized with the first (residues 130–160) of the three WH2 domains of VopL (PDB: 3M1F; Rebowski et al. 2010). The WH2 domain of VopL (*red*) adopts the typical fold of WH2 domains as found, for instance, in WASP and thymosin ß4 and binds identically with its amphipathic helix to the hydrophobic ligand-binding cleft between SD1 and SD3. N- and C-termini are indicated

Aktories 2016; Lang et al. 2016). In contrast, Thr148 ADP-ribosylation interferes with the binding of ABPs, which sever F-actin or stabilize its monomeric form. Thr148 is located between subdomains 1 and 3 representing a major target zone for a number of actin-binding proteins (see Lang et al. 2016).

A different covalent modification of actin leading to interference with its normal cycling is achieved by bacterial toxins, which cross-link actin molecules (e.g. RtxA of *Vibrio cholerae* (Fullner and Mekalanos 2000). These toxins catalyse the cross-linking of actin to dimers and larger oligomers, which are unable to polymerize and thereby interfere with the dynamic behaviour of the actin cytoskeleton (see Kudryashova et al. 2016).

4.1.2 F-actin Dynamics Modifying Bacterial Proteins

During the last years, an increasing number of bacterial effectors has been identified that modify actin filament dynamics (Table 2). These pathogenic factors mimic host nucleation or elongation factors and hold similar structural and functional motifs or follow akin strategies. Some bacteria mimic WH2 domain-containing nucleation factors like the F-actin nucleator TARP (*Chlamydia spp.*), which binds directly with its WH2-like domain to G-actin to initiate new actin filaments upon oligomerization (Jewett et al. 2006). In addition, TARP is able to indirectly activate F-actin nucleation by the Rac1-WAVE-Arp2/3 pathway (Lane et al. 2008) and to bundle actin filaments (Jiwani et al. 2013).

Another group of bacterial effectors mimic host Ena/VASP F-actin-tracking proteins like BimA of *Burkholderia* pathogens (*Burkholderia pseudomallei, B. mallei*). BimA utilizes WH2 motifs and poly-proline-rich regions to nucleate, elongate, and bundle actin filaments (Sitthidet et al. 2011; Benanti et al. 2015). A further example for Ena/VASP mimics are the closely related, dimeric pathogen effectors VopF and VopL (*Vibrio cholera* and *Vibrio parahaemolyticus*). Both contain multiple WH2 domains with actin filament nucleation activity and poly-proline-rich sequences for profilin-actin binding (see Fig. 4c, d for more details; Dziejman et al. 2005; Liverman et al. 2007; Yu et al. 2011). VopF tracks F-actin barbed ends for processive filament elongation and competes with capping proteins to uncap pre-existing filaments (Pernier et al. 2013) like Ena/VASP or formin proteins (Shekhar et al. 2015). SAXS studies of actin-sequestering, dimeric VopF constructs seem to support the role of VopF as barbed end tracker (Avvaru et al. 2015). By contrast, a pointed end nucleation model has been proposed for VopL based on the complex structure of the dimeric VopL-VCD domain with an actin trimer (Fig. 4c; Zahm et al. 2013).

The closest bacterial mimic of a formin described so far is the Sca2 effector of *Rickettsia conorii*. Sca2 possesses strong formin-like F-actin assembly properties and imitates with its N- and C-terminal domains the filament barbed end elongating, dimeric FH2 domains of formins. It possesses, in addition, a profilin-binding FH1 domain for the recruitment of profilin-actin and an actin-binding WH2 domain (Haglund et al. 2010; Madasu et al. 2013).

Conversely, *Salmonella spp.* inject into host cells among many other effectors SipA (invasion-promoting toxin), which promotes actin polymerization and stabilizes existing actin filaments at the cytoplasmic face of the plasma membrane underneath the adhesion site of the bacterium. SipA was shown to clamp two actin subunits from opposing strands leading to localized F-actin stabilization that induces membrane ruffles to internalize the adherent bacterium (Lilac et al. 2003).

A completely different mode of direct actin interaction undergoes the bacterial effector YopO (or YpkA) after injection into host cells by *Yersinia enterocolitica.* Binding of YopO to G-actin had been shown to stimulate the YopO protein kinase activity, which is essential for inhibiting phagocytosis (Trasak et al. 2007). The 3D structure of the 1:1 YopO-actin complex has been solved (Lee et al. 2015). YopO consists of three main domains: a membrane binding, a protein kinase, and a Rho GTPase-binding domain that inhibits guanine-nucleotide dissociation. The kinase and Rho GTPase-binding domains wrap around SD4 of preferentially cytoplasmic G-actin, preventing its polymerization (Fig. 4b). Activated YopO kinase phosphorylates ABPs that interact with the available binding area between SD1 and SD3 (Lee et al. 2015; see also Aepfelbacher and Wolters 2016). Among these ABPs are polymerization-promoting proteins like formins and VASP, and NPFs like WASP, but also F-actin-fragmenting factors such as gelsolin and cofilin. Thus, YopO uses the bound G-actin as bait for proteins involved in phagocytosis, which are subsequently inactivated by YopO (Lee et al. 2015).

4.2 Manipulation of Actin-Binding Proteins by Bacterial Effectors

4.2.1 Recruitment and Regulation of the Host F-actin Nucleation Machinery

Besides mimicking F-actin nucleation function, many pathogens either directly or indirectly target the Arp2/3 complex, the major host actin nucleator (see Table 3). *Listeria* directly recruits the Arp2/3 complex with ActA, an N-WASP-mimicking bacterial effector (Domann et al. 1992; Kocks et al. 1992; Gouin et al. 2005; see also Pillich et al. 2016), to induce F-actin nucleation for actin-based motility in the host cytosol. *Shigella* on the other hand injects the bacterial adaptor IcsA for indirect Arp2/3 complex activation via N-WASP recruitment (Goldberg et al. 1993; Cossart 2000; Suzuki et al. 2002). Other pathogens like *Rickettsia conorii* and *B. thailandensis* mimic NPFs (RickA, BimA) (Gouin et al. 2004, 2005; Welch and Way 2013; Benanti et al. 2015; Sitthidet et al. 2010), while EHEC and EPEC activate N-WASP (TccP, EspF) (Alto et al. 2007; Peralta-Ramírez et al. 2008; Campellone et al. 2004; Cheng et al. 2008) (see Stradal and Costa 2016).

A further, more indirect strategy is the recruitment of membrane-associated scaffold proteins that provide a localization and activation platform for the host F-actin nucleation machinery. A prominent example is the EPEC effector Tir, which induces the recruitment and activation of N-WASP-binding and activating proteins like Nck, IQGAP1, and IRSp53 (Weiss et al. 2009; Campellone 2010; Campellone et al. 2002; Gruenheid et al. 2007; Brown et al. 2008; de Groot et al. 2011; see also Stradal and Costa 2016). Another method to position the Arp2/3 complex is obtained by the recruitment of vinculin, which directly interacts with F-actin and Arp2/3. Virulence factors of several bacteria were found to utilize vinculin for actin cytoskeleton rearrangements including *Rickettsia* (Sca4), *Chlamydia* (TARP), EHEC/EPEC (Tir-talin), and *Shigella flexneri* (IpaA).

4.2.2 Interactions of Bacterial Effectors with Actin-Binding Proteins (ABPs)

Bacterial pathogens have developed effectors that not only manipulate actin filament nucleation and elongation, but also differently affect cellular actin dynamics by hijacking host F-actin capping, bundling, cross-linking, severing, or sequestering proteins. It was recently shown that the alteration of the activity of the F-actin barbed end capping, bundling, and filament severing protein villin by SptP is required for the invasion of *Salmonella* (Lhocine et al. 2015). Annexin 2, a cross-linking protein that connects membrane-bound protein complexes with the actin cytoskeleton, is stimulated by the EHEC/EPEC factor EspL to form pseudopod-like structures (Miyahara et al. 2009). *Heliobacter*'s CagA on the other hand hijacks host signalling pathways to regulate F-actin/plasma membrane cross-linkers like vinculin, ezrin, and cortactin (Tegtmeyer and Backert 2011; Rieder et al. 2005; Stein et al. 2002). Host severing proteins (ADF/cofilin, gelsolin) disrupt actin filaments and release G-actin required for the polymerization of new filaments. The *Salmonella* effector SipA has been found to antagonize with severing proteins for direct F-actin binding and to protect filament ends from severing and depolymerization (Zhou et al. 1999a, b; McGhie et al. 2001; Lhocine et al. 2015). The recently identified Ceg14 effector (*Legionella pneumophila*) might compete with the G-actin-sequestering profilin and directly bind F-actin, which could lead to the inhibition of actin polymerization (Guo et al. 2014).

4.2.3 Manipulation of Host ABP Regulation: Rho GTPases, Kinases, and Phospholipids as Bacterial Targets

In addition to direct interaction with host ABPs, pathogens have developed numerous strategies to upstream manipulate their activity and spatial localization.

Many actin-binding proteins are downstream effectors of Rho GTPases. To modulate the host actin cytoskeleton, bacterial pathogens effectively target Rho GTPase signalling at various stages leading to the activation or inhibition of

GTPase function (Aktories 2001). Altered Rho GTPase activity or availability interferes with downstream Rho GTPase effector proteins and the corresponding signalling pathways responsible for actin cytoskeleton rearrangements. Bacterial toxins usually effectuate Rho GTPase targeting via three different strategies. First, they directly catalyse enzymatic post-translational modifications to block GTPases in their active or inactive states (e.g. ADP-ribosylation of RhoA by the C3 toxin of *C. botulinum*; Aktories 2001; Genth et al. 2003; Chardin et al. 1989). Other bacterial factors manipulate or mimic host regulators of Rho GTPase activity, like the RhoGEF-mimicking virulence factors SopE/E2 (*Salmonella typhimurium*) (Hardt et al. 1998; Friebel et al. 2001). The third strategy is to modulate host Rho GTPase signalling pathways by the stimulation of cell surface receptors (e.g. CadF of *Campylobacter jejuni*; Krause-Gruszczynska et al. 2011; Boehm et al. 2011; Eucker and Konkel 2012).

Several bacterial pathogens interfere with host kinases to indirectly manipulate signalling cascades of the actin cytoskeleton. The *Salmonella* kinase SteC, for example, promotes the formation of an F-actin meshwork by the activation of a MEK/ERK/Myosin IIB signalling pathway (Poh et al. 2008; Odendall et al. 2012), while the *Shigella* virulence factor IpaC localizes the Src kinase at bacterial entry sites, which regulates the activity of several ABPs (Tran Van Nhieu et al. 1999; Mounier et al. 2009).

Another strategy to induce F-actin clearance beneath the plasma membrane is to modify the lipid composition of host membranes by hijacking phosphatidylinositol phosphate metabolism pathways. For example, the *Shigella* phosphatase IpgD facilitates bacterial escape into the cytosol (Niebuhr et al. 2002; Mellouk et al. 2014), while the inositol polyphosphatase SopB of *Salmonella* enables bacterial uptake into host cells (Zhou et al. 2001; Terebiznik et al. 2002; Mallo et al. 2008).

Finally, some bacteria induce actin rearrangements by the recruitment of membrane-associated host scaffold proteins that provide a localization and activation platform for ABPs (as described in Sect. 4.2.2).

5 Conclusions

Due to its high abundance in eukaryotic cells and its conserved structure, actin has become a preferred target of bacterial toxins, which either directly modify actin or mimic or modulate actin-binding proteins, which regulate its supramolecular organization. In addition, a number of bacterial toxins modify signalling cascades that regulate actin cytoskeleton dynamics. Thus, bacteria hijack and divert actin functions for host cell entry, their survival, their transport within the host cell cytoplasm, and intercellular spread. Here, we presented the actin structure, its organization into higher organizational structures, and its basic dynamic behaviour, which might all become modified by the many different bacterial toxins and effectors.

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Formation of Nanotube-Like Protrusions, Regulation of Septin Organization and Re-guidance of Vesicle Traffic by Depolymerization of the Actin Cytoskeleton Induced by Binary Bacterial Protein Toxins

Carsten Schwan and Klaus Aktories

Abstract A large group of bacterial protein toxins, including binary ADP-ribosylating toxins, modify actin at arginine-177, thereby actin polymerization is blocked and the actin cytoskeleton is redistributed. Modulation of actin functions largely affects other components of the cytoskeleton, especially microtubules and septins. Here, recent findings about the functional interconnections of the actin cytoskeleton with microtubules and septins, affected by bacterial toxins, are reviewed.

Contents

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1 Introduction

Actin is an essential protein that plays a central role in numerous cellular processes. It is an abundant, highly conserved protein that is expressed in most cell types in a very large amount (Blanchoin et al. 2014; Dominguez and Holmes 2011). Actin dynamics are regulated by numerous proteins, which play pivotal roles in actin-based functions such as migration, phagocytosis, secretion, adhesion, cell morphology, cell contact, intracellular traffic, cytokinesis and gene regulation (Pollard and Cooper 2009; Dominguez and Holmes 2011; Pollard et al. 1994; Lanzetti 2007; Posern and Treisman 2006; Castellano et al. 2001). Actin is also substrate of numerous posttranslational modifications including methylation, phosphorylation, glycosylation, ubiquitination and ADP-ribosylation (Terman and Kashina 2013). While many of the aforementioned modifications occur catalyzed by endogenous enzymes, modification of actin is also typical for some groups of bacterial protein toxins. Especially ADP-ribosylation of actin is observed with various bacterial protein toxins (Aktories et al. 2011). Modification of arginine-177 was the first toxin-induced covalent modification of actin identified (Aktories et al. 1986b). This modification causes depolymerization of F-actin and blocks actin polymerization. Actin is also ADP-ribosylated at threonine-148, a modification, which causes the opposite effect (Lang et al. 2010). It induces polymerization of actin. This modification by *Photorhabdus* toxins will be discussed in detail in other chapters of this volume. In addition, actin is modified by toxin-induced cross-linking (Satchell 2009; Sheahan et al. 2004). Here, we will briefly review the action of ADP-ribosylation of actin at arginine-177 and will focus on recent findings on the functional consequences of the depolymerization of F-actin on the reorganization of microtubules and septins and the formation of microtubule-based cell protrusions. Moreover, we will discuss recent findings about the consequences of F-actin depolymerization on polarized traffic in epithelial cells.

2 Actin-Depolymerizing Toxins

ADP-ribosylation of actin was first identified with *Clostridium botulinum* C2 toxin (Aktories et al. 1986a, b; Ohishi and Tsuyama 1986). This toxin is normally produced by *C. botulinum* type C and D strains. Both strains are now classified as *C. botulinum* group III type strains. C2 toxin is often produced in parallel with botulinum neurotoxins C (BoNt/C) or D (BoNt/D). Moreover, *C. botulinum* group III type strains often produce in addition to neurotoxins and C2 toxin, the ADP-ribosylating toxin C3, which modifies Rho proteins (Aktories et al. 1987; Aktories and Frevert 1987). Highly related to C2 toxin are toxins from the iota toxin family, which modify actin like C2 toxin (Stiles and Wilkens 1986; Schering et al. 1988; Barth et al. 2004; Stiles et al. 2014; Sakurai et al. 2009). Members of this group are *C. perfringens* iota toxin, *C. spiroforme* toxin and *C. difficile* transferase

CDT (Aktories and Barth 2004; Aktories et al. 2011). Moreover, a novel *C. perfringens* toxin has been described, which shares high similarity with the iota toxin family (Irikura et al. 2015). Also *Bacillus cereus* produces an actin-ADP-ribosylating toxin, termed vegetative insecticidal protein (VIP), belonging to this family (Han et al. 1999). A common thing in all these toxins is that they consist of a binary structure, which means that the binding components and the enzyme components of the toxins are separated proteins (Ohishi et al. 1980; Stiles and Wilkins 1986; Barth et al. 2004; Aktories et al. 1992). This structure of the toxins will be described briefly below. However, actin-modifying toxins are also produced in form of single chain effectors, including *Salmonella Typhimurium* effector SpvB (Otto et al. 2000; Lesnick et al. 2001), *Aeromonas salmonicida/hydrophila* toxin AexT (Fehr et al. 2007; Vilches et al. 2008), *Photorhabdus luminescens toxin* Photox (Visschedyk et al. 2010) and *Aeromonas hydrophila* effector VgrG1 (Suarez et al. 2010). While the binary toxins are able to enter cells by an inbuilt machinery, the single chain toxins are introduced into target cells by type III (e.g., SpvB and AexT) or type VI secretion systems (e.g., VgrG1) and, thus, depend on the direct contact of bacteria with target cells (Gotoh et al. 2003).

2.1 Structure of Binary Toxins

A large group of actin-ADP-ribosylating toxins are binary in structure. This means, they consist of two separate toxin components. One component possesses the biological activity, that modifies actin. The other component is involved in the binding of the toxins to the target cells membrane and the translocation of the enzyme component into the cytosol of host cells. The binding components of all binary toxins are highly similar to the binding component of anthrax toxin, the protective antigen (PA) (Young and Collier 2007). Similar to PA, they form heptamers (whether they can also form octamers like PA is not clear) (Blöcker et al. 2003). In each case, they consist of four domains involved in activation (domain I), oligomerization (domain II), channel formation (domain III) and receptor binding (domain IV) (Barth et al. 2004; Stiles et al. 2014). While domains I–III are highly related between all binary toxins, the binding domain IV is largely different between iota-like toxins and the other toxins. Activation of the binding component occurs by proteolytic cleavage of the N-terminal domain and release of a $\sim$20 kDa fragment. This cleavage allows oligomerization and formation of heptamers. This process might occur in solution or on the surface of target cells after receptor binding.

The enzyme component reveals typical features of an ADP-ribosyltransferase. However, all these enzyme components consist of a doublet of an ADP-ribosyltransferase structure, which has evolved by gene duplication (Han et al. 1999; Schleberger et al. 2006; Margarit et al. 2006; Sakurai et al. 2009; Tsuge et al. 2003). Actually, the N-terminal part of this toxin domain represents an

ADP-ribosyltransferase folding without enzyme activity. This part is mainly involved in interaction with the binding component. In contrast, the C-terminal part represents the catalytic domain, which is able to modify actin.

2.2 Receptors and Uptake

The interaction of the binding components of the binary toxins with their cell receptors is a prerequisite for cytotoxicity (Fig. 1). Quite early it was shown that C2II binds to hybrid and complex carbohydrates on the surface of target cells (Eckhardt et al. 2000). In addition, specific binding proteins might be involved. The receptor for *C. difficile* toxin CDT, iota toxin, and *C. spiroforme* toxin CST has been identified as the lipolysis-stimulated lipoprotein receptor LSR (Papatheodorou et al. 2011, 2012). LSR is a single path membrane protein with a long extracellular part, consisting of an Ig-like V-type domain in the N-terminal part and a cysteine-rich intracellular part (Schmidt et al. 2015). As the name suggest, the LSR receptor protein appears to be involved in lipid metabolism and may play a role in uptake of chylomicrons (Bihain and Yen 1998; Mann et al. 1995; Yen et al. 1994). Moreover, LSR is essential for the organization of tricellular tight junctions that are involved in epithelial barrier function (Furuse et al. 2012; Masuda et al. 2011) and appears to play a major role in tumor development and metastasis (Shimada et al. 2016; Herbsleb et al. 2008).

After binding of the heptameric binding component to its specific receptor, the enzyme component can attach and, thereafter, the whole complex is endocytosed. In low-pH endosomes, the binding component inserts into the vesicle membrane by forming a beta barrel pore, through which the enzyme component is translocated into the cytosol of target cells (Barth et al. 2000; Blöcker et al. 2003; Nagahama et al. 2012, 2014). Translocation depends on chaperones, including HSP90 (Haug et al. 2003), cyclophilins (Kaiser et al. 2009) and others (Kaiser et al. 2012).

3 Modification of Actin by ADP-Ribosylating Toxins

Using NAD^+ as a second substrate, all above-mentioned binary actin-ADP-ribosylating toxins attach ADP-ribose onto actin at arginine-177 (Vandekerckhove et al. 1987, 1988; Gülke et al. 2001). In addition, also single chain toxins (effectors) like *Salmonella* ssp. protein SpvB modify actin at arginine-177 (Hochmann et al. 2006). Although the overall structure of actin is only minimally affected by ADP-ribosylation (Margarit et al. 2006), the toxin-induced attachment of ADP-ribose has major consequences for the polymerization of actin. ADP-ribosylated actin is no longer able to polymerize (Aktories et al. 1986b; Schering et al. 1988; Ohishi and Tsuyama 1986), because the bulky ADP-ribose at this position is not compatible with the structure of the double helix formed by

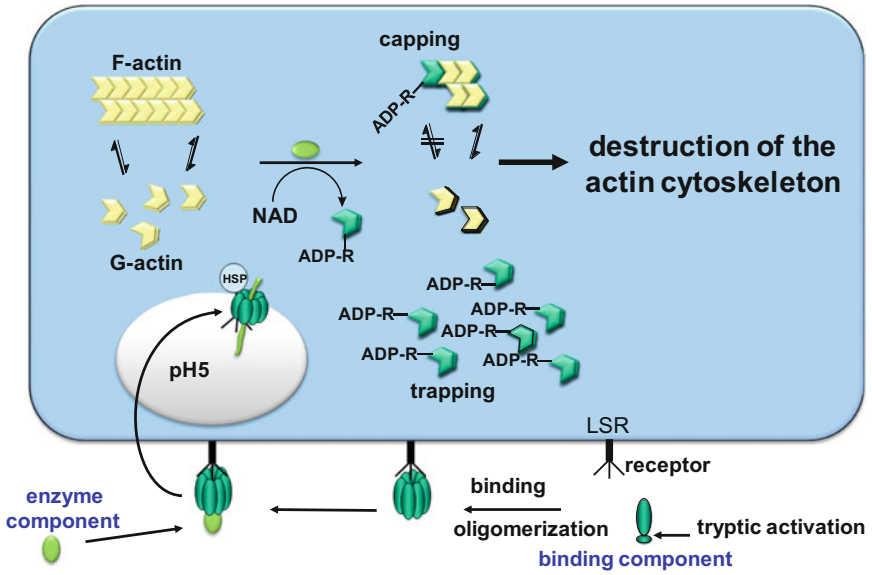

Fig. 1 Actions of binary actin-ADP-ribosylating toxins. The binary actin-ADP-ribosylating toxins (e.g., *C. difficile* toxin CDT or *C. botulinum* C2 toxin) consist of an enzyme component and a binding component, which is activated by proteolytic cleavage. The activated binding components form heptamers and interact with their respective cell membrane receptors [e.g., CDT interacts with lipolysis-stimulated lipoprotein receptor (LSR)]. The toxin–receptor complexes are endocytosed. In low pH endosomes, the binding components undergo conformational changes and insert into membranes to form pores. Through the pores, the enzyme components are translocated into the cytosol. This occurs with the help of chaperones (e.g., HSP90). In the cytosol, the enzyme components of the binary toxins ADP-ribosylate monomeric G-actin. ADP-ribosylated actin is not able to polymerize and trapped in its monomeric form. ADP-ribosylated actin can act as a barbed-end capping protein to inhibit polymerization of non-modified actin. Thereby, the actin cytoskeleton is relocalized and, eventually, depolymerizes

polymerized F-actin (Holmes et al. 1990; Margarit et al. 2006). The only position, where this group can be accommodated, is the barbed end of actin filaments (Wegner and Aktories 1988; Aktories and Wegner 1989). Here, the attachment of ADP-ribose turns the modified actin molecule into a capping protein, which inhibits further binding at this side. In contrast, the pointed end, which favors actin depolymerization, is not affected. Thus, the capping protein-like function inhibits polymerization of still unmodified actin molecules. Moreover, ADP-ribosylated actin changes further properties of the microfilament protein. For example, the modification blocks the ATP hydrolysis by actin, which is especially well observed in the presence of cytochalasin (Geipel et al. 1989, 1990; Margarit et al. 2006). Moreover, the release rate of phosphate from actin is decreased after ADP-ribosylation (Margarit et al. 2006). Also, the interaction of ADP-ribosylated actin with actin-binding proteins is affected. For example, ADP-ribosylation has consequences for the nucleation activity of the gelsolin–actin complex. However, this is only observed when the weakly bound actin of the gelsolin–actin–actin

complex (G-A-A_R) is modified (Wille et al. 1992). Eventually, inhibition of polymerization (e.g., trapping of monomeric ADP-ribosylated actin and capping of F-actin) causes destruction of the actin cytoskeleton and rounding up of cells.

4 Cellular Consequences of the ADP-Ribosylation of Actin in Arginine-177

The functional consequences of the ADP-ribosylation of arginine-177 of actin have been studied in various cells types. Mainly *C. botulinum* C2 toxin was employed for these experiments. By using rather high toxin concentrations, a typical effect is the rounding up of cells under cell culture conditions (Wiegers et al. 1991). Motility, migration and contraction is largely decreased by C2 toxin (Norgauer et al. 1988; Verschueren et al. 1995). The actin-depolymerizing toxins have been used as pharmacological tools to study the role of the actin cytoskeleton in secretory processes, showing that C2 toxin has a bidirectional effect on the release of catecholamines, and time-dependently increases or inhibits the release of noradrenaline by affecting the actin cytoskeleton (Matter et al. 1989). In adrenal Y-1 cells, C2 toxin increases steroid release in a cAMP-independent manner (Considine et al. 1992), while ADP-ribosylation of actin inhibits stimulated release of insulin from HIT-T15 cells or pancreatic islands (Li et al. 1994). These differences most likely depend on the type of secretion, and how and whether vesicles have excess to exocytotic sites (Trifaro et al. 2008). ADP-ribosylation of actin increases the superoxide production (Norgauer et al. 1988). However, recently it was shown that the toxin-induced depolymerization of actin has major functional consequences on other components of the cytoskeleton namely on microtubules and on septins.

4.1 Effects of Actin-Depolymerizing Toxins on Microtubules

Microtubules are filaments, which consist of α- and β-tubulin heterodimers. As known for actin filaments, microtubules are dynamic structures undergoing treadmilling (Waterman-Storer and Salmon 1997). Accordingly, similar to the barbed (plus) and pointed (minus) ends of actin filaments, microtubules are polarized structures, possessing a rapidly growing plus end and a slowly growing minus end (Desai and Mitchison 1997; Waterman-Storer and Salmon 1997; Nogales 2000). The minus ends of microtubules are generally oriented to and stabilized by the microtubule organization center, which is in most cells the centrosome. The plus ends are often directed to the cell membrane, exhibiting high functional dynamics called dynamic instability. Various microtubule-binding proteins are involved in regulation of the dynamic instability. The plus end binding proteins 1 (EB1) or the cytoplasmic linker protein 170 (CLIP170) are so-called *TIPs* that regulate microtubule growth at the plus ends. Interaction of microtubules with the cortical actin

lattice causes growth inhibition and stabilization of microtubules. Here, capture proteins like CLIP-associated protein (CLASP2) and actin-cross-linking-family 7 protein (ACF7) are involved, which bind both to actin and to microtubules (Kodama et al. 2003; Mimori-Kiyosue et al. 2005). When actin is ADP-ribosylated in arginine-177 and F-actin depolymerization is induced, the regulation of microtubule dynamics is significantly affected.

It has been shown that C2 toxin, iota toxin and CDT are able to induce an increase in microtubule growth (Uematsu et al. 2007; Schwan et al. 2009). Moreover, growing microtubules that contact the cell membrane form protrusions, which are filled with microtubules but not with F-actin (Fig. 2). These cell protrusions usually have a diameter of 0.5–1 µm and can reach a length of more than 100 µm. The structures are dynamic and can grow and retract (Schwan et al. 2009).

Long cellular protrusions are a typical feature of filopodia (Blanchoin et al. 2014; Ahmed et al. 2010; Leijnse et al. 2015). Also cytonemes and the various types of *tunneling nanotubes* (TNT) are characterized by long extensions, which appear to be involved in various cellular functions, including cell–cell contact, cargo traffic between cells and cell–cell signaling (Buszczak et al. 2016; Onfelt et al. 2004; Austefjord et al. 2014; Sisakhtnezhad and Khosravi 2015; Fairchild and Barna 2014). However, filopodia and also TNT depend essentially on actin microfilaments and depolymerization of F-actin inhibits formation of filopodia and of TNT. By contrast, the protrusions formed by toxins that cause ADP-ribosylation of actin in arginine-177 depend on the depolymerization of F-actin. Moreover, also latrunculin B and cytochalasin can induce long cell protrusions that exclusively are based on microtubules. Interestingly, in tumor metastasis, cells have been described that posses *cell tentacles* (Matrone et al. 2010; Vitolo et al. 2013), which appear to match the structural features of toxin-induced microtubule-based protrusions.

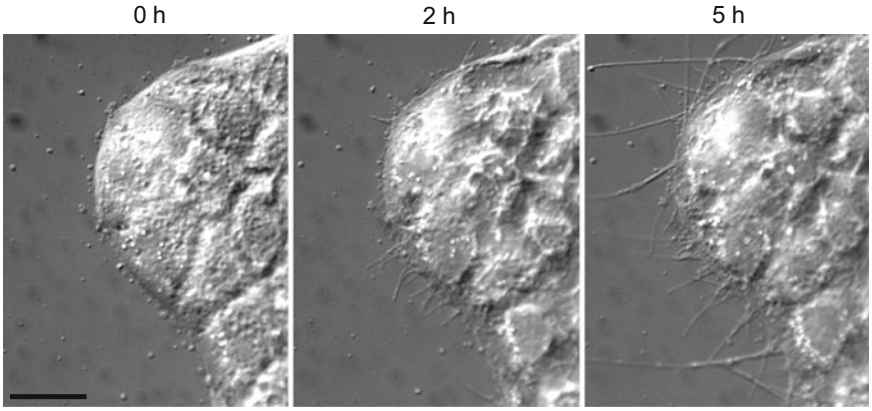

Fig. 2 Formation of microtubule-based cell protrusions induced by ADP-ribosylation of actin. Human adenocarcinoma Caco-2 cells were treated with *C. difficile* toxin CDT (CDTa 20 ng/ml and CDTb 40 ng/ml) for the indicated times. Differential interference contrast microscopy images show protrusion formation over time. *Scale bar* represents 20 µm

Notably, also motile and primary cilia, which are formed on each cell, are based on microtubules and do not contain F-actin (Pedersen et al. 2012; Malicki and Avidor-Reiss 2014). However, the toxin-induced protrusions are generally much longer than cilia and do not share the complex structure of cilia.

4.2 *Mechanisms Involved in Protrusion Formation: A Role for Septins*

Microtubule-based protrusions, which are formed by actin ADP-ribosylation of arginine-177, are well studied in epithelial cells especially human colon adenocarcinoma cells (CaCo-2) (Schwan et al. 2009, 2011, 2014). The protrusions are formed in highly polarized CaCo-2 cells and they are observed in primary mouse intestinal wild-type cells. Protrusions, formed by toxin-induced depolymerization of actin, depend on the growth of microtubules at plus ends. This can be followed by monitoring the dynamics and migration of GFP-labeled EB1 at the top of growing microtubules. Interestingly, Clasp-2 and ACF-7 proteins, which normally are involved in microtubule capture, appear to be redistributed from the actin cortex into the cytosolic fraction after partial depolymerization of actin (Schwan et al. 2009). Although the precise mechanism of protrusion formation is still enigmatic, recent data suggest that septin play a crucial role.

Septins are suggested to be the fourth structural component of the cytoskeleton besides microfilaments, microtubules and intermediate filaments (Mostowy and Cossart 2012). Septins are conserved from yeast to humans. The human genome contains 13 septin genes. Septin proteins can be grouped into four major families which are represented by SEPT1, SEPT3, SEPT6 and SEPT7 (Beise and Trimble 2011). Almost all septins possess a GTPase domain, which is related to other P-loop nucleotide triphosphatases, including Ras proteins (Leipe et al. 2002; Sirajuddin et al. 2007). In addition, they contain a specific domain, which is called SUE (septin unique element). Septin forms hetero-oligomers of 8 septin molecules, which, unlike actin and tubulin monomers, are associated in a unipolar manner, where the GTPase domains associate to form the G–G interface and the N/C terminal parts interact to compose a NC–NC interface. The octamers can form long filaments, bundles and rings. Coiled-coil structures at the C-terminus appear to have finger like projection, which are perpendicular to the septin filaments and might be involved in protein–protein interactions. Septins bind to actin and also to microtubules (Weirich et al. 2008). Formation of higher septin structures appears to be regulated by GTP-binding proteins of the Rho family especially Cdc42.

When cells are intoxicated by binary toxins, partial depolymerization of actin causes redistribution of septins, which are usually associated with cytosol-located actin filaments, to the membrane. There, septins form ring-, chevron- and funnel-like structures that appear to be contact points for microtubule plus ends (Nölke et al. 2016) (Figs. 3 and 4). As shown recently, the time course of septin accumulation and protrusion formation induced by actin-depolymerizing toxins

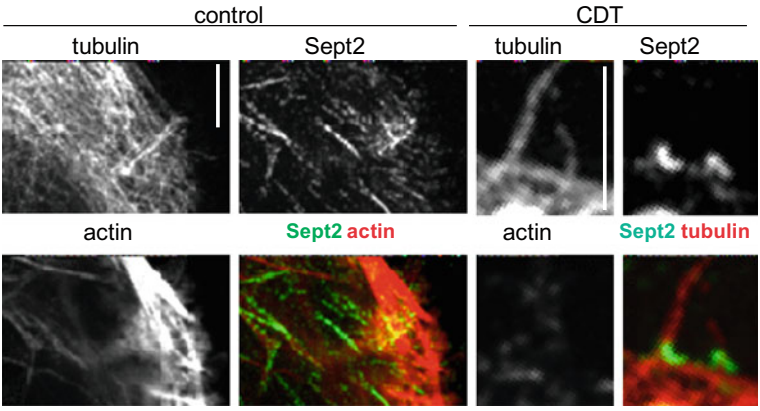

Fig. 3 Septins are involved in toxin-induced protrusions formation. Human adenocarcinoma Caco-2 cells were treated with CDT (CDTa 200 ng/ml and CDTb 400 ng/ml) for 1.5 h. Cells were stained by immunofluorescence for tubulin and septin2. Additionally, F-actin was stained by phalloidin-Atto 568. After CDT-treatment, F-actin is depolymerized and protrusions form at sites of reduced cortical actin. Protrusions have septin2 chevron structures at their base. Scale bar represents 5 μm

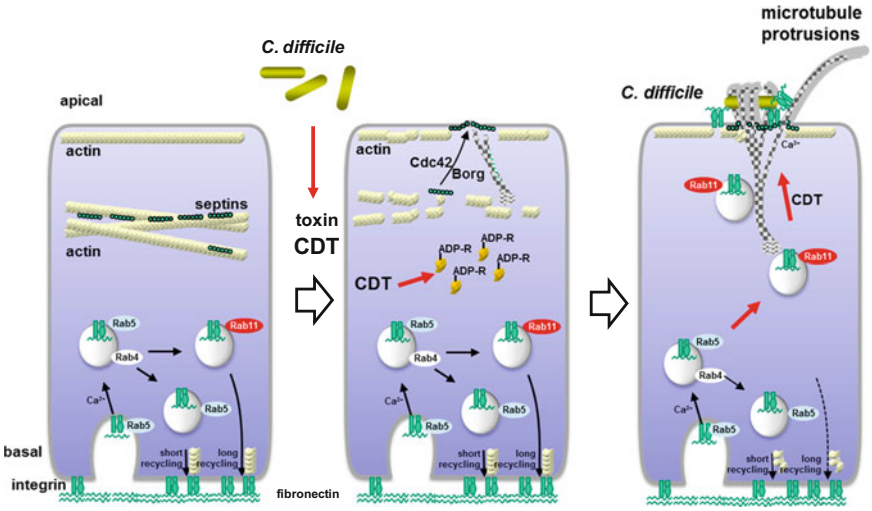

Fig. 4 Model of the formation and function of microtubule-based cell protrusions induced by actin-depolymerizing toxins. *Right panel* "Short" and "long" recycling processes at the basal side of an epithelial cell is shown. The recycling occurs with Rab5- (short recycling) and Rab11- (long recycling) associated vesicles. Septins are mainly localized in the cytosol associated with F-actin. *Middle panel* The binary *C. difficile* toxin CDT enters cells and ADP-ribosylates actin at arginine-177. Thereby, the actin cytoskeleton is partially depolymerized. Septins are released from F-actin and are translocated by means of the small GTPase Cdc42 and its effector Borg to membranes, where the cortical F-actin at the apical side is diminished. At the plasma membrane, septins accumulate in chevron-like structures, interact with microtubules and participate in the formation of microtubule-based protrusions. *Right panel* Depolymerization of F-actin by CDT causes re-guiding of vesicles from the basal membrane to the apical side. The microtubule-based protrusions are involved in interactions with bacteria and increase bacterial adherence

suggest that septins start the restructuring of the membrane. The chevron- or funnel-like structures formed by septin guide microtubules to the membrane and might be essential for protrusion formation, because knockdown of septins largely reduces protrusion formation (Noelke et al. 2016). Similarly, forchlorfenuron, an inhibitor of septin dynamics also blocks protrusion formation. Notably, similar chevron-like structures are observed in neuronal cells at locations where neurite- and axon-branching is observed (Hu et al. 2012). However, neurite-branching mainly depends on actin filaments, whereas (as already mentioned) F-actin is missing in toxin-induced protrusions. EB1 or EB3 proteins apparently regulate the initial interactions of microtubules with septins. These proteins, which control microtubule growth at the plus ends, interact with septins with affinities in the nanomolar range (Nölke et al. 2016). Important questions remain: What are the mechanisms that underlay septin accumulation at the cell membrane caused by depolymerization of F-actin? What defines membrane sites for septin accumulation? Recent studies provided some insights into the regulation of septins at the cell membrane. Septin accumulation at the cell membrane is apparently regulated by the small Rho GTPase Cdc42 and its effector Borg1-5 (Cdc42EP/CEP) (Joberty et al. 2001; Sheffield et al. 2003; Joberty et al. 1999). The current model, suggests that active GTP-bound Cdc42 is involved in trafficking septins to the membrane (Sadian et al. 2013). Fluorescence microscopic studies with a sensor for Cdc42 activation revealed active Cdc42 and Borg co-localization at sites of septin accumulation. Free cycling between the GTP and GDP forms of Cdc42 appears to be necessary for proper septin organization, because activation as well as inhibition of Cdc42 blocks septin accumulation and eventually protrusion formation (Nölke et al. 2016).

4.3 Role and Functions of Toxin-Induced Cell Protrusions

Depolymerization of the actin cytoskeleton by binary ADP-ribosylating toxins usually induces the formation of multiple long protrusions, which results in a web-like structure on the cell surface. What are the functions of these protrusions? From the point of host–pathogen interaction, it was shown that the protrusions might be involved in pathogen colonization, because the toxin-producing bacteria are embedded by this protrusion network, thereby increasing the adherence of the pathogen (Fig. 4). However, the function of the protrusions may be more diverse. Electron microscopic studies reveal that the membrane protrusions induced by toxins contain membranes of endoplasmic reticulum (ER) in addition to microtubule bundles. ER structures are also found in tunneling nanotubes (Zhang 2011; Wang et al. 2011). Apparently, Stim-1 proteins form the functional connection of ER membranes and microtubules. Stim-1 is a crucial regulator of calcium release-activated calcium channels (CRACs), which are based on the channel-forming Orai proteins (Fahrner et al. 2013; Frischauf et al. 2008). Thus, the protrusions appear to form functional Orai calcium channels. This depends on Orai organization in the cell membrane of the protrusions by Stim-1 (Galan et al. 2011; Fahrner et al. 2013).

4.3.1 Re-guidance of Vesicle Traffic

In addition to calcium signaling, the toxin-induced protrusions allow vesicle trafficking. Rab5- as well as Rab11-associated vesicles has been observed within microtubule-based protrusions, which move in antero- and retrograde directions (Schwan et al. 2014). Again vesicle traffic is typical for tunneling nanotubes (Buszczak et al. 2016) and also for cytonemes (Stanganello and Scholpp 2016). However, microfilaments are essential for vesicle transport in tunneling nanotubes, whereas trafficking in toxin-induced protrusions is only dependent on microtubules. In this respect, it is of interest that the depolymerization of actin results in major changes of cellular vesicle traffic. Depolymerization of actin affects the basolateral traffic of integrin receptors, which depends on F-actin. Mellman and coworkers reported that basolateral vesicle recycling was affected by actin-depolymerizing agents (Sheff et al. 2002). Using latrunculin B, several basolateral plasma membrane proteins were found to lose their typical polarized distribution. Depolymerization of F-actin by CDT causes redistribution of α5/β1-integrins associated with Rab-11-labeled vesicles from the basolateral side to the apical side of epithelial monolayers (Schwan et al. 2014) (Fig. 4). Moreover, extracellular matrix proteins like fibronectin and laminin are redistributed from basolateral α5/β1-integrin traffic to the apical surface of toxin-treated cells. Here, the reorganized microtubules serve as roads for vesicle traffic, resulting in membrane fusion of the vesicles at the base of microtubule-formed protrusions. In addition, vesicles from the basolateral side even enter the protrusion at the apical side, resulting in major re-guidance of vesicles. Because fibronectin is a binding protein for the toxin-producing pathogen, also these guiding effects eventually result in increase of bacterial adhesion, because the adherence proteins occur at the apical surface of cells (Schwan et al. 2014).

5 Conclusions

The actin cytoskeleton is a frequent target of bacterial protein toxins and effectors. While a huge group of toxins and effectors manipulate the actin cytoskeleton via Rho proteins (Aktories 2011; Popoff and Geny 2009; Lemichez and Aktories 2013; Lang et al. 2011; Gruenheid and Finlay 2003), some toxins directly modify actin in a bidirectional manner either inducing polymerization by *Photorhabdus* toxin-induced ADP-ribosylation of threonine-148 or depolymerization by ADP-ribosylation of arginine-177 of actin. Studies from recent years suggest that the toxin-induced depolymerization of the actin cytoskeleton has much more functional consequences as suggested previously. The toxin-induced depolymerization of the actin cytoskeleton unmasks regulatory mechanisms, which largely depend on other components of the cytoskeleton, e.g., microtubules and septins, showing the tight interaction of the whole cytoskeleton in numerous cellular functions. Using toxins as tools, specific functional pathways are selectively

suppressed and, so far unknown, regulatory principles uncovered. The recent findings that microtubule-based protrusions are formed by actin-depolymerizing toxins, are paradigmatic for this functional interconnection, which may be related to some extend to cytonemes, tunneling nanotubes, microtentacles or cilia formation. However, the toxin-induced protrusions clearly exhibit unique features, which are not observed in the other forms of cell protrusions. It is tempting to speculate that these novel types of cell structures not only resemble pathophysiological reactions but is also a physiologically important cell response.

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Photorhabdus luminescens Toxins TccC3 and TccC5 Affect the Interaction of Actin with Actin-Binding Proteins Essential for Treadmilling

Alexander E. Lang, Sonja Kühn and Hans Georg Mannherz

Abstract Actin is one of the most abundant cellular proteins and an essential constituent of the actin cytoskeleton, which by its dynamic behavior participates in many cellular activities. The organization of the actin cytoskeleton is regulated by a large number of proteins and represents one of the major targets of bacterial toxins. A number of bacterial effector proteins directly modify actin: Clostridial bacteria produce toxins, which ADP-ribosylate actin at Arg177 leading to inhibition of actin polymerization. The bacterium *Photorhabdus luminescens* produces several types of protein toxins, including the high molecular weight Tc toxin complex, whose component TccC3 ADP-ribosylates actin at Thr148 promoting polymerization and aggregation of intracellular F-actin leading to inhibition of several cellular functions, such as phagocytosis. Here, we review recent findings about the functional consequences of these actin modifications and for the Thr148-ADP-ribosylated actin the subsequent alterations in the interaction with actin-binding proteins. In addition, we describe the effects of ADP-ribosylation of Rho GTPases by the TccC5 component.

Contents

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1 Introduction

A large number of bacterial toxins target actin probably because of its intracellular abundance, highly conserved structure, and participation in many cellular functions. Intracellularly actin exists in monomeric (G-actin) or polymerized, filamentous form (F-actin). Actin filaments are essential for cell migration, phagocytosis, secretion, adhesion, intracellular traffic, cytokinesis, gene regulation, and the maintenance of cell morphology and cell contacts (Pollard and Cooper 2009; Dominguez and Holmes 2011; Pollard et al. 1994; Lanzetti 2007; Posern and Treisman 2006; Castellano et al. 2001). Due to its dynamic behavior, i.e., its constant polymerization and depolymerization cycles, the modification of actin may result in dramatic consequences for these many cellular functions. Bacterial toxins are able to hijack actin functions in order to support their cellular uptake, survival, and intracellular or intercellular transport (see contribution by Kühn and Mannherz).

Many eukaryotic and prokaryotic enzymes posttranslational modify actin by methylation, phosphorylation, glycosylation, ubiquitination, and ADP-ribosylation (Terman and Kashina 2013). In addition, a number of bacterial toxins modulate actin functions by ADP-ribosylation or cross-linking (Aktories et al. 2011; Satchell 2009; Sheahan et al. 2004). The bacterial toxin-induced ADP-ribosylation of actin at Arg177, leading to depolymerization of F-actin and inhibition of polymerization, was the first described toxin-induced modification of actin (Aktories et al. 1986a, b; Ohishi and Tsuyama 1986). Actin Arg177-specific ADP-ribosyltransferases are produced by *Clostridia* (*difficile*, *perfringens*, and *botulinum*), which cause diarrhea, food poisoning, and/or gas gangrene. Most members of this toxin group are binary actin-ADP-ribosylating exotoxins (e.g., *Clostridium botulinum* C2 toxin), although several bacterial ADP-ribosyltransferases (e.g., *Salmonella enterica* SpvB) are not binary in structure (Aktories et al. 2011; see also contribution by Kühn and Mannherz). Actin is also ADP-ribosylated at Thr148 by the *Photorhabdus luminescens* toxin TccC3. These toxins transfer ADP-ribose from NAD^+ to the toxin-specific residues of actin and modify its polymerization behavior (Aktories et al. 2011; Lang et al. 2011). Whereas ADP-ribosylation at Arg177 leads to inhibition of actin polymerization, ADP-ribosylation at Thr148 induces actin polymerization and aggregation. TccC3-catalyzed Thr148-ADP-ribosylation and subsequent actin polymerization appears to be aggravated through ADP-ribosylation of Rho GTPases by the *P. luminescens* TccC5 toxin (Lang et al. 2010). Both toxins are part of the tripartite Photorhabdus Tc (toxin complex) toxins, which consist of the three components TcA, TcB, and TcC. These Tc toxins are highly toxic for insects.

Here, we will briefly review the life cycle of *P. luminescens*, the modified interaction of Thr148-ADP-ribosylated actin with actin-binding proteins (ABPs) and the functional consequences of the toxin-induced polymerization of F-actin on the cytoskeleton and actin treadmilling. In addition, we will summarize the effects of the TccC5-catalyzed ADP-ribosylation of Rho GTPases on the actin cytoskeleton.

2 Life Cycle and Tc Toxins of *Photorhabdus luminescens*

Photorhabdus luminescens are motile Gram-negative entomopathogenic enterobacteria, which live in symbiosis with nematodes of the family *Heterorhabditidae* (Forst et al. 1997; Joyce et al. 2006; Waterfield et al. 2009). The nematodes, carrying the Photorhabdus bacteria in their gut, invade insect larvae, where the bacteria are released from the nematode gut by regurgitation into the open circulatory system (hemocele) of the insect (Fig. 1). In the larvae, the bacteria replicate and start to produce a large number of different virulence factors, including various toxins that kill the insect host within few days (Ciche et al. 2008; Waterfield et al. 2009). Then, the cadaver is used as a food source for the bacteria and the nematodes (Ffrench-Constant et al. 2003; Waterfield et al. 2009). After the death of the insect and advanced tissue degradation, the characteristic bioluminescence of

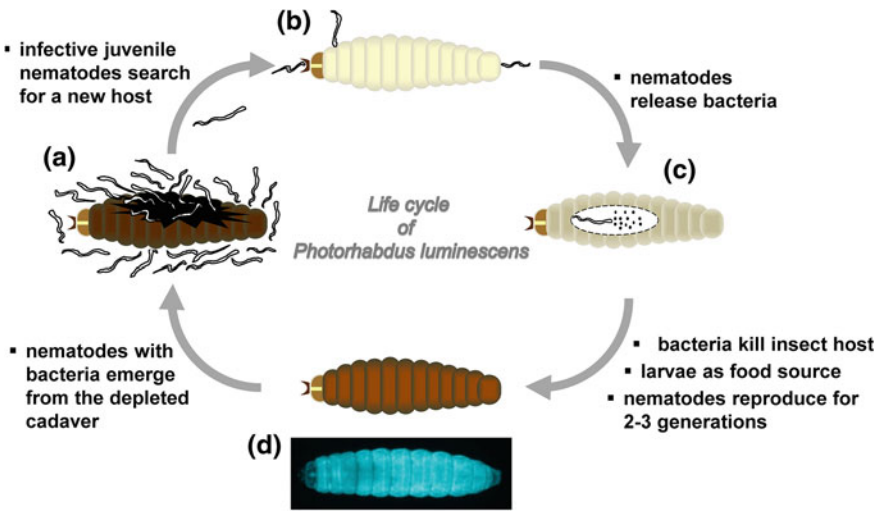

Fig. 1 Life cycle of *Photorhabdus luminescens.* Nematodes with *Photorhabdus luminescens* in their gut leave a dead insect larva (a) and search for new prey. They are taken up by a new larva or intrude it (b). Within the insect hemocoel, they release the bacteria by regurgitation. The bacteria release their toxins which kill the insect larva (c) and a luciferase leading to the bioluminescence of the infected insect larva (d). For further details see the text

P. luminescens can be detected (Daborn et al. 2001) (Fig. 1d). When the insect cadaver is depleted, the replicated nematodes take up bacteria again and leave the insect cadaver to find and invade new insect prey. Therefore, nematodes harboring *P. luminescens* are also used as biological insecticides.

Photorhabdus luminescens produces a large array of toxins, which are only partially characterized. One of the most potent agents produced by the bacteria is the tripartite Tc (toxin complex) toxin, which occurs in several homologues and isoforms. These high molecular mass toxin complexes ($\sim$1.7 mDa) consist of three components TcA, TcB, and TcC (Ffrench-Constant and Waterfield 2006; Meusch et al. 2014). TcA is the pentameric binding and membrane translocation component, which inserts into the endosomal membrane after acidification of endosomes. Membrane insertion is triggered not only at low pH, but also at high pH, explaining why TcA is able to act directly through the midgut of insects (Bowen et al. 1998; Gatsogiannis et al. 2013). TcB and TcC form together a large cage-like structure (cocoon), which harbors the TcC components (Meusch et al. 2014). The TcC components TccC3 and TccC5 posses at their C-terminal domains an ADP-ribosyltransferase, which are auto proteolytically cleaved and reside inside the cocoon before their intracellular translocation through the TcA complex (Meusch et al. 2014). TccC3 ADP-ribosylates actin at Thr148 while the component TccC5 modifies Rho proteins at Gln61/63 (Lang et al. 2010). Thus, ADP-ribosylated Rho proteins are persistently activated and strongly induce the formation of stress fibers and lamellipodia. Both modifications alter the dynamic behavior of the actin cytoskeleton leading to an increased polymerization and clustering of F-actin, which finally inhibit crucial cellular functions such as phagocytosis (Lang et al. 2010).

Tc toxins were first described in the insect pathogens *Photorhabdus* and *Xenorhabdus*. Due to the increasing number of genome sequencing projects, it became obvious that Tc gene homologues are widely distributed among the other pathogens such as the Gram-negative human pathogens *Yersinia* and *Burkholderia* or Gram-positive insect pathogens such as *Paenibacillus* and *B. thuringiensis* (Yang and Waterfield 2013). Homologues of TcB and TcC proteins are also found in other bacteria sucha as *Wolbachia* or *Mycobacteria*, and even in fungi, indicating a much wider role of this toxin family beyond insect toxicity (Yang and Waterfield 2013). Even infections of humans by Photorhabdus have been reported (Peel et al. 1999).

3 ADP-Ribosylation of Actin by *P. luminescens* TccC3

3.1 Thr148-ADP-Ribosylation Promotes Actin Polymerization

In contrast to Arg177-ADP-ribosylation, Thr148-ADP-ribosylated actin is still able to polymerize. Arg177-ADP-ribosylation is performed by a number of toxins from different bacterial origins. Arg177 is located in subdomain 3 of actin facing the

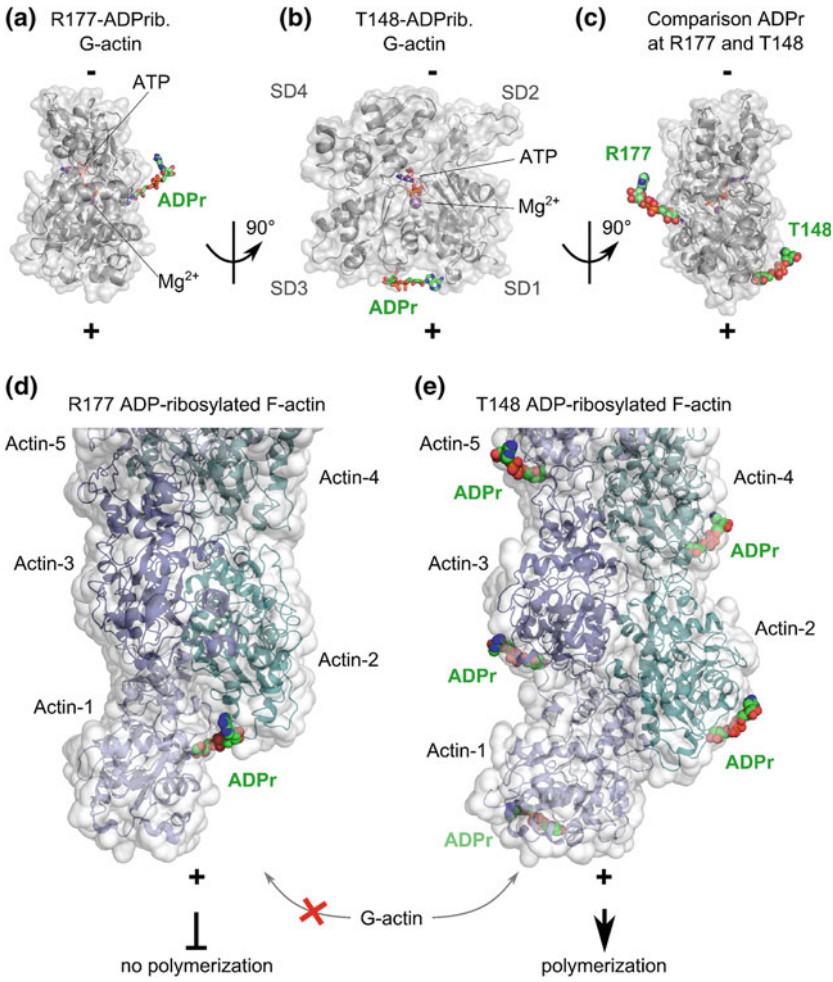

Fig. 2 Model of ADP-ribosylated G-actin and its effect on F-actin polymerization. **a** 'Side-view' of Arg177-ADP-ribosylated (R177-ADPrib.) G-actin (*light gray*) in cartoon-surface representation. The ADP-ribosyl moiety (ADPr) has been modeled on R177/T148 (PDB 1ATN) to illustrate the position of this modification on actin. The pointed (−) and barbed face (+) of G-actin are indicated. **b** 'Front-view' of Thr148-ADP-ribosylated (T148-ADPrib.) G-actin. The ADP-ribosyl modification is located at the hydrophobic cleft between actin subdomains SD1 and SD3. **c** Comparison of the locations of the ADP-ribosyl moieties attached to either Arg177 or Thr148. **d**, **e** Effect of actin-ADP-ribosylation on its polymerization behavior. Depicted are F-actin filaments (PDB 4A7N) in cartoon-surface representation. **d** Arg177-ADP-ribosylated G-actin binds the barbed end (+) of F-actin but prevents the addition of G-actin monomers like a capping protein (Aktories and Wegner 1988). It potentially impairs the formation of interstrand contacts between actin protomer 1 of F-actin (actin-1) and the incoming G-actin. **e** In contrast, Thr148-ADP-ribosylated actin is able to polymerize into F-actin filaments (Lang et al. 2010) with the ADP-ribosyl moiety decorating the outer surface of the filament. Thr148-ADP-ribosylation appears to not interfere with the interstand and intrastrand actin-actin contacts in F-actin

opposite parallel strand (Fig. 2a–c). After its ADP-ribosylation, actin polymerization is impaired because the adenine–ribose moiety appears to interrupt the interstrand actin-actin contacts (Fig. 2d).

In contrast, Thr148-ADP-ribosylated actin shows a high tendency to polymerize. It retains its native state as judged by its ability to inhibit the enzymatic activity of deoxyribonuclease I (DNase I) in monomeric state (Lang et al. 2016). No difference was observed in the critical concentration of polymerization between native and Thr148-ADP-ribosylated actin (Lang et al. 2010). The ADP-ribose moieties covalently attached to Thr148 are located on the outer surface of F-actin and therefore do not interfere with the actin-actin contacts within the filament (Fig. 2c, e). Upon polymerization, Thr148-ADP-ribosylated F-actin is even able to stimulate the myosin subfragment 1 ATPase activity (unpublished data).

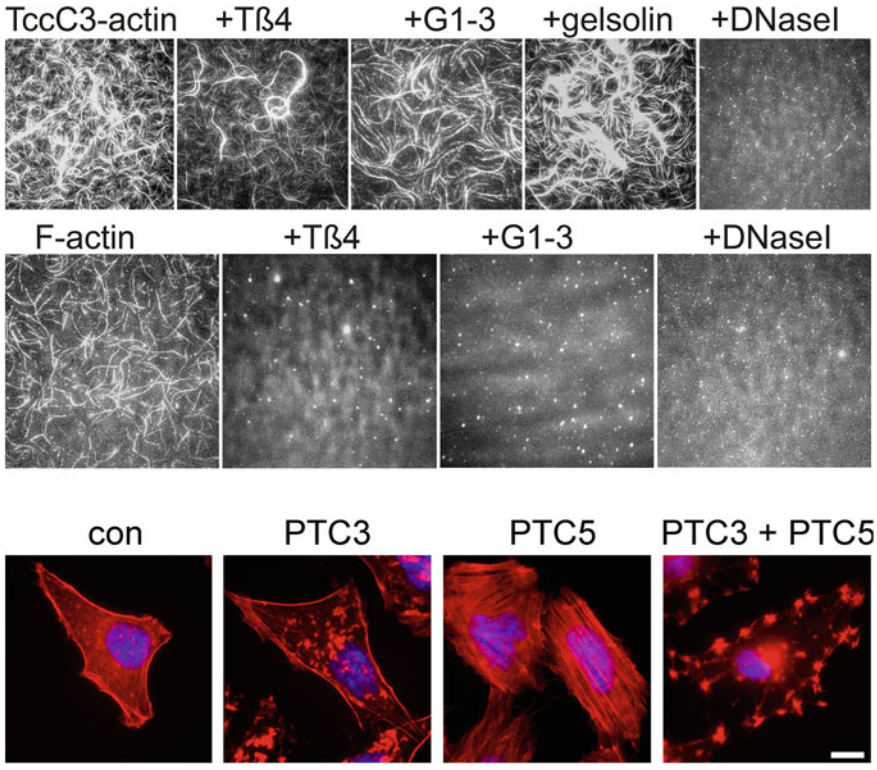

Fig. 3 Polymerization behavior of Thr148-ADP-ribosylated actin as determined by TIRF microscopy. *Upper row* Thr148-ADP-ribosylated actin at 5 µM was polymerized by addition of 1 mM $MgCl_2$ and 50 mM KCl (Lang et al. 2016). The extent of F-actin polymerization is shown 15 min after initiating without an ABP or with addition of 10 µM of Tß4, 5 µM gelsolin G1–3, 3.66 µM full length gelsolin, or 5 µM DNase I as indicated. *Middle row* Identical experiment with unmodified actin. *Lower row* Effect of intoxicating HeLa cells with *P. luminescens* toxins (PTC3, PTC5 and both simultaneously). Images show TRITC-phalloidin staining of HeLa cells 4 h after intoxication. Note F-actin aggregate formation after TccC3 (PTC3) intoxications and the increase in cytoplasmic stress fibers after TccC5 (PTC5) intoxication

When the polymerization behavior of Thr148-ADP-ribosylated actin was visualized by total internal reflection fluorescence (TIRF) microscopy (Fig. 3; Lang et al. 2016), it was noted that the rapid polymerization of Thr148-ADP-ribosylated actin at concentrations above 5 μM was accompanied by the formation of curled F-actin bundles. The strong tendency for bundle formation was not observed for

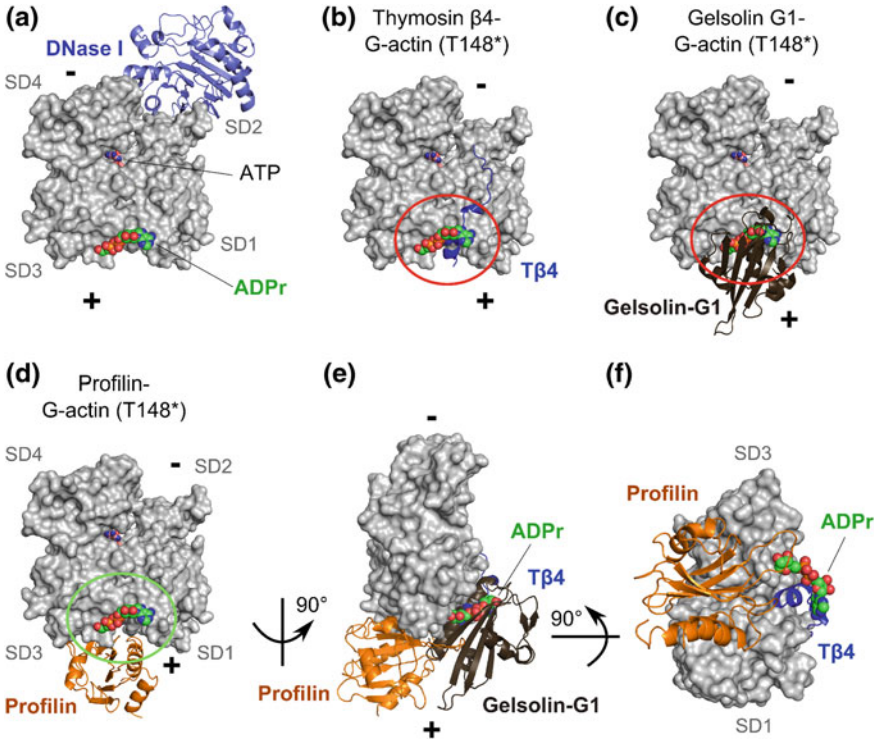

Fig. 4 Thr148-ADP-ribosylation of actin interferes with its binding to certain actin-binding proteins (ABPs). **a** The binding site of DNase I (*slate blue*, PDB 1ATN) on the pointed face of G-actin is separated from the ADP-ribosylated Thr148 amino acid side chain of actin (T148*). Therefore, the ADP-ribosyl moiety (ADPr) does not prevent the formation of the actin: DNase I complex. The ADPr was modeled on T148 as in Fig. 2 to illustrate the location of the modification in the actin: ABP complex. **b** Tß4 (*blue*, PDB 4PL8) binds with its amphipathic helix into the hydrophobic cleft between actin subdomains SD1 and SD3. The impaired binding of Tß4 to Thr148-ADP-ribosylated actin seems to occur due to its steric hindrance with ADPr (see also **f**). **c** The N-terminal G1 segment of gelsolin (*dark brown*, PDB 1EQY) interacts like Tß4 with the same surface at the barbed face of actin. Its binding to actin appears likewise to be impaired by the ADP-ribosylated Thr148. **d** In contrast, biochemical data indicate that Thr148-ADP-ribosylation does not impair binding of profilin (*orange*, PDB 2PBD) to actin. This may be explained by a further rearward binding of profilin into the cleft between SD1 and SD3. The different binding regions of Gelsolin-G1 and profilin are further demonstrated by a side-view of actin (**e**) and between profilin and Tß4 on a direct view onto the actin's barbed face (**f**). The translational rotation axes are indicated. *Red circle* clash between ABP and ADP-ribosylation, *green circle* no steric hindrance between the ABP and the ADP-ribosyl moiety

native F-actin under otherwise identical conditions (Fig. 3). The polymerization of Thr148-ADP-ribosylated actin, however, is not inhibited by thymosin-ß4 (Tß4), a small actin-binding protein that normally inhibits actin polymerization [as shown by the fluorescence assay using pyrene-actin (Lang et al. 2010)] or TIRF microscopy (Fig. 3; Lang et al. 2016). Indeed TIRF microscopy revealed no change in the polymerization behavior or bundle formation of Thr148-ADP-ribosylated actin in the presence of Tß4. Only DNase I was able to inhibit the polymerization of Thr148-ADP-ribosylated actin like native actin (Fig. 3) that was most probably due to its binding to the so-called D-loop, which is spatially separated from the Thr148-ADP-ribose moiety (Fig. 4a).

Bundle formation was predominant in the test tube when using purified actin; however, it was only transiently detected in HeLa cells intoxicated with the TccC3 toxin. Instead, the cellular actin condensed into aggregates of varying sizes, which were positively stained by TRITC-phalloidin suggesting the aggregation of short actin filaments (Fig. 3; Lang et al. 2010, 2016). The different behavior of F-actin condensation (predominantly bundles with purified actin versus aggregates in cells) might be due to additional regulatory mechanisms present in intact cells like, for instance, other ABPs or further modified not yet identified target proteins aiming at the disposal of the Thr148-ADP-ribosylated actin.

3.2 Impaired Interactions of Thr148-ADP-Ribosylated Actin with a Number of Actin-Binding Proteins

Thr148 is located at the base of subdomain 3 (SD3) of actin close to the hydrophobic cleft between SD1 and SD3 (Fig. 4; see also contribution of Kühn and Mannherz), which constitutes a main binding area for a number of actin-binding proteins (ABPs) (Dominguez and Holmes 2011), in particular for ABPs, which sever F-actin or maintain actin in monomeric, non-polymerized state. The bulgy ADP-ribose attached to this residue might hinder the access of these ABPs to their binding region (Fig. 4). Therefore, the interaction of a number of ABPs targeting this area of native and Thr148-ADP-ribosylated actin was further analyzed (see below).

This appeared to be indeed the case for ABPs with G-actin sequestering or F-actin fragmenting activity. The first ABP, for which reduced affinity to Thr148-ADP-ribosylated actin was shown, was thymosin-ß4 (Tß4). Tß4 is a small peptide of 5 kDa and the main intracellular monomeric actin sequestering protein (Fechheimer and Zigmond 1993). It forms a 1:1 complex with G-actin, whereby actin polymerization is inhibited. Since, however, it binds G-actin with relatively low affinity (Kd about 1–3 μM), it is easily displaced by other ABPs with higher affinity to G-actin (Mannherz and Hannappel 2009; Mannherz et al. 2010) or by free barbed ends of F-actin or F-actin fragments. Only due to its high intracellular concentrations (up to 500 μM; Cassimeris et al. 1992), Tß4 can appreciably sequester G-actin (Fechheimer and Zigmond 1993). Thr148-ADP-ribosylated actin

was shown to have an eightfold reduced affinity to Tß4 resulting in a marked reduction in its ability to inhibit the polymerization of Thr148-ADP-ribosylated actin (Lang et al. 2010). This effect is most probably due to the structural proximity of the ADP-ribose moiety attached to Thr148 to the N-terminal helix of Tß4 responsible for binding to the SD1–SD3 cleft of actin (Fig. 4b). Therefore, Thr148-ADP-ribosylation will shift the intracellular equilibrium between G- and F-actin (normally 1 to 1 in quiescent cells) toward F-actin. Indeed, TIRF microscopy demonstrated that Tß4 had no polymerization inhibitory effect on Thr148-ADP-ribosylated actin in contrast to native actin (Fig. 3; Lang et al. 2016).

Intracellularly, the polymerization promoting effect by the TccC3 toxin might be further enhanced by the reduction in affinity of ABPs to Thr148-ADP-ribosylated actin that have F-actin severing activity. Gelsolin and ADF (actin depolymerizing factor)/cofilin and profilin were tested as examples for this class of ABPs (Lang et al. 2016). These experiments were in most cases performed using purified skeletal muscle actin, since no difference in the rate and extent of Thr148-ADP-ribosylation of skeletal muscle and cytoplasmic ß-actin had been detected (Lang et al. 2016). The F-actin fragmenting activity of gelsolin is mainly dependent on the high affinity binding of its N-terminal segment G1 to the target area between SD1 and SD3 (McLaughlin et al. 1993; Nag et al. 2013). Comparing the extent of TccC3-catalyzed Thr148-ADP-ribosylation of free actin or after complexing with gelsolin and its fragments, it was shown that intact gelsolin and the gelsolin fragments comprising G1 inhibited Thr148-ADP-ribosylation (Lang et al. 2016). A similar inhibition was shown for ADF and cofilin. Again these effects will be most probably due to steric clashes between the ADP-ribose attached to Thr148 and the binding helices of gelsolin G1 and ADF/cofilin (McLaughlin et al. 1993; Paavilainen et al. 2008).

In contrast, complexing of actin with profilin did not inhibit Thr148-ADP-ribosylation, although it also binds to the cleft between subdomain 1 and 3 (Schutt et al. 1993). A detailed structure analysis may explain these diverse effects. The ADP-ribose attached to Thr148 is located at the entrance of this cleft. Since G1 and ADF/cofilin were shown to bind to the front part of this cleft, a structural clash between the ADP-ribose and G1 (Fig. 4c) or ADF/cofilin is easily conceivable (not shown). In contrast, profilin binds to the rear part of this cleft (Schutt et al. 1993). Therefore, profilin binding to actin will not be affected by Thr148-ADP-ribosylation (see Fig. 4d–f). Of note, the profilin:actin complex is preferentially added to the plus end of growing actin filaments or used by, for instance, the nucleating proteins of the formin family for F-actin elongation (Goode and Eck 2007). Indeed, profilin is still able to bind to Thr148-ADP-ribosylated actin as shown by chemical cross-linking (Lang et al. 2016). Therefore, profilin binding to Thr148-ADP-ribosylated actin might further support its general tendency to polymerize.

Conversely, polymerized Thr148-ADP-ribosylated actin is resistant against the severing activity of gelsolin or its fragment G1-3 and cofilin as shown by TIRF (total internal reflection fluorescence) microscopy (Fig. 3; Lang et al. 2016). Furthermore, Thr148-ADP-ribosylated F-actin appears to possess a lower rate of

cycling or treadmilling as indicated by a lower ATPase activity and rate to exchange its bound nucleotide (ADP) (Lang et al. 2016). In contrast to the effects of these severing proteins on Thr148-ADP-ribosylated F-actin is the observed effect of deoxyribonuclease I (DNase I). DNase I binds G-actin with high affinity by interacting with the so-called D-loop of subdomain 2 (Kabsch et al. 1990) and is able to depolymerize F-actin (Mannherz et al. 1980). Therefore, DNase I binding is not impaired by Thr148-ADP-ribosylation, and consequently, DNase I is able to depolymerize Thr148-ADP-ribosylated F-actin (Fig. 3; Lang et al. 2016). However, DNase I is an extracellular protein and therefore not able to reverse the intracellular apparently irreversible actin polymerization and aggregation after Thr148-ADP-ribosylation.

4 ADP-Ribosylation of Rho GTPases by *Photorhabdus luminescens* TccC5

Rho proteins are a family of small GTP-binding proteins with about 20 family members (Hall 1993; Nobes and Hall 1994). Best studied are Rho, Rac, and Cdc42 isoforms. They are the master regulators of the cytoskeleton and involved in motility functions, cellular traffic, but also transcription or proliferation (Jaffe and Hall 2005). Rho proteins are regulated by a GTPase cycle (Hall 1994) (Fig. 5). They are active in the GTP-bound form and inactive with GDP-bound form. Activation occurs by GDP to GTP exchange, which is facilitated by guanine nucleotide exchange factors (GEFs) and allows interaction with effectors like the formins or Rho-dependent kinases, which then cause polymerization of actin and formation of stress fibers (Schmidt and Hall 2002; Pruyne et al. 2002; Kimura et al. 1996). The inactive state is induced by GTP hydrolysis, caused by their endogenous GTPase activity, and accelerated by GTPase-activating proteins (GAPs) (Fig. 5). Rho-regulating proteins (GDIs) stabilize the inactive GDP-bound form of Rho proteins and keep Rho proteins in inactive form in the cytosol (DerMardirossian and Bokoch 2005). Because of their multifunctional regulatory roles are Rho proteins preferred targets for bacterial toxins and effectors.

TccC5 as part of the Photorhabdus toxin complex PTC5 ADP-ribosylates Rho GTPases leading to their irreversible activation (Lang et al. 2010) that indirectly should promote polymerization of actin and formation of stress fibers. This assumption is indeed supported by data obtained after selective intoxication of HeLa cells with TccC5, which led to a prominent increase in long stress fibers within their cytoplasm (see Fig. 3). Though the exact mechanism of this effect is still unclear, it appears highly likely that the activated Rho proteins stimulate actin nucleation and elongation activating proteins such as formins and the Arp2/3 complex (see contribution by Lemichez). In combination with *P. luminescens* TccC3, TccC5 will thus increase the effects on the cytoskeleton. It was, however,

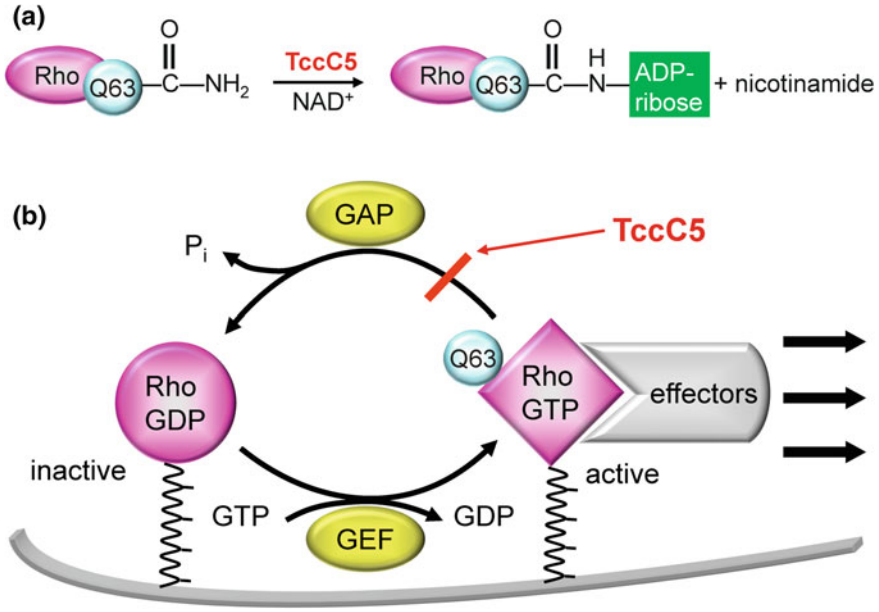

Fig. 5 Mechanism of Rho-ADP-ribosylation by TccC5 toxin. **a** TccC5 ADP-ribosylated Rho at Gln63 (Gln61, not shown), which (**b**) leads to permanent activation of the Rho-GTPase, because binding of Rho-GAP (GTPase-activating protein) is inhibited by the attached ADP-ribosyl moieties. The Rho-GTPase remains in the GTP-bound, i.e., active state

noted that only TccC3 intoxication alone or in combination with TccC5 led to strong clustering of the intracellular actin (Fig. 3; Lang et al. 2010).

Like TccC3, TccC5 functions as an ADP-ribosyltransferase, but instead of modifying actin, TccC5 modifies Rho GTPases (Lang et al. 2010; Pfaumann et al. 2015). The ADP-ribosyltransferase domain of TccC5 belongs to the subfamily of ADP-ribosyltransferase also designated as ARTC family of ADP-ribosylating toxins (Hottiger et al. 2010; Lang et al. 2010; Pfaumann et al. 2015). This subfamily contains a conserved RSE motif with an arginine and serine residue involved in NAD-binding and the so-called catalytic glutamate (Domenighini et al. 1994). By mutational analyses, we recently showed that in TccC5 these residues are Arg774, Ser809 and Glu886 (Pfaumann et al. 2015).

Rho proteins belong to the Ras superfamily of GTP-binding proteins and the Rho family comprises about 20 proteins, of which the Rho, Rac, and Cdc42 isoforms are best known (Madaule and Axel 1985; Wennerberg et al. 2005). Of these, RhoA and B, Rac1–3, Cdc42, and the plant Rac-like protein Rop4 are major substrates of TccC5-induced ADP-ribosylation. Minor substrates are RhoC and TC10 (Pfaumann et al. 2015). Interestingly, TccC5-catalyzed ADP-ribosylation of Rho proteins occurs at Gln63 and Gln61, which is also modified by deaminating cytotoxic necrotizing factors (CNFs) from *E. coli* and *Yersinia* species (Schmidt et al. 1997; Flatau et al. 1997; Hoffmann and Schmidt 2004). This glutamine

residue is essential for GTP hydrolysis by Rho proteins (Wittinghofer and Vetter 2011). ADP-ribosylation of Gln63/61 prevents GTP hydrolysis by Rho proteins even in the presence of GAPs (Fig. 5). Therefore, Rho proteins are persistently in the active state and cause activation of effector proteins such as Rho kinase and formins, which cause polymerization of actin and formation of stress fibers or lamellipodia (the latter due to Rac activation; Lang et al. 2010; Pfaumann et al. 2015). Thereby, TccC5 will also contribute to the inhibition of phagocytosis by insect hemocytes and death of larvae (Lang et al. 2010).

5 Conclusions

Among the many bacterial toxins, which modulate the intracellular actin cytoskeleton, two groups of bacterial toxins directly and covalently modify actin by ADP-ribosylation. Actin Arg177-specific ADP-ribosyltransferases are produced by *Clostridium difficile*, *perfringens*, and *botulinum*, which cause diarrhea, food poisoning, and gas gangrene. Bacterial toxins, which transfer adenine–ribose to Arg177, inhibit actin polymerization finally leading to depolymerization of all intracellular actin. Thus, impaired cells will undergo programmed cell death or apoptosis and the cell remnants might be used as nutrients for the multiplication of the bacterium or impair immune cells. In contrast, the TccC3 toxin from *P. luminescens* ADP-ribosylates actin at Thr148 leading to polymerization/aggregation of the intracellular actin. *Photorhabdus* bacteria live in the gut of nematodes, which after invading insect larvae release these bacteria into their open circulatory system, where they infect insect hemocytes and subsequently evade their elimination by inhibiting phagocytosis. Since this actin modification reduces also its interaction with F-actin depolymerizing ABPs, Thr148-ADP-ribosylation appears to be practically irreversible. In PTC5-intoxicated cells the Rho GTPases are additionally activated after ADP-ribosylation by the TccC5 component inducing a signaling cascade that will further stimulate actin polymerization. Irreversible polymerization of most intracellular actin will lead to an arrest of most cellular functions and inevitably to cell death.

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Comparative Studies of Actin- and Rho-Specific ADP-Ribosylating Toxins: Insight from Structural Biology

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Abstract Mono-ADP-ribosylation is a major post-translational modification performed by bacterial toxins, which transfer an ADP-ribose moiety to a substrate acceptor residue. Actin- and Rho-specific ADP-ribosylating toxins (ARTs) are typical ARTs known to have very similar tertiary structures but totally different targets. Actin-specific ARTs are the A components of binary toxins, ADP-ribosylate actin at Arg177, leading to the depolymerization of the actin cytoskeleton. On the other hand, C3-like exoenzymes are Rho-specific ARTs, ADP-ribosylate Rho GTPases at Asn41, exerting an indirect effect on the actin cytoskeleton. This review focuses on the differences and similarities of actin- and Rho-specific ARTs, especially with respect to their substrate recognition and cell entry mechanisms, based on structural studies.

Contents

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1 Introduction

Some bacterial protein toxins exert deleterious effects on target cells by entering cells and modifying an intracellular target. These toxins recognize a cellular receptor, translocate across the target cell membrane, and then exert their toxic effect against their targets. Iota toxin, produced by *Clostridium perfringens* type E, is a member of the clostridial binary toxin group, which includes *C. botulinum* C2 toxin, *C. difficile* toxin (CDT), and *C. spiroforme* toxin (CST). These toxins are structurally related to the vegetative insecticidal proteins (VIP) produced by *Bacillus cereus* and *B. thuringiensis* (Barth et al. 2004). Recently, novel enterotoxins from non-CPE (*C. perfringens* enterotoxin) producing *C. perfringens* were found in humans. These toxins were named CPILE (*C. perfringens* iota-like enterotoxin) or BEC (binary enterotoxin of *C. perfringens*) (Lyras and Rood 2014; Yonogi et al. 2014; Irikura et al. 2015; Monma et al. 2015). All of these binary toxins consist of two unlinked proteins, A and B. The A component has ADP-ribosyltransferase (ART) activity toward actin, and the B component is involved in binding to a membrane receptor and translocation of the toxin into the cell (Table 1). These ARTs ADP-ribosylate globular actin at Arg177, leading to the destruction of the filamentous actin and intoxicated cell.

In contrast, the Rho-specific C3-like ARTs produced by *C. botulinum*, *Clostridium limosum*, *B. cereus*, and *Staphylococcus aureus* lack B components and are single domain enzymes (Aktories et al. 1989; Sekine et al. 1989). *C. botulinum* C3 (C3bot) ADP-ribosylates RhoA, RhoB, and RhoC at Asn41 and also modifies Rac1 very weakly. *S. aureus* C3 (C3stau) modifies RhoE and Rnd3 (Wilde et al. 2001). *B. cereus* C3 (C3cer) ADP-ribosylates RhoA, RhoB, and RhoC but does not modify Rac1 or Cdc42 at all (Wilde et al. 2003). Though Asn41 is conserved in Rac1 and Cdc42, they are not good substrates of C3-like toxins. Rho GTPases are master regulators of the actin cytoskeleton (Etienne-Manneville and Hall 2002), and their C3-catalyzed ADP-ribosylation causes their biological inactivation and thus the inhibition of downstream signaling and its consequences.

This review focuses on the similarities and differences of actin-specific ARTs from binary toxins, and Rho-specific ARTs. Comparative studies of these ARTs are very interesting. One reason is that their tertiary structures are very similar, but their targets are totally different. Recently, the crystal structure of an actin-specific ART (Ia) in complex with actin was solved (Tsuge et al. 2008; Tsurumura et al. 2013). Furthermore, the structure of the C3–RhoA complex was also solved (Toda et al. 2015). Comparison of the structures of these two complexes provided novel insight concerning substrate protein recognition and the mechanism of ADP-ribosylation. A second reason is that the cell entry mechanisms of two toxins seem totally different. Although Iota-like ARTs, which are binary toxins, are dependent on their B components, C3-like ARTs lack a B component. In contrast to the information

Table 1 Characterization of bacterial ART subfamilies to modulate actin cytoskeleton

Toxin	Bacterium	Uniprot	EXE	Eukaryotic substrate	Target	Cellular receptor delivery
Iota-like binary toxins						
Iota toxin	*Clostridium perfringens*	Q46220	EYE	G-Actin	Arg177	LSR
CPILE/BEC	*Clostridium perfringens*	X5I2D7	EYE	G-Actin	Arg177	?
C2 toxin	*Clostridium botulinum*	D4N871	EQE	G-Actin	Arg177	N-linked carbohydrates
CDT	*Clostridium difficile*	Q9KH42	EYE	G-Actin	Arg177	LSR
CST	*Clostridium spiroforme*	O06497	EYE	G-Actin	Arg177	LSR
VIP	*Bacillus cereus*	G8C882	EKE	G-Actin	Arg177	?
C3-like toxins						
C3bot	*Clostridium botulinum*	P15879	QLE	Rac1, RhoA, B and C	Asn41	Vimentin
C3stau/EDIN	*Staphylococcus aureus*	Q9ADS9	QQE	Rnd3, RhoA, B, C and E	Asn41	NA
C3lim	*Clostridium limosum*	Q46134	QLE	Cdc42, RhoA, B and C	Asn41	NA
C3cer	*Bacillus cereus*	Q8KNY0	QYE	RhoA, B and C	Asn41	NA

available about ART, the cell entry mechanisms used by both of these toxins remain largely unknown. Comparison of the cell entry mechanisms used by these two different toxins is an important area for future study.

2 Functional and Structural Studies of Actin- and Rho-Specific ADP-Ribosylating Toxins

Significant functional studies of the enzymatic A component have been conducted. The actin-specific iota toxin A component (Ia) and the RhoA-specific C3-like toxin, which belong to the same ARTC subfamily (related to C2 and C3 Clostridial Toxins), contain a conserved R-S-E motif (Hottiger et al. 2010) (Fig. 1a).

The actin-specific, iota-like ARTs, including Ia (Vandekerckhove et al. 1987), C2-I (Aktories et al. 1986), CSTa (Popoff et al. 1989), CDTa (Popoff et al. 1988), and CPILEa/BECa (Yonogi et al. 2014; Irikura et al. 2015) ADP-ribosylate actin at

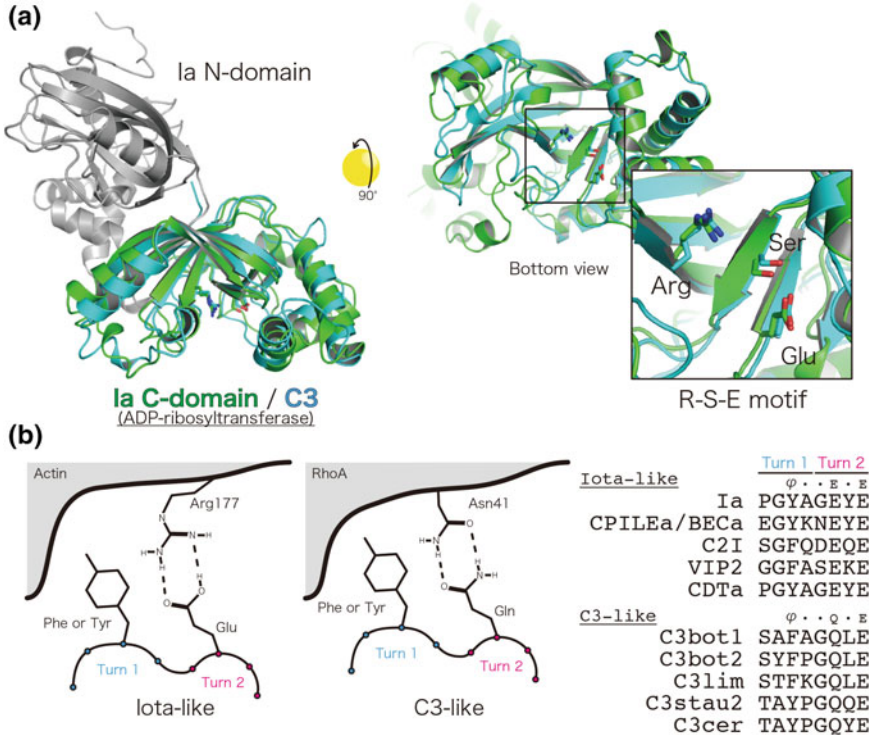

Fig. 1 Superimposed structures and proposed substrate recognition schemes of Ia and C3. **a** Superimposed structures of Ia (*green*) and C3 (*cyan*). *Black box* is a detailed view around the R-S-E motif. **b** Schematic diagram of target recognition by the ARTT loop and sequence alignment of the ARTT loop among Iota- and C3-like ARTs

Arg177. ADP-ribosylated G-actin can bind to F-actin at the plus (barbed) end of the filament as a capping protein. However, depolymerization occurs at the minus (pointed) end, and the newly released G-actin is then ADP-ribosylated by the toxin. Toxin-induced actin depolymerization has a dramatic effect on target cells, including the remodeling of their microtubules and destruction of the actin cytoskeleton with subsequent apoptosis (Aktories et al. 2011).

Since the first actin-specific toxin C2 was identified (Aktories et al. 1986), numerous functional studies of the iota-like ARTs have been carried out. The enzymatic components of iota-like toxin consist of two similar repeated domains. The C-terminal domain harbors NAD^+ binding and ART activity, while the *N*-terminal domain lacks both NAD^+ binding and ART activity but binds the complementary B component. Based on the reported structures of the enzymatic components of cholera toxin and diphtheria toxin, it was proposed that three motifs

are conserved among ARTs; these include the (i) aromatic-Arg/His motif, (ii) STS motif, and (iii) the E(Q)XE motif (Domenighini and Rappuoli 1996). Replacement of the Arg in the aromatic-Arg/His motif with Ala leads to a complete loss of the ART and NADase activities, as well as their cytotoxic activities and their lethality (Perelle et al. 1996; Nagahama et al. 2000). Mutation of the first glutamate in the EXE motif prevents ART activity but not NADase activity, while mutation of the second glutamate diminishes both the ART and NADase activities (Perelle et al. 1996; Barth et al. 1998; Nagahama et al. 2000). Mutation of the first serine in the STS motif drastically reduces ART activity (Barth et al. 1998; Nagahama et al. 2000).

The functional studies outlined above were subsequently confirmed by X-ray structural studies. The first solved structure of an iota-like ART, VIP2 from *B. cereus*, revealed that it is a mixed α/β protein that is divided into an N-terminal domain and a C-terminal domain with similar structures (Han et al. 1999). VIP2 belongs to the binary toxin family, which comprises VIP1 (membrane binding unit) and VIP2 (enzymatic unit), as well as iota toxin (Ib and Ia) and C2 (C2-II and C2-I). Prior to the VIP2 structural analysis, Ia and C2-I had been characterized using mutagenesis and biochemical methods (Perelle et al. 1996; van Damme et al. 1996; Barth et al. 1998; Nagahama et al. 2000). Next paper examining the structure of C3 exoenzyme from *C. botulinum* (C3bot1) have shown that the structure of C3 is very similar to that of the C-terminal domain of VIP2 (Han et al. 2001). The crystal structure analyses of VIP2 and C3 have provided not only a common structural framework for the enzymes but also a possible reaction mechanism (Fig. 1a). The structures of the iota-like and C3-like toxins imply that both ARTs involve a bipartite recognition specificity motif that comprises residues from two adjacent protruding turns, turn 1, and turn 2 (Han et al. 2001). In iota-like toxins, a hydrophobic residue (Tyr or Phe) on turn 1 is thought to interact with actin, and the initial glutamate of the EXE motif is thought to recognize the substrate amino acid, forming a salt bridge with Arg177 of actin (Fig. 1b). In C3-like toxin, a hydrophobic residue (Tyr or Phe) on turn 1 would interact with RhoA, and the glutamine of the QXE-motif would recognize substrate amino acid, forming a salt bridge with Asn41 of RhoA (Fig. 1b). It was speculated that the hydrophobic patch of RhoA formed by Leu72, Trp58, and Phe39 is important for binding to the solvent-exposed Phe of turn 1 in C3. The precise position of the hydrophobic patch of RhoA was later revised in the structure of the C3–RhoA complex (Toda et al. 2015). Proof of these hypotheses awaits analysis of the crystal structure of the toxin-substrate protein complex. Structural studies of several more iota-like toxins in this class, including Ia from *C. perfringens* (Tsuge et al. 2003), C2-I from *C. botulinum* (Schleberger et al. 2006) and CDTa from *C. difficile* (Sundriyal et al. 2009), are presently available. On the other hand, in addition to various conformational analyses of the C3bot structure (Menetrey et al. 2002, 2008), structures of C3 from *S. aureus* (C3stau2), *C. limosum* (C3lim), and *Paenibacillus larvae* (C3larvin: the target is unknown in honey bee) have been solved (Evans et al. 2003; Vogelsgesang et al. 2008; Krska et al. 2015) (Fig. 2).

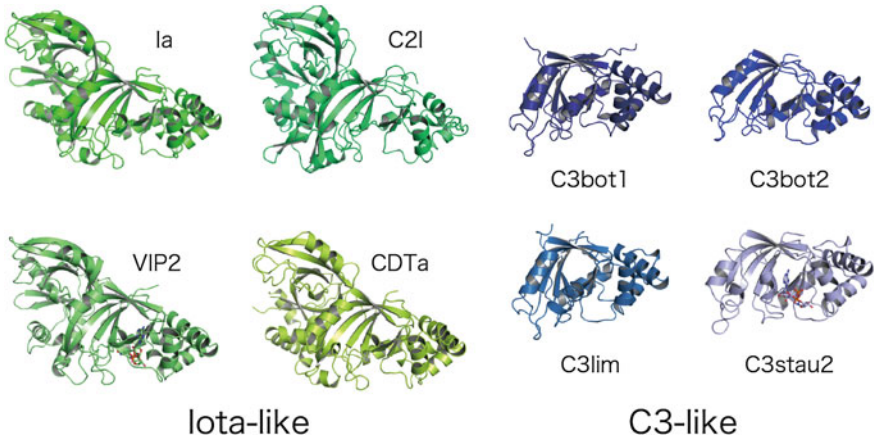

Fig. 2 Structures of Iota- and C3-like ARTs. Iota-like ARTs: Ia (1GIQ), C2I (2J3X), VIP2 (1QS2), and CDTa (2WN4). C3-like ARTs: C3bot1 (PDB: 1UZI), C3bot2 (1R45), C3lim (3BW8), and C3stau2 (1OJZ)

3 Comparative Studies of Substrate Recognition by Actin- and Rho-Specific ARTs

Since we solved the crystal structure of Ia in 2003, we have tried to solve the structure of an ART-substrate protein complex (Tsuge et al. 2003). In the first crystallization trial of the complex of Ia and α-actin, we thought two factors, latrunculin A and βTAD, would be necessary to obtain the crystals of the complex because latrunculin A and βTAD prevent the actin polymerization and ART reaction, respectively (Tsuge et al. 2008). However, later we recognized that it was possible to obtain crystals of the complex without latrunculin A and βTAD. In 2008, we obtained βTAD-Ia-actin crystals and revealed the first Ia-actin structures (Tsuge et al. 2008) (Fig. 3a). In 2013, we soaked apo-Ia-actin crystals with NAD^+ under different conditions. This crystal soaking allowed the determination of the structures of the pre- and post-reaction state of ADP-ribosylation in the Ia-actin complex (Tsurumura et al. 2013). The binding region of actin comprises loop I (Tyr60 and Tyr62 in the *N*-terminal domain), loop II (active site loop), region III (adenine binding region), loop IV (the phosphate nicotinamide (PN) loop), and loop V (ARTT loop) (Figs. 3b and 4). Although it was possible to predict that three of these loops (active site, PN, and ARTT loops) would be used for substrate recognition, it was surprising that loop I of the *N*-terminal domain plays an important role in binding to substrate actin, because the *N*-terminal domain was thought to be the region necessary for Ib binding. The 3D structures of these complex suggested a mechanism of ART-substrate recognition; five regions around NAD^+ bound to the surface of actin around the residue Arg177. They also brought the first visualization of the ADP-ribosylation of actin Arg177.

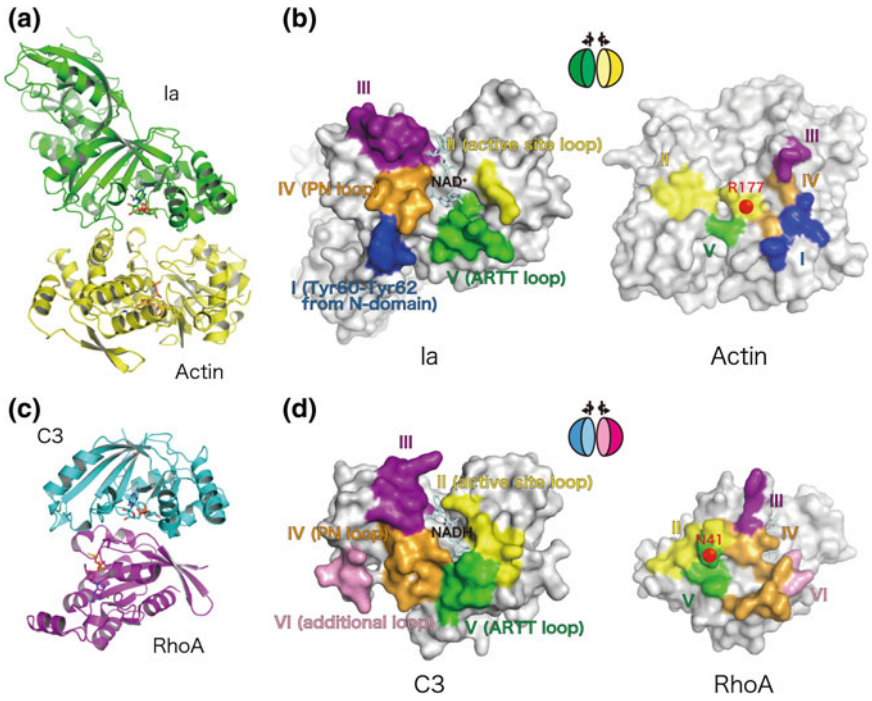

Fig. 3 Target recognition by Ia and C3 based on their crystal structures. **a** Structure of the Ia (*green*)-actin (*yellow*)-NAD^+ complex (PDB: 4H03). **b** Butterfly representation of the Ia and actin interfaces. *Colored regions* show the binding sites of each molecule. *Red sphere* indicates the position of the target amino acid Arg177. **c** Structure of the C3cer (*cyan*)-RhoA (GTP) (*magenta*)-NADH complex (PDB: 4XSH). **d** Butterfly representation of the C3cer and RhoA interfaces. *Red sphere* indicates the position of the target amino acid Asn41

Last year, the first structure of the C3–RhoA complex was determined (Toda et al. 2015) (Fig. 3c). The structure of this complex was determined using C3cer, which specifically ADP-ribosylates RhoA, RhoB, and RhoC, but not Cdc42 or Rac1. The structural components of C3cer that mediate RhoA binding consist of four regions around the bound NAD^+ and one additional loop. They include loop II, composed of residues 45–52 (active site loop) (the loop numbers are derived from the Ia-actin complex structure (Tsuge et al. 2008)); region III, composed of residues 100–110 (adenine binding region); loop IV, composed of residues 148–156 (PN loop); loop V, composed of residues 175–183 (ARTT loop); and loop VI, composed of residues 206–209 (additional loop in C3cer) (Figs. 3d and 4). There is no interaction loop via the *N*-terminal domain analogous to loop I in Ia, because C3 lacks the *N*-terminal domain. Additional loop interactions may be seen only in C3cer. Thus, four regions (active site loop, adenine binding region, PN loop, ARTT

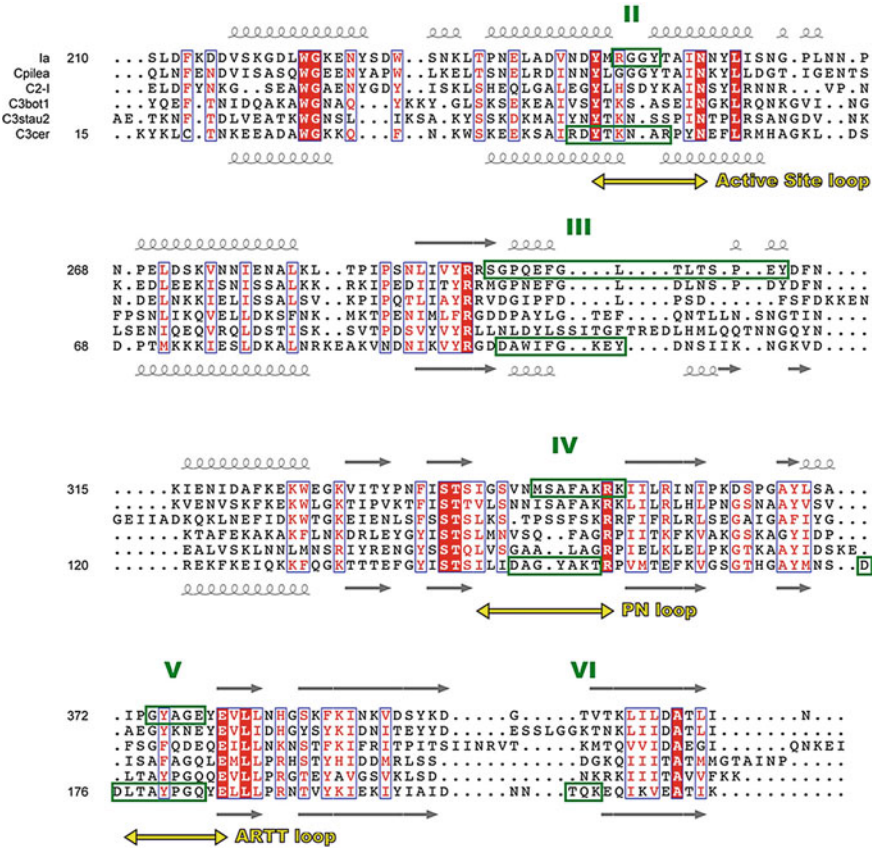

Fig. 4 Sequence alignment of Ia, CPILE-a, C2-I, C3bot1, C3stau2, and C3cer. *Boxes* with *roman numbers* (II, III, IV, V, and VI) show the interaction region with the substrate protein (*green*). *Gray coils* and *arrows above* or *below* the sequence show the helices and β-strands, respectively. Three conserved loops were shown as *yellow*

loop) are common to both Ia and C3 and seem most important for substrate recognition in these two different ARTs (Fig. 4). Surprisingly, the structures of the C3-RhoA complexes directly demonstrate that the glutamine (QXE) of C3 grips the Asn41 of RhoA (Toda et al. 2015) as predicted before (Han et al. 2001) (Fig. 5). We can summarize our knowledge concerning ARTT loop recognition of C3 as follows. The hydrophobic residue (Tyr180) on turn 1 interacts with a hydrophobic patch on RhoA composed of Val43, Ala56, and Trp58. As describe above, Asn41 of RhoA forms a hydrogen bond with Gln183 in the QXE-motif on turn 2 of the ARTT loop. The distance between NC1 of the *N*-ribose and N_{D2} of Asn41 is only 2.9 Å. It seems that the relationship of Asn41 of RhoA, glutamine of QXE, and NC1 of the *N*-ribose of NAD is ideal for the ADP-ribosylation reaction.

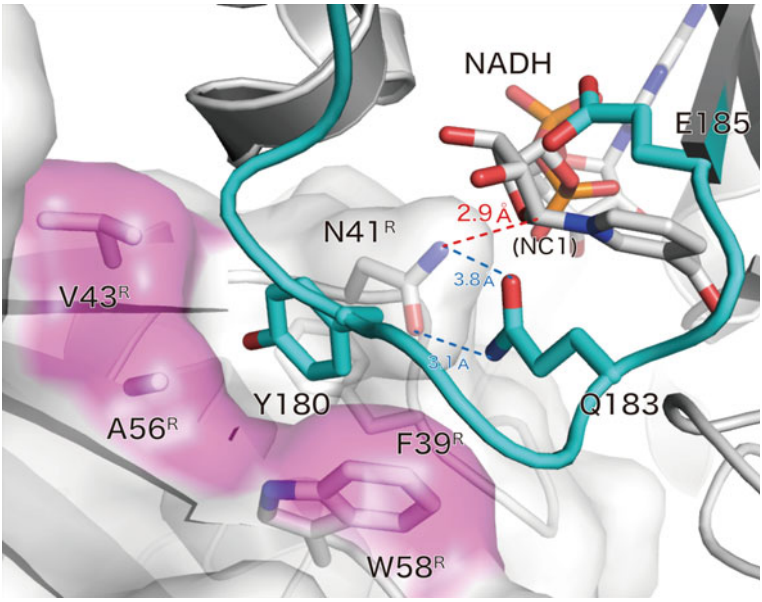

Fig. 5 RhoA recognition by the ARTT loop of C3. The ARTT loop of C3 is colored in *cyan*. RhoA residues recognized by the ARTT loop are depicted as a *magenta* surface. The distances between Asn41 of RhoA and Gln183 of C3cer and between Asn41 of RhoA and the electrophile [NC1 of NAD(H)] are shown as *dotted lines* in *blue* and *red*, respectively

From the snapshot structural analysis of pre-ADP-ribosylation and post-ADP-ribosylation obtained from the structures of the Ia-actin complex, we proposed an SN1 strain-alleviation model of ADP-ribosylation (Jank and Aktories 2013; Tsurumura et al. 2013). The ADP-ribosylation reaction starts with the release of nicotinamide from the NAD donor, followed by the formation of an oxocarbenium cation intermediate in a 'strained' conformation. Rotation around the phosphodiester bond forms a second oxocarbenium cation intermediate, which results in relief from the strained conformation. Finally, the intermediate reacts with the acceptor residue to complete the ADP-ribose transfer. This model fully explains the ADP-ribosylation reaction in the Ia-actin complex structure. In successive analyses of Ia-actin structures, no direct structural evidence has been found that the initial glutamate of the EXE motif on the ARTT loop of an iota-like ART can grip Arg177 of actin. However, in view of the structure analysis of C3-RhoA, we think that this type of interaction will occur transiently in the ADP-ribosylation reaction of also actin to allow the specific recognition of the amino acid to be modified. We would like to watch and trace whether the strain-alleviation model is applicable in other actin- and Rho-specific ARTs.

4 Cell Entry Mechanism Between Actin- and Rho-Specific ARTs

Structural studies of the B component of these binary toxins remain largely unknown, except for a partial structure of C2-II. However, from the sequence similarity between binary toxin B component and anthrax protective antigen (PA), it was postulated that similar cell entry events happen via the B component or PA. Recent advances in the study of the PA structure also provide a clue relating to the binary toxin B component. Anthrax toxin PA shares significant sequence identity with Ib (33.9 %) (Perelle et al. 1993, 1995) (Fig. 6). PA comprises four different domains, which are thought to be reflected in the structures of Ib and the other B components of clostridial binary toxins. PA mediates adenyl cyclase (LF) and metalloprotease (EF) entry into target cells. It binds to either of two cell surface receptors: capillary morphogenesis protein 2 (CMG2) or anthrax toxin receptor/tumor endothelial marker 8 (Petosa et al. 1997; Bradley et al. 2001; Scobie et al. 2003). Proteolytic activation by furin and furin-like proteases removes a 20 kDa *N*-terminal propeptide (Klimpel et al. 1992; Molloy et al. 1992). Activated PA_{63} self-associates to form a ring-shaped heptamer (prepore) (Milne et al. 1994; Petosa et al. 1997). The activated *N*-terminal domain [domain I′ (residues 168–258)] contains a binding site for EF and LF (Cunningham et al. 2002). Domain II (residues 259–487) has a β-barrel core structure and lines the heptamer lumen, and a large amphipathic loop between beta2 and beta3 strands (Domain IIs) is predicted to peel away from the domain II core and form an extended β-barrel to span the membrane (Benson et al. 1998; Nassi et al. 2002). Domain III (residues 488–595) is located on the outside of the heptamer and contains several key residues for oligomerization (Mogridge et al. 2001). Domain IV (residues 596–735) has been thought to function in host-cell receptor binding (Brossier et al. 1999; Varughese et al. 1999; Mourez et al. 2003; Rosovitz et al. 2003). The crystal structure of the PA monomer-CMG2 and PA prepore-CMG2 complex gave us invaluable information about how PA binds CMG2 via domain II and domain IV (Lacy et al. 2004; Santelli et al. 2004). Furthermore, the PA pore structure, which was determined at 2.9 Å by cryo-electron microscopy, confirmed the predicted structure, which is similar to other α-hemolysin and *Vibrio cholerae* cytotoxin structures, but there is a significant difference between them (Jiang et al. 2015). This structure delineates the protein translocation mechanism via a charge-state-dependent Brownian ratchet using a phi-clamp composed of Phe427 (Jiang et al. 2015).

The B components of iota-like toxins also undergo limited proteolysis, from the Ib precursor (Ibp) to Ib, to gain functional activity (Gibert et al. 2000). Activated Ib recognizes a specific cell surface receptor, then oligomerizes (heptamer) and mediates the binding of Ia (Papatheodorou et al. 2011). It has been shown that the C-terminal residues (domain IV: 656–665) of Ib are critical for cell surface binding (Marvaud et al. 2001). The cell surface receptor recognized by C2II and Ib is unique (Blocker et al. 2000; Eckhardt et al. 2000; Stiles et al. 2000). Recently, it was revealed that the Ib receptor is the lipolysis-stimulated lipoprotein receptor (LSR) (Papatheodorou et al. 2011). It has been reported that CDT and CST also use

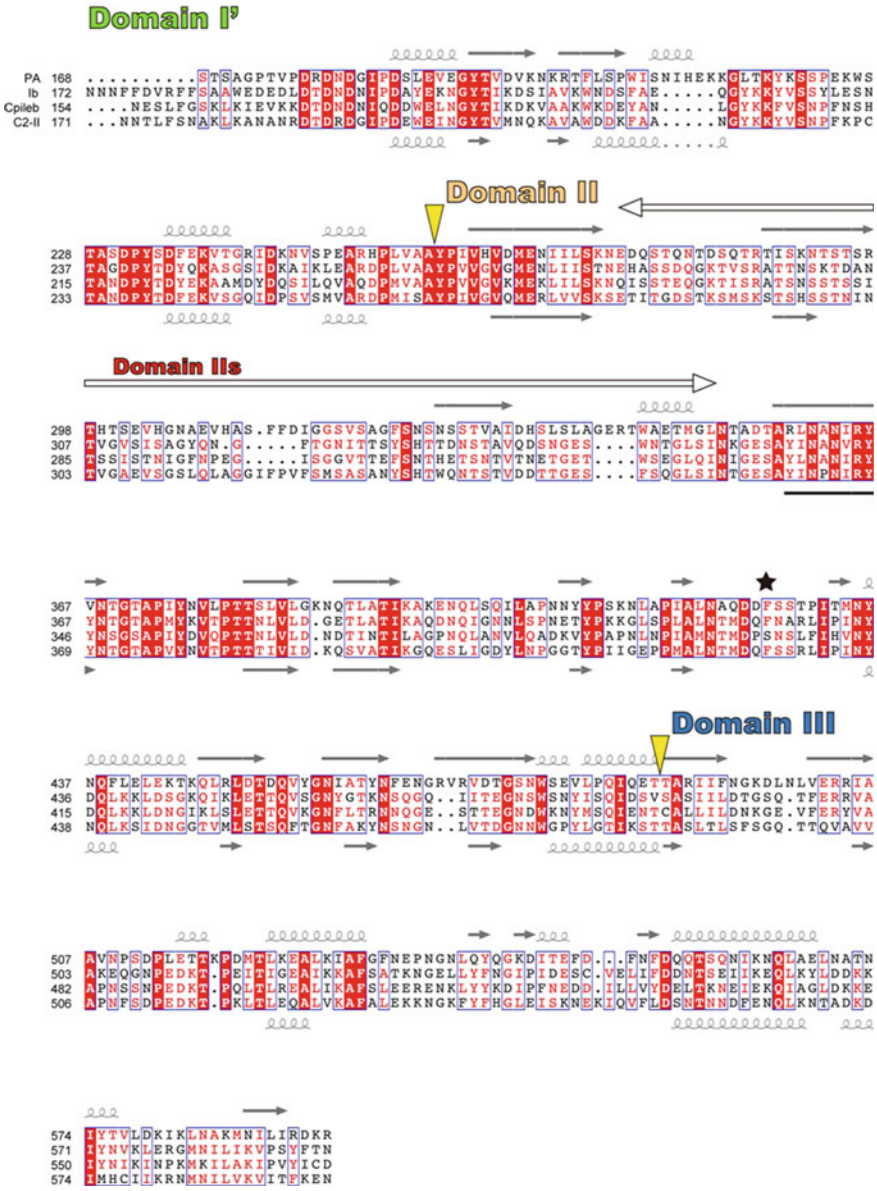

Fig. 6 Sequence alignment of domains I′, II, and III of PA, Ib, CPILEb, and C2-II. *Gray coils* and *arrows above* or *below* the sequence show the helices and β-strands, respectively. The secondary structure of PA_{83} monomer (PDB ID, 4H2A) is shown above the sequence. The secondary structure of C2-II monomer (PDB ID, 2J42) is shown below the sequence. *Yellow triangles* identify the borders between each domain. The *white double-headed arrow* shows the region of domain IIs. The *star* identifies the Phe residue forming the β-clamp

the LSR for the cell entry; however, C2 toxin does not (Papatheodorou et al. 2011, 2012). A previous study showed that an *N*-linked complex (or hybrid) carbohydrate is essential for C2 toxin binding (Eckhardt et al. 2000). This difference is explained by the low sequence similarity (12 %) of the C-terminal receptor-binding domain (domain IV) of Ib with the same region of C2II.

From the sequence similarity shared by the B component of binary toxin and PA, it is predicted that iota-like toxins undergo similar cell entry events, as shown in Fig. 7. The structure of C2-II is the only available structure of a binary toxin B component, up to now (Schleberger et al. 2006) (Fig. 7). The structure was solved at 3.1 Å resolution with the molecular replacement method using PA as the model. The structure includes three domains (domain I, domain II, and domain III), but domain IV is missing in the electron density. This means domain IV is highly flexible, which leads to unsatisfactory Rfree value (43.3 %). Domain II of C2-II has a compact structure to maintain the monomer (closed conformation) seen in PA_{63}. It is interesting that there were no large domain rearrangements between the crystal structure of C2-II obtained at pH 4.3 and the crystal structure of PA_{83} obtained at pH 6.0. The change in conformation from closed to open may require low pH and other factors, such as cleavage of the pro-region, oligomerization, and membrane binding. Future structural studies of binary toxins may provide answers for the following questions: (1) how the B component binds to the membrane receptor,

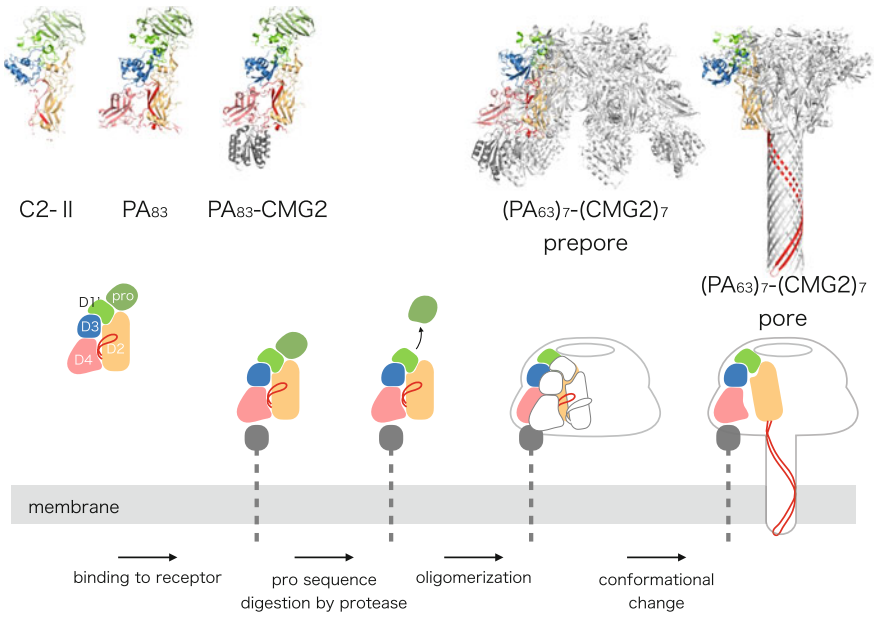

Fig. 7 Schematic model of the pore formation. Known structures and representing each step are shown as the schematic model. PDB IDs: C2-II monomer, 2J42; PA_{83}, 4H2A; PA_{83}-CMG2, 1T6B; $(PA_{63})_7$-$(CMG2)_7$, 1TZN; $(PA_{63})_7$ pore, 3J9C

(2) how the A component binds to the B component, (3) how different the prepore and pore structures of binary toxins are, and (4) the translocation mechanism of the A component. It would be interesting to know whether Ib does interact with LSR via domain II and domain IV as well as with PA-CMG2. Furthermore, we expect that these receptor interactions may enhance the stability of domain IV, allowing the determination of the full structure of an iota-like B component.

How about the cell entry mechanism of C3 exoenzyme? The cellular receptor and the exact endocytosis process have been unknown for a long time, because C3 lacks a cell binding and translocation domain. The C3 membranous binding partner is proteinaceous, and the glycosylation and phosphorylation state are critical for efficient C3 binding (Rohrbeck et al. 2014a, b). Recently, it was shown that the uptake of C3 in macrophage-like cells was inhibited by bafilomycin, which is an inhibitor of the vacuolar ATPase. This indicates the involvement of an acidic compartment during translocation (Fahrer et al. 2010). Poor cell accessibility has been overcome by generating fusion constructs that enhance cell entry. However, some cell types, such as macrophages, neutrophils, astrocytes, and neurons, are sensitive to C3. C3 at nanomolar concentrations is able to efficiently enter cells and cause the ADP-ribosylation of Rho, resulting in morphological changes (Holtje et al. 2011; Rohrbeck et al. 2012; Rotsch et al. 2012). Surprisingly, the intermediate filament component vimentin was identified as the cell surface receptor of C3bot (Rohrbeck et al. 2014a, b). C3 exclusively binds to the rod domain of vimentin, which comprises three domains: the head (1–101), rod (102–410), and tail (411–466). In this story, after C3 binds cell surface vimentin, C3 is endocytosed via vimentin-mediated endocytosis. Further analysis will be expected to explore the exact molecular mechanism of the C3–vimentin interaction.

5 Conclusion

Recently, non-typical ARTs were discovered using bioinformatics approaches (Simon et al. 2014). SpvB from *Salmonella enterica* and VahC from *Aeromonas hydrophila* lack B components; thus, SpvB is delivered via a type III secretion system, and VahC is delivered via a Type VI secretion system (Margarit et al. 2006; Shniffer et al. 2012). Both toxins ADP-ribosylate actin at Arg177; however, they belong to the subclass of iota-like toxins because they contain a 20-residue insertion (α3 helix) situated in the binding region of actin. Furthermore, novel insect large tripartite toxin complexes from *Photorhabdus luminescens*, which consist of the three components TcA, TcB, and TcC, have been identified and their structure solved using cryo-electron microscopy and X-ray crystallography (Gatsogiannis et al. 2013; Meusch et al. 2014). The toxin complex comprises the TcA cell binding and translocation component TcdA1, the TcB linker component TcdB2, and one of the TcC components TccC3 or TccC5. TccC3 and TccC5 are unique ARTs, which ADP-ribosylate actin at Thr-148 and Rho GTPase at Gln63/61, respectively (Lang et al. 2010). TcA forms a large, bell-shaped pentameric structure and enters the

membrane like a syringe, forming a translocation channel through which the cytotoxic domain is transported into the cytoplasm. This unique mechanism differs from the cell entry mechanism of PA or iota-like toxins, but it is intriguing that the cleaved TccC3 (ART domain) resides inside the cocoon before translocation and is not resolved in the structure, indicating the protein is either unfolded or in static disorder (Meusch et al. 2014).

Comparative studies of typical iota-like and C3-like toxins give important clues to understand the mechanism of ARTs, including these novel toxins. The two topics treated in this review (substrate recognition and cell entry mechanism) are important because their structural analysis is beyond *in silico* analysis (Simon et al. 2014). Substrate recognition or ART specificity can be divided into two meanings. One is recognition of the substrate protein, and the other is target residue recognition. The structures of the Ia-actin and C3–RhoA complexes are model structures for understanding both protein recognition and residue recognition. Residue recognition determines which residue is modified. In the C3–RhoA complex, the modified residue Asn is recognized by the Gln residue of the QXE-motif. Furthermore, we think this kind of recognition determines also the arginine specificity of the actin-specific ARTs. However, a variety of residues are the target of the ADP-ribosylation, including Cys, Thr, Glu, Asp, Gln, and Lys (Aravind et al. 2015). What drives the recognition and specificity of these residues? As an example, the unique DXE motif in TccC3 might be responsible for the recognition of Thr-148 of actin (Aktories et al. 2015). These questions will be answered by solving more structures of toxin-substrate protein complexes. On the other hand, we expect from future structural studies of the B component of binary toxins important contributions to our understanding of the cell entry mechanism of these ARTs.

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Pathogenic Mechanisms of Actin Cross-Linking Toxins: Peeling Away the Layers

Elena Kudryashova, David B. Heisler and Dmitri S. Kudryashov

Abstract Actin cross-linking toxins are produced by Gram-negative bacteria from *Vibrio* and *Aeromonas* genera. The toxins were named actin cross-linking *domains* (ACD), since the first and most of the subsequently discovered ACDs were found as effector domains in larger MARTX and VgrG toxins. Among recognized human pathogens, ACD is produced by *Vibrio cholerae*, *Vibrio vulnificus*, and *Aeromonas hydrophila*. Upon delivery to the cytoplasm of a host cell, ACD covalently cross-links actin monomers into non-polymerizable actin oligomers of various lengths. Provided sufficient doses of toxin are delivered, most or all actin can be promptly cross-linked into non-functional oligomers, leading to cell rounding, detachment from the substrate and, in many cases, cell death. Recently, a deeper layer of ACD toxicity with a less obvious but more potent mechanism was discovered. According to this finding, low doses of the ACD-produced actin oligomers can actively disrupt the actin cytoskeleton by potently inhibiting essential actin assembly proteins, formins. The first layer of toxicity is *direct* (as actin is the immediate and the only target), *passive* (since ACD-cross-linked actin oligomers are toxic only because they are non-functional), and *less potent* (as bulk quantities of one of the most abundant cytoplasmic proteins, actin, have to be modified). The second mechanism is *indirect* (as major targets, formins, are not affected by ACD directly), *active* (because actin oligomers act as "secondary" toxins), and *highly potent* [as it affects scarce and essential actin-binding proteins (ABPs)].

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Contents

1 Introduction

Enzymatic protein toxins are among the most potent effectors produced by bacteria to compromise immunity and viability of their neighbors, either predators or preys. Emergence of toxins coevolved with the ability of targeted organisms to neutralize them, leading to a never-ending chain of mutual adjustments and creating a pressure for exceptional efficiency of bacterial toxins. An unrivaled efficiency of bacterial toxins, when delivery of one molecule of a toxin can be lethal for an affected cell, is often achieved by targeting essential and scarce molecules, many of which are highly conserved and shared by numerous organisms across various kingdoms.

The actin cytoskeleton is a common target for numerous bacterial toxins for several reasons. The actin cytoskeleton is a complex and highly versatile structure involved in many aspects of host immunity as a motor, a substrate for other motors (i.e., myosins), and/or a structural cytoskeleton element. Therefore, using toxins that impair the actin cytoskeleton lowers the host immune barriers and makes the pathogenic bacteria less vulnerable, whereas hijacking actin polymerization machinery enables bacteria to move within the cell and spread between host cells entirely at the expense of the host. Since actin is a ubiquitous and highly conserved eukaryotic protein, an actin-targeting toxin, once emerged in evolution, can be used to target the cytoskeleton of diverse host organisms from amoebae and yeast to plants and humans.

In most cases, actin-targeting toxins affect a delicate equilibrium between monomeric and filamentous actin either by promoting or preventing actin polymerization. Both effects can be achieved by acting directly, i.e., on actin (Aktories et al. 2011), or indirectly, e.g., on signaling cascades and polymerization promoting factors (Lemichez and Aktories 2013). The latter group of toxins is large and

diverse, whereas all known toxins of the former group act via only three conserved covalent modifications of actin: (1) ADP-ribosylation of actin residue R177 (e.g., by *Clostridium* C2 and iota toxins, *Salmonella* SpvB), (2) ADP-ribosylation of actin residue T148 (by *Photorhabdus luminescens* TccC3 toxin), and (3) covalent intermolecular cross-linking of actin monomers between residues E270 and K50 into oligomers of various size (by ACD toxins of *Vibrio* and related species). The goals of the current review are to provide a historical perspective on the current state of our understanding of actin cross-linking pathogenic enzymes, their origin, the substrates they utilize, and the mechanisms of catalysis and toxicity.

2 Discovery of ACD

A toxin with an ability to covalently cross-link actin was first identified in pandemic strains of pathogenic *Vibrio cholerae* (Fullner and Mekalanos 2000). It was noticed that live attenuated strains of *V. cholerae* devoid of the major virulence factor, the ADP-ribosylating cholera toxin (CT), still caused mild-to-moderate diarrhea (Morris 2003; Levine et al. 1988). Hence, it had been proposed that additional toxins contribute to the pathogenesis of cholera disease as well as to a prevalence of the bacteria in the environmental reservoirs leading to high risks of cholera outbreaks upon transmission to humans through seafood and/or water sources. Particularly, treatment of a variety of cultured mammalian cells with *V. cholerae* resulted in the secretion of a soluble factor that rapidly induced cell rounding and complete loss of phalloidin-stainable cytoplasmic actin (Fullner and Mekalanos 2000). Western blot analysis of actin from affected cells showed the loss of monomeric actin and the formation of actin oligomers of various lengths (Fullner and Mekalanos 2000). *V. cholerae* strains with the deletion of the CT gene still induced cell rounding across many cell lines (Lin et al. 1999), suggesting that other effector proteins produced by the pathogen are responsible for this activity. These strains were also found to contain a toxin gene cluster encoding the repeat-in-toxin (RTX) family found in several pathogenic Gram-negative bacteria that produce a variety of exotoxins (Lin et al. 1999). Deletion of the *rtxA* gene resulted in loss of actin cross-linking activity (Fullner and Mekalanos 2000). To identify the domain responsible for the actin cross-linking, truncated fragments of the *rtxA* gene were fused to GFP and transfected into eukaryotic cells. A construct corresponding to the toxin's amino acids 1963–2375 was determined to be responsible for the cell rounding effect and the covalent cross-linking of actin and was therefore named the actin cross-linking domain (ACD; Sheahan et al. 2004). In the following studies, borders of the catalytically active part of ACD were narrowed to residues 1963–2301 (Durand et al. 2012; Geissler et al. 2009), but high-sequence homology between different ACD orthologs extends beyond this point, suggesting that the C-terminus may play an unknown but essential role in vivo.

3 ACD-Containing Toxins and ACD-Producing Organisms

3.1 ACD Protein Family

3.1.1 Originally Identified ACD Toxins

ACD was first discovered and characterized as an effector domain of two major toxin families from aquatic and human pathogens of *Vibrio* and *Aeromonas* species: (1) multifunctional auto-processing repeats-in-toxin (MARTX) toxin (Satchell 2011, 2015; Roig et al. 2011; Sheahan et al. 2004) and (2) valine–glycine repeat protein G1 (VgrG1) toxin (Pukatzki et al. 2007; Sheahan et al. 2004; Hachani et al. 2016).

The first family of ACD-containing toxins, MARTX, is encoded by the *rtxA* gene and characterized by a large size ($\sim$0.5 MDa), conserved glycine-rich repeats at the N- and C-termini, and a cysteine protease domain responsible for auto-processing and release of a variety of effector domains in the cytosol of host cells (Satchell 2007). Ten effector domains (including ACD) conferring distinct cytotoxic activities have been identified in the MARTX family, with different combinations of one to five domains being present in each individual toxin (Gavin and Satchell 2015). The emergence of *rtxA* variants is aided by horizontal gene transfer leading to the mosaic structure and a large variety of the effector domain regions of MARTX toxins (Roig et al. 2011; Kwak et al. 2011; Dolores and Satchell 2013). The remarkably large size and the characteristic amino- and carboxy-terminal repeat regions distinguish the MARTX toxins from other pore-forming RTX toxins (Satchell 2007). The essential role of the N-terminal repeats in translocation of the effector domains across the eukaryotic plasma membrane to the cytoplasm of target cells has been demonstrated (Kim et al. 2015), while it is proposed that both N- and C-terminal repeats are implicated in the formation of a putative pore in the target cell (Satchell 2007; Kim et al. 2015). Notably, *V. vulnificus* biotype 2 (BT2), besides a chromosomal *rtxA,* carries an additional plasmid-encoded *rtxA* copy (Valiente et al. 2008; Lee et al. 2008; Roig et al. 2011) allowing facilitated exchange of this toxic element between bacteria; while on the *V. cholerae* chromosome, similar (but not identical) ACD sequences are present in both *rtxA* and *vgrG1* genes (Sheahan et al. 2004).

The members of the second ACD-containing toxin family, VgrG, are components of a multiprotein secretion complex [the type VI secretion system (T6SS)], which serves to puncture membranes of target cells similar to bacteriophage tail-like injection machinery (Pukatzki et al. 2007). At the C-terminus, some VgrG proteins bear one of the distinct catalytic domains (ACD is among them), which is injected into the cytoplasm of the targeted cell (Hachani et al. 2016). Endocytosis of bacteria by eukaryotic host cells is required for the translocation of *V. cholerae* VgrG1 effector domain (i.e., ACD) into target cell cytosol to exert its cytotoxic activity (Ma et al. 2009).

Interestingly, while both MARTX and VgrG1 toxins have intricate translocation mechanisms for delivery of their effector domains into a host cell, a hypothetical protein from an environmental *Vibrio* sp. AND4 has been described as a putative stand-alone ACD with no associated translocation domains (Satchell 2009). It remains to be established whether this ACD retains its specific functional activity and whether/how it is delivered to a host cell. For example, it has been recently discovered that many bacterial effector proteins contain conserved MIX motifs, which mediate their delivery to host cells via interaction with components of the T6SS (Salomon et al. 2014). Similarly, transport of AND4 ACD into a host cell could be mediated via interaction with a yet to be identified delivery machinery.

3.1.2 Extended List of ACD Toxins

The current list of the ACD orthologs was updated using the Basic Local Alignment Search Tool [BLAST; specifically, protein–protein BLAST (blastp)]. In addition to previously recognized ACD sequences [MARTX and VgrG1 domains and a stand-alone ACD from *Vibrio* sp. AND4; (Satchell 2009, 2011, 2015)], the search revealed novel putative ACD-containing proteins (including some from unexpected sources) sharing 24–90 % identity with *V. cholerae* MARTX ACD ($ACD_{MARTXVc}$) (Fig. 1). Specifically, there are four hypothetical proteins from *Streptomyces* spp. containing a putative ACD-like domain sharing 56 % similarity (40 % identity) with $ACD_{MARTXVc}$. In three of them, the ACD-like domain is preceded by a domain homologous to the putrescine/ornithine antiporter PotE catalyzing the uptake and excretion of putrescine (Igarashi and Kashiwagi 1999). Furthermore, complementary to *Vibrio* sp. AND4 ACD, several putative individual ACD-like proteins from *Grimontia marina*, *Saccharothrix syringae*, *Actinokineospora inagensis*, *Hamadaea tsunoensis*, and *V. campbellii* have been identified. While *V. campbellii* ACD shares 80 % similarity (72 % identity) with $ACD_{MARTXVc}$, *H. tsunoensis*, *A. inagensis*, *S. syringae*, and *G. marina* ACDs are only 24–55 % identical to $ACD_{MARTXVc}$. In addition to previously described *V. cholerae* VgrG1 ($VgrG1_{Vc}$) (Ma et al. 2009; Pukatzki et al. 2006; Sheahan et al. 2004), ACD-containing VgrG toxins were found in *V. albensis*—a non-O1 serovar of *V. cholerae* (Hada et al. 1985) (99 % identical to $ACD_{VgrG1Vc}$) and *V. ordalii* (70 % identical to $ACD_{VgrG1Vc}$).

This search has also extended the list of *Vibrio* and *Aeromonas* species known to produce ACD-containing MARTX toxins (Fig. 1). Thus, in addition to *V. cholerae*, *V. vulnificus*, *V. splendidus*, and *V. anguillarum* (Roig et al. 2011; Satchell 2015), ACD domains were found in MARTX of two other *Vibrio* spp. (*V. ordalii* and *V. harveyi*). In *Aeromonas* spp., in addition to *A. hydrophila* (Roig et al. 2011; Satchell 2011; Suarez et al. 2012), ACD is an effector domain of MARTX toxins from *A. enteropelogenes* [aka *A. trota* (Huys et al. 2002)], *A. salmonicida*, and *A. dhakensis*. Moreover, *Photobacterium marinum* and *Moritella dasanensis* have emerged as

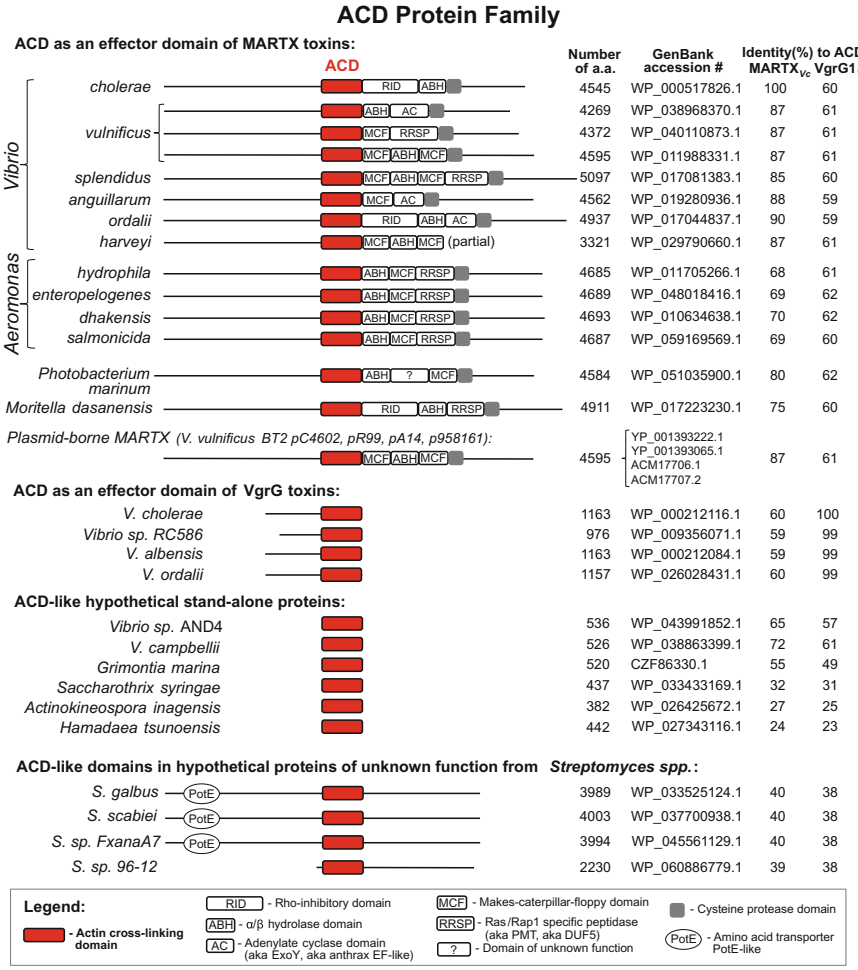


Fig. 1 ACD protein family. The NCBI protein BLAST search identified ACD orthologs present in different microorganisms either as part of larger proteins (both, already known toxins and previously uncharacterized proteins with ACD-like domains) or as stand-alone entities. Proteins are shown in scale with the total number of amino acids indicated for each protein. When available, reference sequence (RefSeq) non-redundant protein accession numbers are provided (O'Leary et al. 2016). The percent of identity of each protein with $ACD_{MARTXVc}$ and $ACD_{VgrG1Vc}$ is shown

novel members of MARTX-producing organisms with ACD as one of the effector domains homologous to $ACD_{MARTXVc}$ (75–80 % identity). This list of ACD-containing toxins will certainly grow as more sequences of bacterial genomes become available.

3.2 ACD-Producing Organisms

Vibrio and *Aeromonas* spp. producing ACD-containing toxins are Gram-negative pathogens causing human and marine life diseases. *Vibrio* spp. inhabit warm surface waters worldwide with at least ten species pathogenic to humans (Morris and Black 1985), among which *V. cholerae* and *V. vulnificus* are particularly important as the causative agents of infections associated with high mortality rates (Igbinosa and Okoh 2008). The majority of *V. cholerae* strains, except for the classical O1 biotypes, carry the *rtxA* gene (Chow et al. 2001; Dolores and Satchell 2013; Lin et al. 1999; Cordero et al. 2007). While CT is the major determinant associated with cholera disease in humans, non-O1 strains missing CT have been also associated with gastroenteritis, septicemia, and wound infections usually after consumption of contaminated shellfish or exposure of broken skin to contaminated water (Daniels and Shafaie 2000; Morris and Black 1985). Marine organism pathogens *V. vulnificus, V. anguillarum*, and *V. ordalii* are responsible for vibriosis, lethal hemorrhagic septicemic disease in fish (Schiewe et al. 1981; Frans et al. 2011; Amaro et al. 2015), while *V. vulnificus* is also associated with high-mortality-rate human infections typically linked to consumption of raw or undercooked oysters (Strom and Paranjpye 2000). *V. splendidus* is associated with mortality of juvenile oysters (Lacoste et al. 2001). *V. harveyi* and *V. campbellii* are marine fish and invertebrate (particularly, shrimp) pathogens (Austin and Zhang 2006) causing luminescent vibriosis, as many strains are bioluminescent (Defoirdt et al. 2008). Although considered to be a marine animal pathogen, *V. harveyi* has recently been found in human wound infections from seawater exposure (Hundenborn et al. 2013; Akram et al. 2015). Warming of sea surface and coastal waters, which enhances growth and persistence of *Vibrio* spp., has been proposed to contribute to the emergence of *Vibrio* infections worldwide (Vezzulli et al. 2013). *G. marina* isolated from Yellow Sea (Choi et al. 2012) is one of three known members of the genus, which was reclassified from *Vibrio* in 2003 (Thompson et al. 2003). The most famous specie of the genus is a human pathogen *Grimontia hollisae*, which causes severe gastroenteritis (Abbott and Janda 1994; Hinestrosa et al. 2007).

Aeromonas spp., psychrotrophs and mesophiles from aquatic and soil environment, cause human and marine animal diseases: gastroenteritis, septicemia, skin, and soft tissue infection in humans and furunculosis with hemorrhages and septicemia in fish (Janda and Abbott 2010). *P. marinum* is a novel Gram-negative member of the genus isolated from sediment samples collected from Palk Bay, India (Srinivas et al. 2013). *Photobacterium* spp. are mainly found in marine habitats living in symbiotic association with fish; some members of the genus are disease agents, while others are decomposers of dead marine organisms. *M. dasanensis* is an aerobic, motile, Gram-negative, cryo-protective ice-active substance (IAS) producing psychrophile (with the optimal growth temperature $\sim$9 °C) isolated from Arctic Ocean (Kim et al. 2008). Therefore, ACD is a part of MARTX toxins produced by mesophilic, psychrotrophic, and psychrophilic bacteria.

Finally, ACD-like proteins (sharing ~25–40 % identity with $MARTX_{Vc}$ and $VgrG1_{Vc}$ ACD toxins) were found in *Streptomyces, Saccharothrix, Actinokineospora,* and *Hamadaea* spp., which, in contrast to MARTX- and VgrG1-producing Gram-negative *Vibrio* and *Aeromonas* spp., are mainly saprophytic Gram-positive bacteria from the *Actinobacteria* phylum. Similar to many *Streptomycetes*, *Streptomyces galbus* is known to produce antibiotics (Paul and Banerjee 1983). *Streptomyces scabiei*, unlike the majority of *Actinobacteria*, is a plant pathogen causing common scab, an economically important potato disease (Lerat et al. 2009). Given relatively low homology with actin cross-linking ACD toxins, the role of the ACD-like domains in these bacteria as well as their ability to cross-link actin are unknown. It is an intriguing possibility that the ACD-like domain of *S. scabiei* may contribute to the common scab pathogenesis by acting on plant actin.

3.3 ACD Pathogenesis

3.3.1 Pathological Role of ACD-Containing Toxins in Animal Models

ACD-containing MARTX and VgrG1 toxins are recognized as important virulent factors contributing to the pathogenesis of infectious diseases of marine organisms and humans. Thus, in vivo actin cross-linking by $VgrG1_{Vc}$ has been associated with inflammatory diarrhea enabling replication of the bacteria within the intestine in an infant mouse model (Ma and Mekalanos 2010). A detectable level of actin cross-linking was evident in the intestinal tissue samples isolated from the infected mice. This is rather surprising given that $VgrG1_{Vc}$ can target the cytoplasmic actin only upon engulfment of the bacteria by phagocytic cells, which, therefore, are thought to be the toxin's primary targets (Ma et al. 2009). The authors speculated that transcytosis of the bacteria [i.e., transport across the interior of a cell (Yu 2015)] by intestinal epithelial cells can make them vulnerable to $ACD_{VgrG1Vc}$ toxicity. This hypothesis may also explain the observed abundant infiltration of the intestine by macrophages and other immune cells (Ma and Mekalanos 2010), since actin cross-linking compromises epithelial border by loosening epithelial tight junctions and allowing penetration by immune cells. This hypothesis also resonates with the recently proposed *amplified toxicity mechanism* of ACD pathogenesis (see Sect. 5.2).

In a murine lung infection model, $MARTX_{Vc}$ contributes to increased inflammation and tissue damage (Fullner et al. 2002; Haines et al. 2005), whereas in an intestinal model, MARTX promotes prolonged colonization of the mouse intestine by *V. cholerae*, most likely preventing clearance of the bacteria by inhibiting phagocytosis (Olivier et al. 2007a, b, 2009). A role of secreted accessory toxins (including MARTX) in the evasion of innate immune cells in the intestine has been proposed, since bacterial strains void of these toxin genes were efficiently cleared

from the intestine in the neutrophil-dependent manner (Queen and Satchell 2012). $MARTX_{Vc}$ has been linked to inflammasome activation in murine macrophages (Toma et al. 2010), but not in human monocytes (Queen et al. 2015). ACD-containing MARTX from *V. vulnificus* BT2 ($MARTX_{Vvbt2}$) is a lethal factor for eels, a virulent factor for mice, and can partially protect the bacteria from phagocytosis by eel phagocytes and murine macrophages (Lee et al. 2013). Overall, MARTX toxins are believed to play a role in the environmental fitness of *Vibrio* and related bacteria (Rahman et al. 2008). It has been proposed that $MARTX_{Vvbt2}$ and other ACD-containing MARTX toxins may promote survival of the bacteria in the environment by providing defense against natural predators, such as amoebae, and allowing them to infect marine animals (Lee et al. 2013). Yet, given that MARTX toxins carry several diverse effector domains, elucidation of the exact roles of individual effectors in the disease pathogenesis is a challenging topic that remains to be investigated.

3.3.2 Cellular Toxicity of ACD

Cytopathic role of ACD as an effector domain of MARTX and VgrG1 toxins has been illuminated in several studies, with $ACD_{MARTXVc}$ being the most studied. ACD causes cell rounding in a variety of cultured cells (Cordero et al. 2006; Dolores et al. 2015; Fullner and Mekalanos 2000; Heisler et al. 2015; Sheahan et al. 2004; Kudryashova et al. 2014b, 2015; Lin et al. 1999) due to adverse effects on the cytoskeleton without affecting the plasma membrane integrity (Fullner and Mekalanos 2000). In intestinal monolayers, ACD compromises the barrier function and causes rapid drop in trans-epithelial electrical resistance (TEER) due to the loss of integrity of the tight junctions (Dolores et al. 2015; Fullner et al. 2001; Heisler et al. 2015). In macrophages, ACD is sufficient to inhibit phagocytosis (Dolores et al. 2015). $ACD_{VgrG1Vc}$ is cytotoxic for phagocytic cells, e.g., *Dictyostelium* amoebae and mammalian macrophages (Ma et al. 2009; Pukatzki et al. 2006, 2007). ACD from *A. hydrophila* MARTX ($ACD_{MARTXAh}$), similarly to $ACD_{MARTXVc}$, induces host cell rounding (Suarez et al. 2012). Apoptosis reported upon $ACD_{MARTXAh}$ overexpression in transfected cells (Suarez et al. 2012) should be taken with caution due to the production of unrealistically high levels of the toxin, which are unlikely to be achievable under conditions of actual infections.

A progress in understanding of the roles of individual MARTX effector domains should be facilitated with a recent methodological development: a system, which allows modifying the *rtxA* gene on the *V. cholerae* chromosome and expressing the secretion- and translocation-competent MARTX toxin carrying a single effector domain or different domain combinations (Dolores et al. 2015). This new tool has enabled studying the individual effectors' roles without potential adverse effects pertinent to alternative delivery mechanisms, e.g., use of anthrax toxin delivery machinery (Cordero et al. 2006; Heisler et al. 2015) or protein overexpression upon transfection (Sheahan et al. 2004; Suarez et al. 2012).

4 Mechanism of the Cross-Linking Reaction

4.1 Substrate and Cofactors of ACD

In eukaryotic cells, actin exists in a delicately regulated equilibrium between monomeric (G-actin) and filamentous (F-actin) states. Shifting this equilibrium in either direction, whether via covalent modification of actin or by indirectly acting on actin-binding proteins (ABPs) and signaling cascades, is exploited by numerous bacterial toxins. In general, such shifts disorganize the cytoskeleton and compromise the immune barriers of the host via various mechanisms. In the study that linked the *rtxA* gene product with actin covalent cross-linking activity and cell rounding, it has been proposed that *only G-actin is the substrate of the toxin's enzymatic activity* (Fullner and Mekalanos 2000). The conclusion was reached based on the observation that the cross-linking prevailed even after disassembly of F-actin upon cell treatment with a small molecule drug cytochalasin D (Fullner and Mekalanos 2000) or, in a later study, with latrunculin B, whereas stabilization of the F-actin with dolastatin-11 completely abolished the formation of ACD-cross-linked actin oligomers (Cordero et al. 2006). The question was readdressed on a different level after the discovery of ACD as the effector domain within the $MARTX_{Vc}$ toxin responsible for the cross-linking (Sheahan et al. 2004). Use of a variety of factors stabilizing either G- or F-actin with purified actin and recombinant ACD in vitro unambiguously demonstrated that *monomeric, but not filamentous, actin is the substrate for the toxin* (Kudryashov et al. 2008a).

Covalent cross-linking of a host protein is the unique mechanism of toxicity pertinent solely to ACD toxins; therefore, neither the exact nature of the covalent bond nor the enzymatic identity of ACD could be deduced by comparison with a known analog. At the time of the discovery, sequence analysis of $ACD_{MARTXVc}$ showed no substantial homology to any other protein, but a putative *V. cholerae* protein later recognized as an effector domain of VgrG1 (Pukatzki et al. 2007; Sheahan et al. 2004). A hypothetical involvement of the host's own cross-linking proteins from the transglutaminase (TG) family was ruled out by using a TG-deficient cell line and demonstrating that purified actin can be cross-linked efficiently in the presence of a recombinant ACD (Cordero et al. 2006).

In addition to actin, two other factors, Mg^{2+} *cations* and *ATP*, appeared to be *required to support the reaction* (Cordero et al. 2006), but their exact role was obscured by the fact that both molecules have strong and specific influence on the structure of the substrate, i.e., actin. ATP is an irreducible integral part of actin structure, while binding of Mg^{2+} cations at the high-affinity site (in complex with ATP) and several low affinity sites promote conformational changes favoring actin polymerization. Furthermore, actin is an ATPase that hydrolyzes ATP upon polymerization. Hence, replacing ATP with a non-hydrolyzable analog, AMP-PNP, strongly reduced, but did not abolish the ACD-cross-linking reaction, possibly due to a residual ATP leakage from the nucleotide-binding cleft of actin (Cordero et al. 2006).

This uncertainty was resolved by using a complex of actin with gelsolin segment 1 (GS1)—a recombinant fragment of a human actin-binding protein, gelsolin. The ability of GS1 to block the nucleotide release from actin (Kudryashov and Reisler 2003; Bryan 1988) allowed to clearly separate the ATP prerequisite for actin versus that for the ACD-catalyzed cross-linking. Regardless of the nucleotide locked at the nucleotide cleft of actin (ATP or AMP-PNP), the presence of ATP in the experimental buffer was essential to support the cross-linking reaction (Kudryashov et al. 2008a). The fact that ACD requires ATP to fuel the cross-linking was further confirmed by demonstrating that the amount of inorganic phosphate released upon ATP hydrolysis in the course of the reaction directly correlates with the amount of new bonds formed between actin molecules (Kudryashov et al. 2008a).

4.2 Nature of the Cross-Link

The role of ATP in ACD-cross-linking revealed that ACD belongs to a class of ligases—the least common type of enzymes that utilize the energy of ATP hydrolysis for the formation of a new covalent bond. The type of the bond and the identity of the cross-linked actin residues were determined using a combination of several biochemical, analytical, and structural biology approaches. Specifically, limited proteolysis of isolated ACD-cross-linked actin dimers by several proteases demonstrated that the covalently linked residues are located in the peptides 46–68 and 227–375 (Kudryashov et al. 2008b). Crystallization of the dimers in complex with either DNase I alone or both DNase I and GS1 strongly suggested, but did not reveal explicitly, due to a structural disorder in the areas of interest, that the cross-linked residues are K50 and E270. Finally, extensive cleavage of the isolated dimers with trypsin and enrichment of the cross-linked peptides on strong cation-exchange microcolumns provided high-quality material for mass spectrometry. LTQ-FT mass spectrometry analysis unequivocally demonstrated that *E270 and K50 are indeed the cross-linked residues covalently linked by an iso-peptide bond*, i.e., amide bond between the side chain carboxylic and amine groups of the glutamate and lysine residues, respectively (Kudryashov et al. 2008b). This finding was further confirmed by mutagenesis on both yeast and mammalian actins. Thus, mutation of either of the two residues abolished cross-linking of purified yeast actin mutants, suggesting that the cross-linking reaction is highly specific and the identified actin residues are the only ones that can be cross-linked by ACD. Mixing together both individual mutants limited the cross-linking reaction at the level of actin dimers, as the only available E270 or K50, each provided by only one of the mutants, was consumed in the reaction and did not allow elongation of the chain. In mammalian cells, actins with either K50A or E270D mutations and myc-tagged for identification purposes formed shorter chains of oligomers due to their cross-linking to wild-type cellular actin; the mutant encompassing both mutations was not cross-linked by ACD (Kudryashov et al. 2008b).

4.3 *Structure of ACD and Its Homology to Glutamine Synthetases*

4.3.1 ACD Homology to Glutamine Synthetases

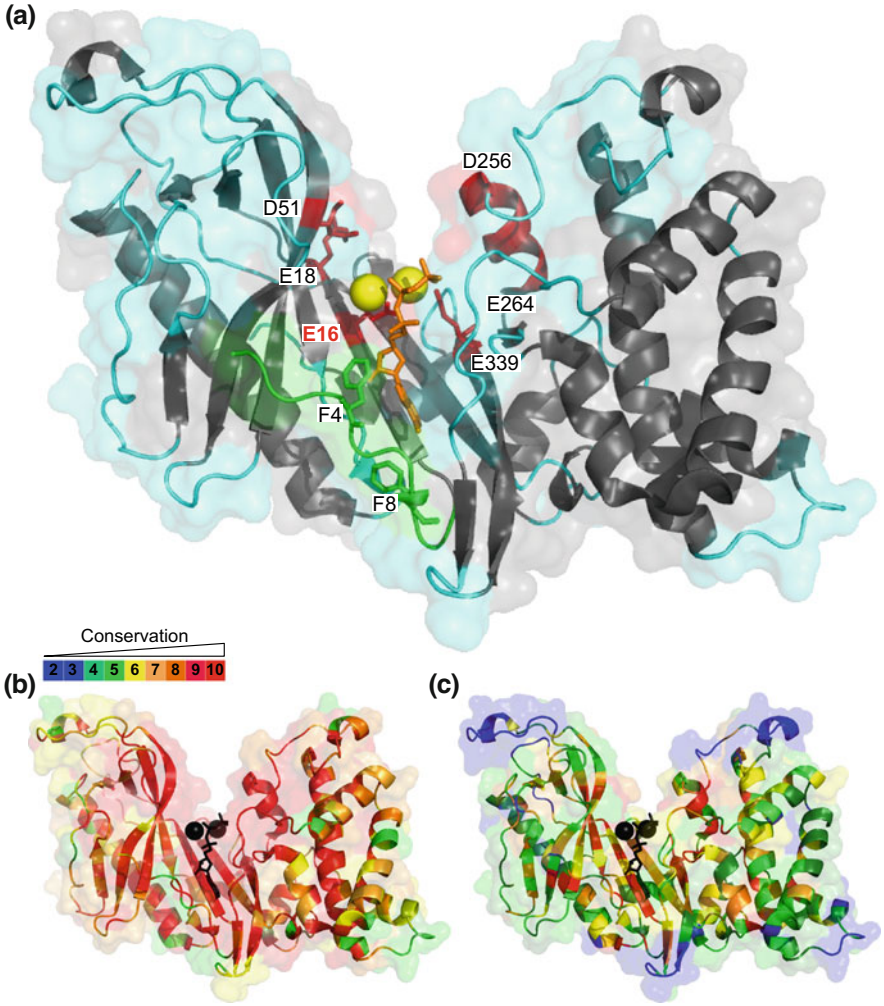
A lack of substantial sequence similarity to any known protein precluded homology-based identification of the ACD active site and called for different approaches. In an elegant and extensive study, Geissler and colleagues employed a combination of linker scanning mutagenesis and error-prone PCR mutagenesis approaches to test the cross-linking activity of ACD mutants on mammalian and yeast cell backgrounds (Geissler et al. 2009). This strategy identified (i) functionally essential regions with low tolerance to inserts and (ii) four residues involved in the catalysis (Fig. 2a; red sticks). These new constraints allowed to recognize the homology of the ACD active site to those of glutamine synthetase (GS) and glutamylcysteine synthetase (GCS) families of enzymes. These enzymes of amino acid biosynthesis covalently link glutamate to either ammonia (GS) or cysteine (GCS) using the energy of ATP hydrolysis. In both enzymes, binding of ATP to the active site is coordinated by Mg^{2+} or Mn^{2+} cations (Eisenberg et al. 2000; Orlowski and Meister 1971), in agreement with the biochemical data obtained for ACD (Cordero et al. 2006; Durand et al. 2012). Residues of GS/GCS enzymes involved in binding to ATP and Mg^{2+}/Mn^{2+} as well as residues essential for interaction with the substrate (i.e., glutamate) were found to be highly conserved in $ACD_{MARTXVc}$ (Geissler et al. 2009).

The first step in the reactions catalyzed by GS/GCS enzymes is the activation of glutamate by transferring the terminal phosphoryl group from ATP to the glutamate's side chain carboxyl with the formation of an acyl-phosphate intermediate (Midelfort and Rose 1976; Orlowski and Meister 1971). Based on the overall similarity of the active sites of these enzymes with the active site of ACD (Durand et al. 2012; Geissler et al. 2009), it had been first proposed and then confirmed experimentally (Kudryashova et al. 2012) that in a similar manner *ACD activates the side chain of E270 of one actin monomer for its subsequent cross-linking to the amine group of K50 on another actin molecule*. In vitro, treatment of K50C yeast actin mutant with ACD in the presence of γATP^{32} resulted in the incorporation of the radioactive phosphoryl group into the mutant. The resulted *acyl-phosphate derivative* can be detected as a radioactive band on an SDS-gel. The addition of the phosphoryl group to E270 was confirmed by the lack of radioactivity in the E270Q yeast actin mutant, while the role of the acyl-phosphate in the catalysis was corroborated by an observation that the radioactive phosphate was removed from E270 of K50C-actin upon its cross-linking to K50 of the E270Q-mutant with a formation of actin dimer (Kudryashova et al. 2012).

4.3.2 Crystal Structure of ACD

Initial attempts of several groups to crystallize $ACD_{MARTXVc}$ failed, while the homologous $ACD_{VgrG1Vc}$ was successfully crystallized and the structure solved at a 2.5 Å resolution (Durand et al. 2012). Structures were solved with ADP, ATP, AMP-PNP, or in the nucleotide-free state in the presence of either Mg^{2+} or Mn^{2+} and confirmed a high structural homology of the ACD active site with those of GS/GCS enzymes. ACD has a characteristic butterfly-like shape with two well-defined relatively flat domains ("wings") and the nucleotide positioned in the cleft between the domains (Fig. 2a). The N-terminal domain is largely composed of β-strands, while the C-terminal domain is largely α-helical.

At the core of the enzyme is an eight-stranded antiparallel β-sheet, six strands of which belong to the N-terminal domain and two others are contributed by the C-terminal domain, which also contains seven α-helices. One of these helices (residues 256–264 in ACD_{VrgGVc} crystallized sequence) is highly conserved in all ACD orthologs from MARTX and VgrG toxins and throughout different species as it comprises part of the catalytic site and is involved in coordination of the substrates (Fig. 2a; red helix). The ATP molecule (Fig. 2a; orange sticks) is bound in the "saddle" formed by the core β-sheet and coordinated by two divalent cations, which can be either Mg^{2+} or Mn^{2+} (Fig. 2a; yellow spheres). In the apo-state, the structure of ACD is stabilized upon binding of a conserved N-terminal peptide (Fig. 2a; shown in green) to the nucleotide cleft region between the domains. Given the overall low stability of ACD (ΔG = 11.5 kJ/mol; Kudryashova et al. 2014a), such stabilization can be critical for protein integrity in the absence of the nucleotide (e.g., upon secretion into extracellular milieu before entering into the host cell cytoplasm). Recombinant removal of the peptide destabilizes ACD to the extent that it cannot be produced in *E. coli* (Durand et al. 2012). In the presence of ATP, the peptide is displaced from the cleft, but in this state, it contributes to the formation of the front-bottom part of the nucleotide-binding pocket, particularly with its two highly conserved phenylalanine residues (Fig. 2a; green sticks) contacting the nucleoside moiety of ATP. It is tempting to speculate that the mechanism of protein destabilization by dislodging the N-terminal peptide from the cleft is essential for spontaneous unfolding of ACD toxins, critical for crossing the host cell membranes by MARTX effector domains via narrow pores. Notably, in all MARTX toxins containing ACD, it always precedes all other effector domains (Fig. 1), suggesting that it may play an important role in priming the unfolding of the entire effector domain region needed for the translocation process. This hypothesis is indirectly supported by the observation that the ACD domain of MARTX from psychrotrophic *A. hydrophila* is less stable (by ~10 °C) than its counterpart from mesophilic *V. cholerae*, whereas all other effector domains from MARTX toxins of both bacteria share similar thermal stability (Kudryashova et al. 2014a).



It is noteworthy that over 40 % of ACD residues are not folded in secondary structure elements and are represented by long loops connecting most of the β-strands and some α-helices (Fig. 2a; sown in cyan). The loops are not part of the catalytic site, but given that high interacting versatility is a common property of weakly ordered regions, they are likely to participate in binding to actin. Accordingly, most of the loops are highly conserved among the three toxins known to cross-link actin (ACDs of $MARTX_{Vc}$, $MARTX_{Ah}$, and $VgrG1_{Vc}$) as well as ACD domains of all other MARTX toxins, VgrG toxins, and stand-alone ACDs from *Vibrio* spp. (Fig. 2b). Therefore, it can be predicted that all of these ACDs are likely to share the same substrate, i.e., actin. High content of loosely ordered elements may also assist in protein unfolding required for crossing the membrane. Given the overall low degree

◀ **Fig. 2** Structure and conservation of ACD toxins. **a** PyMOL-generated image of the X-ray structure of $ACD_{VgrG1Vc}$ (PDB 4DTH; Durand et al. 2012). Four ACD active site residues (E16, E18, D51, and E339, numbered as in crystallized $ACD_{VgrG1Vc}$) critical for catalysis (Geissler et al. 2009) are shown as *red sticks*; essential catalytic residue E16 is highlighted in *red*. Highly conserved helix (residues 256–264) comprising part of the catalytic site is shown in red. N-terminal peptide is colored in *green*, with two highly conserved phenylalanine residues (F4 and F8; *green sticks*). ATP molecule is represented as *orange sticks*; Mg^{2+} cations are shown as *yellow spheres*. **b**, **c** The conservation scoring was performed using the PRALINE multiple sequence alignment program (Simossis and Heringa 2005) and color-coded on PyMOL-generated images of the X-ray structure of $ACD_{VgrG1Vc}$ (PDB 4DTH). Residues that are 100 % conserved across all compared sequences are colored in *red*; those with the lowest degree of conservation are in *blue* (color scheme is provided). ATP molecule and Mg^{2+} cations are shown in *black*. **b** Conservation of highly homologous ACD orthologs from all MARTX and VgrG1 toxins as well as stand-alone ACDs of *Vibrio* spp. (AND4 and *V. campbellii*). **c** Conservation of newly identified, distantly related ACD-like proteins of *Streptomyces* spp. and non-*Vibrio* stand-alone proteins from *S. syringae*, *Grimontia marina*, *A. inagensis*, and *H. tsunoensis*, as compared to each other and to $ACD_{VgrG1Vc}$

of homology of non-*Vibrio,* stand-alone ACD-like proteins, and ACD-like domains from *Streptomyces* spp., it is unlikely that actin is a substrate for these proteins, despite the active site residues being highly conserved in all of them (Fig. 2c).

4.3.3 Metal Cofactors

Out of the several divalent cations tested experimentally (Ca^{2+}, Mg^{2+}, Zn^{2+}, and Mn^{2+}) only Mg^{2+} and Mn^{2+} supported the cross-linking reaction (Cordero et al. 2006; Durand et al. 2012). However, one study reported that the $ACD_{VgrG1Vc}$ toxin was more active in the presence of Mn^{2+} (Durand et al. 2012), whereas another group found that $ACD_{MARTXVc}$ was notably more active in the presence of Mg^{2+} (Cordero et al. 2006). To resolve this uncertainty, we reexamined the specific activity of the ACD domains from $MARTX_{Vc}$, $MARTX_{Ah}$, and $VgrG1_{Vc}$ sharing 60–68 % identity and 74–80 % homology. We found that at concentrations of the divalent cations below 250 μM, all three ACD orthologs were more active in the presence of Mn^{2+}. The situation was completely reversed, however, at higher concentrations of the cations, as the reaction was strongly potentiated by Mg^{2+} concentrations above 1 mM, but strongly inhibited by equal concentrations of Mn^{2+}, either in the absence or presence of Mg^{2+}. As a result, the rates were 17- to 30-fold higher at 2 mM of Mg^{2+} as compared to the ones at equal concentration of Mn^{2+} (data not shown). Interestingly, similar inhibition by high Mn^{2+} was reported for brain avian and mammalian glutamine synthetases (Tholey et al. 1987; Yamamoto et al. 1987) suggesting that these properties are not accidental but rather dictated by a common enzymatic mechanism. Such dependence reflects a higher affinity of Mn^{2+} to both glutamine synthetases (Wedler et al. 1982) and ACDs, as confirmed in thermal denaturation experiments by a very prominent 8–10 °C raise in the enzyme stability in the presence of Mn^{2+}/ATP as compared to only 1–2 °C stabilization by Mg^{2+}/ATP (our unpublished data). Even though our results indicate higher activity of all three

ACD orthologs at 2 mM Mg^{2+} as compared to 2 mM Mn^{2+}, the opposite has been reported for $ACD_{VrgG1Vc}$ (Durand et al. 2012). Such discrepancy can be explained by underestimating actin polymerization as a factor that: (i) happens spontaneously in the presence of monovalent (K^+ or Na^+) and divalent (Mg^{2+} or Mn^{2+}) cations present in the experimental buffer and (ii) efficiently competes with the cross-linking reaction by depleting the substrate (i.e., G-actin). This supposition is indirectly confirmed by the fact that the reported activity of $ACD_{VrgG1Vc}$ was overall very low, but slightly higher in the presence of Mn^{2+} (Durand et al. 2012), tentatively reflecting weaker abilities of this cation to maintain actin in the filamentous state.

5 Layers of the ACD Pathogenesis: Toxicity Amplification by Actin-Binding Proteins

5.1 Role of G-Actin-Sequestering Proteins in the ACD Pathogenesis

The actin cytoskeleton is a complex hierarchy of interdependent structures brought together through regulated interactions of G- and F-actin with hundreds of ABPs. Taking these interactions into consideration is essential for understanding of pathogenic mechanisms triggered by actin-targeting toxins. Manipulation with the G/F-actin equilibrium in cultured cells and experiments with purified actin unequivocally pointed on G- rather than F-actin as the substrate for ACD. Yet, G-actin is not a likely physiological substrate as in the cell it is rarely found in a pure form. The monomeric pool of cellular actin is maintained by several ABPs, mainly profilin and thymosin-β4. Importantly, complexes of both proteins with actin can be cross-linked by ACD as efficiently or better than pure G-actin (Kudryashov et al. 2008a), likely owing to the ability of these proteins to inhibit spontaneous polymerization and thereby preserve the cross-linking-competent form of actin. Accordingly, both proteins bind to actin away from the E270 and K50 residues and do not interfere with binding of actin to ACD in the model proposed by Durand et al. (2012) (Fig. 3). Therefore, the actual physiological substrates of ACD are complexes of G-actin with one of the actin-sequestering proteins. In contrast to profilin and thymosin-β4, another essential actin partner, cofilin, strongly inhibited the cross-linking of monomeric actin, while accelerating the formation of oligomers from prepolymerized actin (Kudryashov et al. 2008a). Cofilin is both a G- and F-actin-binding protein, whose major cellular function is to promote high rates of actin dynamics via accelerated severing and depolymerization of aged filaments (Bernstein and Bamburg 2010). The facilitated cross-linking of polymerized actin can be explained by faster filament recycling (i.e., a higher amount of actin passing through the monomeric state per unit of time). The inhibited cross-linking of initially monomeric actin likely has two components: (1) the ability of cofilin to accelerate polymerization (and thus, promptly deplete the

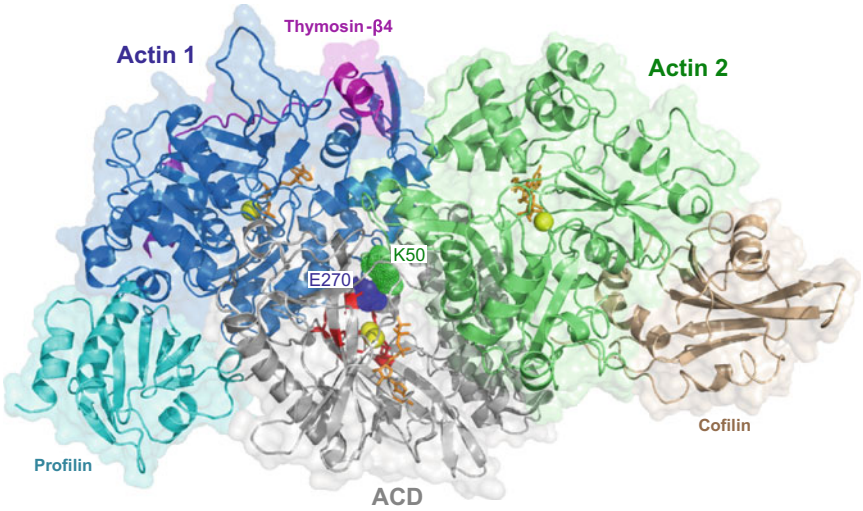


Fig. 3 Model of ACD binding to two molecules of the substrate (actins). As proposed and modeled by Durand et al. (2012), actin molecule donor of E270 (Actin 1 colored in *dark blue*) binds at one face of ACD (*gray*), whereas the actin molecule donor of K50 (Actin 2 in *green*) binds to the opposite face of ACD. The orientation of the actin monomers is such that the H-plug of one and the D-loop of another are positioned in the ACD nucleotide-binding cleft with K50 and E270 residues (*green and blue spheres*, respectively) oriented toward each other and in proximity to the terminal phosphoryl group of ATP (*orange sticks*). Notice that major G-actin-sequestering proteins profilin (*cyan*) and thymosin-β4 (*magenta*) as well as a G-/F-actin-binding protein cofilin (beige) do not clash with ACD, permitting cross-linking of physiologically relevant G-actin complexes. Four ACD active site residues (E16, E18, D51, and E339; Geissler et al. 2009) are shown as *red sticks*. ATP molecules bound to actin and ACD are represented as *orange sticks*; Mg^{2+} cations are shown as *yellow spheres*. The image was generated in PyMOL by aligning a model of $ACD_{VgrG1Vc}$ bound to two actin monomers (generously provided by Dr. Cambillau) with the following PDB structures: $ACD_{VgrG1Vc}$ (PDB 4DTH; Durand et al. 2012) and G-actin complexes with profilin (PBD 2BTF; Schutt et al. 1993), thymosin-β4 (PDB 4PL7; Xue et al. 2014), and cofilin (PDB 3DAW; Paavilainen et al. 2008). Profilin and thymosin-β4 are positioned at the same actin monomer for presentation purposes and do not represent an actual physiological complex

pool of monomeric actin) and (2) its tentative ability to allosterically induce unfavorable conformational changes in actin regions involved in cross-linking. The latter possibility is supported by a similar but less efficient inhibition observed in the presence of twinfilin (Kudryashov et al. 2008a), a protein related to cofilin, but lacking its ability to accelerate actin polymerization (Paavilainen et al. 2004). At the cellular level, the net effects of cofilin are expected to be promoting the ACD toxicity as only a small fraction of G-actin would be complexed with cofilin due to its low affinity to physiological ATP-G-actin; whereas its rapid dissociation from freshly depolymerized ADP-G-actin and the exchange of ADP to ATP are promoted by a coordinated action of profilin, Aip1, and Srv2/CAP proteins (Balcer et al. 2003).

5.2 *Role of Actin Assembly Factors in the Mechanisms of ACD Toxicity*

Early observations that actin cross-linking causes rounding of cells treated with MARTX-producing *V. cholerae* suggested that the resulted oligomeric species are not fully functional and cannot support the cell shaping functions of the actin cytoskeleton (Fullner and Mekalanos 2000). Subsequent in vitro experiments confirmed this conclusion by demonstrating that the ACD-cross-linked actin oligomers fail to polymerize and do not sustain stable filaments (Kudryashov et al. 2008b). The cross-linked E270 and K50 residues are located in the hydrophobic loop (H-plug) and DNase I-binding loop (D-loop) of actin, regions critical for the formation of inter-subunit interfaces in actin filaments (Fig. 4a). In all recent models of F-actin, the two residues are separated by 20–21 Å (Fig. 4a) and the

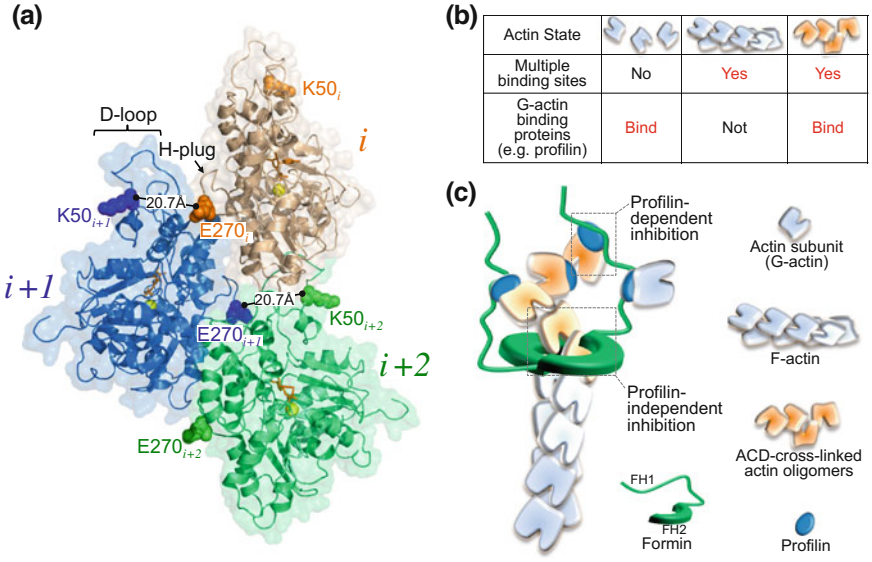


Fig. 4 Two layers of ACD toxicity. **a** Three individual actin subunits [*i* (*cream/orange*), *i* + 1 (*blue*), and *i* + 2 (*green*)] are shown in PyMOL-generated image of the F-actin structure (PDB 3J8I; Galkin et al. 2015). K50 and E270 residues of each actin subunit are labeled and represented by colored spheres (in accordance with the individual subunit coloring). In F-actin, E270 and K50 residues of two laterally adjacent subunits are separated by ~21 Å and their mutual reorientation driven by the ACD-catalyzed covalent cross-linking is not compatible with the formation of stable inter-subunit contacts. According to the *passive mechanism of toxicity*, accumulation of bulk quantities of non-polymerizable actin leads to a failure of all major functions of the actin cytoskeleton in the cell. **b** Actin oligomers possess a unique combination of properties that render their high affinity to multivalent G-actin-binding proteins, e.g., formins. **c** *Active mechanism of toxicity* direct binding of actin oligomers to FH2 domains of formins and multivalent, profilin-mediated binding to FH1 domains of formins confer high-affinity inhibitory interaction with sub-nanomolar affinities. **b** and **c** are adopted from supplemental materials of Heisler et al. (2015)

constraints applied by a zero-length covalent bond prevent the loops to form F-actin contacts. Therefore, the initial hypothesis that ACD acts by sequestering actin in the form of non-polymerizable oligomers (i.e., sequestering, or *passive mechanism of toxicity*) appeared to be confirmed by structural and functional in vitro assays.

However, two lines of thoughts prompted us to question the sequestering mechanism as the only and the most efficient one. *Firstly*, the potency of many bacterial toxins is such that they can harm/kill the cell when present in few or even a single copy [e.g., diphtheria, Shiga/verotoxin, botulinum, and tetanus toxins (Yamaizumi et al. 1978; Tam and Lingwood 2007; Gill 1982)]. Such outstanding efficiency is dictated by the need to overcome the host immune responses (Kudryashova et al. 2014b) and is achieved by acting on both essential and relatively scarce elements (signaling proteins, ribosomes, cytoskeletal elements of a synapse, etc.), cellular concentrations of which rarely exceed several micromolar. In striking contrast, actin is one of the most abundant proteins, present in the cell at hundreds of micromolar (Pollard et al. 2000). Therefore, achieving efficiency similar to other toxins would require much higher doses of an actin-sequestering toxin to be delivered to a cell. Thus, extrapolation of the ACD kinetic parameters from in vitro studies (Kudryashova et al. 2012) to cellular conditions suggests that a single ACD molecule per cell (~ 1 pM) would require several months to cross-link half of the cellular pool of actin. *Secondly*, we noticed that cross-linking of only a small fraction (<6 %) of the total cellular actin leads to a dramatic loss of the integrity of intestinal cell monolayers, evoking that ACD-cross-linked actin oligomers may exert an *active,* rather than passive, *toxicity mechanism* (Heisler et al. 2015).

To explain an unexpectedly *potent toxicity of the cross-linked actin*, we hypothesized that the *oligomers possess a unique combination of properties* that distinguish them from both G- and F-actin (Fig. 4b). Particularly, unlike F-actin, oligomers can bind to G-actin-binding proteins; and unlike G-actin, they can bind to several copies of such proteins or several G-actin-binding domains within the same protein complex. The resulting avidity of the ACD-produced actin oligomers to such proteins would be a product of individual affinities, i.e., orders of magnitudes higher than that of G-actin monomer (Fig. 4b). Several essential actin assembly factors possess an appropriate architecture to bind oligomers as they have several actin-binding domains organized in tandem (e.g., Cobl) and/or brought together by oligomerization (e.g., Spire, WASP, Ena/Vasp, and formins). In particular, *formins* are one family of such proteins governing the actin cytoskeleton dynamics behind numerous cellular processes, including *phagocytosis* (Colucci-Guyon et al. 2005) and *regulation of cell–cell contact stability within epithelial sheets* (Grikscheit and Grosse 2016). The main functional domains of formins, formin homology domains 1 (FH1) and 2 (FH2), cooperate in nucleation and elongation of actin filaments (Kovar 2006). A non-covalent FH2/FH2 homodimer nucleates an actin filament and remains at the polymerizing end to facilitate processive filament elongation (Fig. 4c). Tandem poly-proline stretches within the FH1 domains attract profilin–actin complexes, accelerating elongation as much as ten-fold (Kovar et al. 2006). In agreement with the proposed *active mechanism of toxicity*, it has been shown that

several human formins bind to the ACD-cross-linked actin oligomers with abnormally high affinity in cultured cells and that actin polymerization controlled by formins was inhibited by sub-nanomolar concentrations of actin oligomers (Heisler et al. 2015). Mathematical modeling of bulk actin polymerization using kinetic parameters extracted from a single filament-level imaging revealed that *the oligomers potently inhibit both nucleation and elongation steps of actin filament assembly controlled by formins* (Fig. 4c; Heisler et al. 2015).

6 Conclusions and Future Perspectives

Being tuned to their eukaryotic hosts through a long history of coevolution, bacterial pathogens and their toxins have been and remain invaluable tools that aid in our understanding of eukaryotic machineries. Most of the highly efficient toxins work on host substrates (often enzymes or enzymatic complexes) that are present in the cell in relatively low concentrations (signaling molecules, ribosomes, etc.). However, dealing with the highly abundant protein actin presents a major challenge for actin-targeting bacterial toxins to overcome in order to be efficient. This challenge is tackled by different toxins in various ways. Thus, many toxins amplify their efficiency by working not on actin per se, but modulating and/or mimicking activity of signaling molecules, such as Rho family of small GTPases. Another efficient strategy shared by many toxins is promoting actin polymerization, when a single molecule of a toxin can initiate assembly of thousands of actin monomers. On the other hand, toxins targeting monomeric actin have to engage other *toxicity amplification mechanisms*.

Recent studies discovered that the actin cross-linking toxin ACD employs a novel type of toxicity: Not only does it simply destroy a function of a targeted protein, but converts normal cellular proteins into potent toxins with a disruptive "gain-of-function" mode of operation. The ACD-produced actin oligomers are toxic because they can bind with abnormally high affinity and potently inhibit ABPs, formins. Formins are proteins that are essential, but much less abundant than actin and, therefore, represent a much potent target. This high affinity is achieved via multivalent binding of the oligomers to the regions on formins capable of binding several monomeric actins. Similarly to formins, many other actin assembly factors are capable to binding several molecules of G-actin, either to nucleate a new filament, or to increase local concentration of polymerization-competent actin monomers. Therefore, it is likely that additional layers of the ACD pathogenesis involving other actin-binding partners remain to be discovered.

In a broader prospective, the sophisticated pathogenic mechanism employed by ACD shows that on their way to efficiency, toxins not only can compromise existing pathways, but initiate new toxicity cascades (in this case with the de novo produced cross-linked actin species as "second messengers") with a disruptive "gain-of-function" mode of operation.

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ActA of *Listeria monocytogenes* and Its Manifold Activities as an Important Listerial Virulence Factor

Helena Pillich, Madhu Puri and Trinad Chakraborty

Abstract *Listeria monocytogenes* is a ubiquitously occurring gram-positive bacterium in the environment that causes listeriosis, one of the deadliest foodborne infections known today. It is a versatile facultative intracellular pathogen capable of growth within the host's cytosolic compartment. Following entry into the host cell, *L. monocytogenes* escapes from vacuolar compartments to the cytosol, where the bacterium begins a remarkable journey within the host cytoplasm, culminating in bacterial spread from cell to cell, to deeper tissues and organs. This dissemination process depends on the ability of the bacterium to harness central components of the host cell actin cytoskeleton using the surface bound bacterial factor ActA (actin assembly inducing protein). Hence ActA plays a major role in listerial virulence, and its absence renders bacteria intracellularly immotile and essentially non-infectious. As the bacterium, moving by building a network of filamentous actin behind itself that is often referred to as its actin tail, encounters cell–cell contacts it forms double-vacuolar protrusions that allow it to enter the neighboring cell where the cycle then continues. Recent studies have now implicated ActA in other stages of the life cycle of *L. monocytogenes*. These include extracellular properties of aggregation and biofilm formation to mediate colonization of the gut lumen, promotion and enhancement of bacterial host cell entry, evasion of autophagy, vacuolar exit, as well as nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) activation. These novel properties provide a new view of ActA and help explain its role as an essential virulence factor of *L. monocytogenes*.

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1 Introduction

Listeria monocytogenes is a gram-positive, motile and non-spore forming bacterium that is ubiquitously distributed in the environment and can survive extreme conditions of temperature, pH and salt (Murray et al. 1926; Vázquez-Boland et al. 2001). *L. monocytogenes* causes listeriosis, the clinical manifestations of which include meningitis, septicemia, gastroenteritis, and abortion. Ingestion of contaminated dairy products, salads, sausages and foods consumed without cooking or reheating can lead to listeriosis (Vázquez-Boland et al. 2001). Pregnant women, neonates, the elderly and immunocompromised adults are primarily at the risk of contracting infection. Although the incidence is low, listeriosis is one of the leading causes of mortality (ranging from 20 to 30 %) as a consequence of food poisoning (Lomonaco et al. 2015; Ramaswamy et al. 2007). The dose of infection is not known and the incubation period is often longer than 60 days (Lomonaco et al. 2015). *L. monocytogenes* is capable of growth in the cytosol of its host cells, under saprophytic conditions and at refrigerator temperatures (Vázquez-Boland et al. 2001).

L. monocytogenes is a facultative intracellular bacterium. It can infect and survive within macrophages, dendritic cells, epithelial cells, endothelial cells, fibroblasts, hepatocytes and neurons (Dramsi et al. 1998; Drevets et al. 1995; Gaillard et al. 1987; Guzman et al. 1995; Mackaness, 1962; Sun et al. 1990; Wood et al. 1993). The pathogen relies on several virulence factors for its intracellular survival. Following the current model of the intracellular life cycle of *L. monocytogenes*, internalin A (InlA) and InlB are the key players that regulate its internalization by host cells, albeit with different host cell specificities (Dramsi et al. 1995; Gaillard

et al. 1991; Khelef et al. 2006; Lecuit et al. 1999). Thus, InlA is specific in its interaction with the human host protein E-cadherin (CDH1), and InlB interacts with the c-Met receptor tyrosine kinase (MET) (Mengaud et al. 1996; Lecuit et al. 1999; Shen et al. 2000). In addition, the virulence protein (Vip), the invasion associated secreted endopeptidase (Iap) and listeriolysin O (LLO) are implicated in host cell entry (Cabanes et al. 2005; Kuhn and Goebel 1989; Vadia et al. 2011). Following internalization *L. monocytogenes* are enclosed within a single-membrane phagocytic vacuole (Tilney and Portnoy 1989). The cytolysin LLO of *L. monocytogenes* and its two phospholipases (PlcA and PlcB) are required for disruption of the phagocytic vacuole membrane, and thus facilitate its escape into the cytoplasm (Camilli et al. 1993; Gaillard et al. 1987; Geoffroy et al. 1987; Vázquez-Boland et al. 1992). Cytosolic bacteria multiply and recruit the host cell actin machinery by means of ActA, which enables their intracellular motility (Kocks et al. 1992). Motile *L. monocytogenes* infect neighboring cells wherein they are enclosed in a double-membrane phagocytic vacuole (Tilney and Portnoy 1989). This ActA-dependent cell-to-cell spreading event enables the bacterium to cross even the placental–fetal barrier (Bakardjiev et al. 2005). LLO, PlcA and PlcB help *L. monocytogenes* to escape out of the secondary phagocytic vacuole, and the cycle continues. Abundant research in the past two decades has established *L. monocytogenes* as a good model system to study the molecular mechanisms of host cell parasitism (Disson and Lecuit 2013).

2 ActA as a Virulence Factor of *L. monocytogenes*

Mutant bacteria unable to move intracellularly led to the discovery of ActA, a surface protein of *L. monocytogenes* that is responsible for bacterial movement in the cytosol and cell-to-cell spread. Since its discovery over 25 years ago, ActA has remained unique in that there are no known homologues present in any other bacterial species apart from *Listeria* (Domann et al. 1992; Kocks et al. 1992). The 639 amino acid ActA protein is encoded by the *act*A gene, and is composed of four distinct domains: (a) the residues 1–29 encode for a signal peptide required for directed transport of ActA to the bacterial membrane, (b) the N-terminal domain (amino acids 30–263) contains many cationic residues and is responsible for the regulation of actin assembly, filament elongation and interaction with F-actin, (c) the central domain (amino acids 264–423) harbors the proline-rich repeats that bind the actin-associated proteins vasodilator-stimulated phosphoprotein (VASP) and mammalian enabled protein (Mena), and (d) the C-terminal domain (amino acids 424–639) comprises of a hydrophobic region which attaches ActA to the bacterial surface (Vázquez-Boland et al. 2001) (Fig. 1). An ActA-negative *L. monocytogenes* mutant still escapes from the phagosome and replicates within the cytoplasm. However, as a consequence of its inability for actin recruitment this mutant strain forms microcolonies which are localized close to the nucleus (Domann et al. 1992).

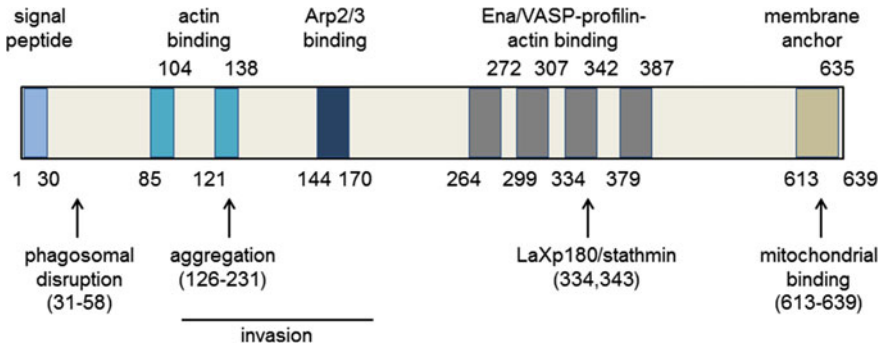

Fig. 1 The structure of ActA protein

L. monocytogenes is classified into at least 13 serotypes which are combined to four closely related lineages (I–IV) (Orsi et al. 2011). *act*A is present in all lineages (Doumith et al. 2004; Tsai et al. 2011). Bacterial strains belonging to lineage I and III exhibit the truncation of an identical repeat within the *act*A sequence in comparison to a strain of lineage II. This kind of truncation occurs in 77 or 51 % in strains of the serotype 1/2b and serotype 4b (lineage I) and in 7.5 % in strains of the serotype 1/2a (lineage II) (Hain et al. 2012). The polymorphism of *act*A gene was also shown in *L. monocytogenes* strains isolated from food and working environments (Conter et al. 2010). Moreover, the production levels of ActA differ between several *L. monocytogenes* serotypes of different lineages grown in selective as well as in nonselective medium (Lathrop et al. 2008). A functionally analogous gene from *L. ivanovii,* a pathogen of animals, shares only 46 % amino acid sequence homology with the ActA of *L. monocytogenes* (Gerstel et al. 1996; Kreft et al. 1995).

Recent studies have described novel roles and functions of ActA, and it has now been proposed to be involved in nearly all the stages of the intracellular life cycle of *L. monocytogenes* (Fig. 2). ActA can mediate *L. monocytogenes* entry into host cells by binding to the cell surface heparan sulfate proteoglycans (HSPGs) (Alvarez-Domínguez et al. 1997; Suárez et al. 2001). ActA has also been implicated in the rupture of the phagocytic vacuole membrane and provides a major mechanism for autophagic evasion (Poussin and Goldfine 2010; Yoshikawa et al. 2009). Moreover, ActA causes aggregation of *L. monocytogenes* in the cecum and colon lumen of mice, thereby it favors long-term intestinal colonization and transmission (Travier et al. 2013). The aim of this article is to collate and summarize the multifarious roles and functions of the ActA protein as an essential virulence factor of *L. monocytogenes*.

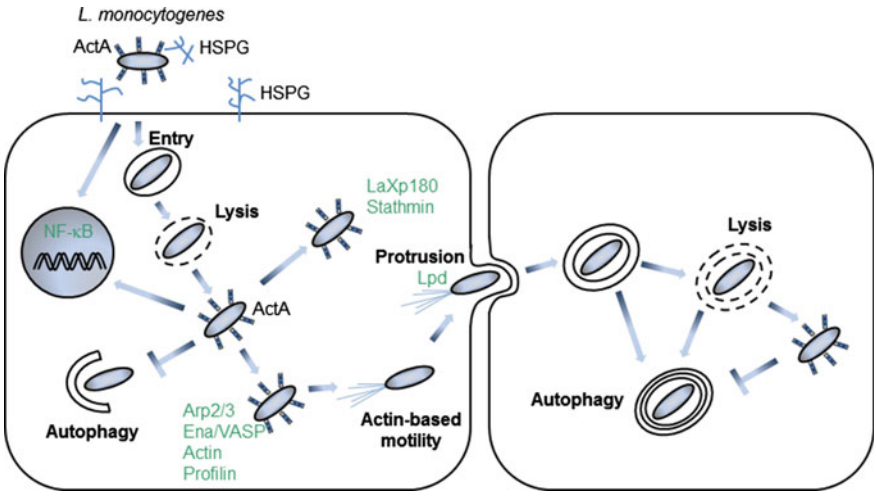

Fig. 2 The role of ActA in the intracellular cell cycle of *L. monocytogenes*. *L. monocytogenes* enters the host cell mainly by InlA and InlB. In addition, ActA interacts with heparan sulfate proteoglycans (HSPGs) leading to bacterial uptake. LLO, PlcA and ActA promote lysis of the formed vacuole. Within the cytoplasm, the pathogen expresses ActA which recruits Arp2/3, Ena/VASP, actin and profilin mediating actin-based motility and protrusion formation, a process which is promoted by interaction of actin with lamellipodin (Lpd). Invasion into neighboring cells results in entrapment of bacteria in a double-membrane vacuole which is lysed by LLO, PlcA, PlcB and ActA. Thus, a new infection cycle starts. Furthermore, ActA recruits the host protein LaXp180, which interacts with stathmin, allows autophagosomal escape and activates NF-κB

3 *act*A Expression and Its Localization

3.1 Regulation of act*A Expression*

Expression of *act*A is fine-tuned and it is dependent on growth conditions such as glucose and salt concentrations as well as buffering capacity. Thus, selective media (buffered *Listeria* enrichment broth, University of Vermont medium and Fraser broth) promote ActA expression of several *L. monocytogenes* strains whereas a subset of these strains express ActA to a lower extent in a nonselective media (Luria-Bertani and brain heart infusion) (Lathrop et al. 2008). In addition, iron, which is essential for bacteria and not available in the host in a free form, decreases the mRNA levels of *act*A (Conte et al. 2000). The expression of ActA is also determined by the growth temperature being low at 4 °C and high at 37 °C (Lathrop et al. 2008). Better production was also demonstrated in a study comparing the *act*A levels of bacteria growing at 30 °C to that present in the intestinal lumen (Toledo-Arana et al. 2009). The transcription levels of *act*A further increase when *L. monocytogenes* is located in the blood and is elevated when the pathogen is growing intracellularly in host cells (Moors et al. 1999; Toledo-Arana et al. 2009).

These reports illustrate that during the transition from the environment to the host, the expression of *act*A is induced.

The weak expression of *act*A at low temperatures is related to its transcriptional regulation. The *act*A gene is located in the virulence gene cluster (*vgc*) consisting of *prf*A, *plc*A, *hly*, *mpl*, *act*A and *plc*B (Chakraborty et al. 2000). The master transcriptional regulator of these genes is PrfA (Leimeister-Wächter et al. 1990; Mengaud et al. 1991). Expression of PrfA is thermoregulated where it is not expressed at temperatures below 30 °C (Johansson et al. 2002). The levels of PrfA increase during exponential phase as well as in the beginning of the stationary phase at 37 °C (Johansson et al. 2002; Mengaud et al. 1991). Transcriptional regulation of *act*A occurs at two promoters: both upstream of the *mpl* gene and upstream of the *act*A gene. The polycistronic messenger deriving from the *mpl* promoter contributes to the expression of *act*A when bacteria grow extracellularly while the *act*A promoter is primarily involved in promoting expression of *act*A during its intracellular growth (Shetron-Rama et al. 2002). Hence, the $actA_p$ promoter represents one of the most exquisitely regulated promoter in the transition from extra- to intra-cytosolic growth. Activation of PrfA enables the expression of *act*A (Port and Freitag 2007; Toledo-Arana et al. 2009). However, at lower temperatures, for instance at 23°C, expression of *act*A requires an additional transcription factor: the alternative sigma factor σ^B. Strikingly, ActA expression is involved in σ^B activity (Tiensuu et al. 2013). Thus, *act*A expression is regulated by σ^B when bacteria are found in the environment and by PrfA when the pathogen enters the host.

3.2 *Modification of ActA*

During extracellular growth of *L. monocytogenes*, phosphorylation of the ActA protein occurs at five serine residues located in its C-terminal region at S356, S365, S470, S482 and S546, respectively. Phosphorylation of S470 is mediated by the protein kinase Lmo1820 (Misra et al. 2014). It is not known which protein kinase/kinases phosphorylate the remaining serine residues. In addition, the role of this modification is not clear to date.

Phosphorylation of ActA takes also place when *L. monocytogenes* replicates within the host. The modification of ActA results in generation of three distinct ActA forms (Brundage et al. 1993). In contrast to the phosphorylation sites of ActA in bacteria grown extracellularly, intracellular phosphorylation of ActA takes place at S155 and S157. Phosphorylation at S155 occurs via the host cell serine/threonine casein kinase 2 (CK2). This modification is essential for intracellular motility (see below) (Chong et al. 2009).

3.3 Localisation of ActA

The C-terminal region of ActA (amino acids 613–635) comprises a membrane spanning region that is also associated to the peptidoglycan bacterial surface (Garcia-del Portillo et al. 2011; Kocks et al. 1992). In addition, ActA is released into the supernatants of bacterial cultures which might be due to a proteolytic cleavage of the membrane bound protein (Garcia-del Portillo et al. 2011). Curiously, eukaryotic cell-based expression of ActA showed that it is targeted to mitochondria which are subsequently decorated by components of the host cell cytoskeleton such as α-actinin. The mitochondrial targeting signal of ActA is located at the C-terminal 26 amino acids which displays motifs characteristic of mitochondrial import targeting sequences (Pistor et al. 1994).

During intracellular growth, ActA is concentrated at one pole of the bacterial surface. This polar distribution is essential for intracellular motility where actin nucleation is initiated at one pole of the bacterium to propel its movement (Dabiri et al. 1990). During bacterial growth ActA is disseminated asymmetrically on the surface and is absent from the site of septation (Kocks et al. 1993). Thus, ActA must move towards the "old" pole. Indeed, as described by Rafelski and Theriot (2006) there are specific steps in the localization during bacterial growth that can be divided into four stages (I–IV). Stage I that is characterized by the appearance of one to four precise spots predominantly along the sides of bacteria occurs after 20 min of growth. Stage II, which follows 30–90 min later, is defined by irregular accumulation of ActA in larger patches along the sides of bacteria. The coverage of almost the whole bacterial surface with ActA is described as stage III. Further bacterial cell division is accompanied with the growth of the peptidoglycan subsequently pushing surface located ActA to the "old" pole of the bacterium. This phase is defined as stage IV and it is achieved over several generation times of the bacteria. Thus, the polarization of ActA is dependent on the movement as well as peptidoglycan growth and explains why ActA may not be observed at the region of septation (Rafelski and Theriot 2006).

4 ActA Interaction with Actin

4.1 ActA-Mediated Intracellular Movement

When ActA had first been identified, the presence of proline-rich motifs similar to those present in the actin-binding protein vinculin was noted (Domann et al. 1992). Subsequently it was established that this singular factor ActA, when present on the surface of bacteria, mitochondria or latex beads, displayed all of the properties required for actin-based motility (Cameron et al. 1999; Domann et al. 1992; Kocks et al. 1992; Pistor et al. 1994). Landmark experiments with this system revealed that

it required the insertion of newly polymerized actin at the bacterial surface, which was considered the driving force for propulsion.

Initial studies with intracellular bacterial motility mediated by ActA led to the discovery of many host-cell actin-binding proteins that were present in the bacterial comet tail. Notable discoveries included the identification of the actin-related protein (Arp2/3) complex, the enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) and the actin-related proteins (Arps) as important motility motifs (Chakraborty et al. 1995; Lasa et al. 1997; Welch et al. 1997, 1998) (Fig. 1). These experiments also provided information on the dynamic interactions with host cytoskeletal proteins. Within the cytosol, actin tails contained α-actinin, which was not present in the actin tails seen in cellular protrusions primarily composed of short branched arrays of actin filaments (Dabiri et al. 1990).

Major findings were made using cell-free extracts enriched in cytoskeletal components, which were uncharacterized at that time. Further biochemical characterization and purification led to the discovery that ActA was an activator of the activity of the Arp2/3 complex, which initiates branches from the sides of existing actin filaments but by itself is a weak catalyzer of actin assembly (Goley and Welch 2006; Welch et al. 1997). The Arp2/3 complex regulates the initiation of actin polymerization as well as the organization of formed filaments. This complex consists of seven subunits: Arp2, Arp3, actin-related protein complex protein-1 (ArpC1), ArpC2, ArpC3, ArpC4 and ArpC5 (Goley and Welch 2006). Arp2, Arp3, ArpC1, ArpC2, ArpC3 and ArpC4 are required for ActA-driven actin tail formation, whereas ArpC5 is dispensable. ArpC4 is probably only necessary during initial stages of actin polymerization and not at the stage of tail elongation (Kühbacher et al. 2015). Interaction of ActA with the Arp2/3 complex occurs via its N-terminal region (amino acids 144-170) thereby nucleating actin polymerization (Skoble et al. 2000; Welch et al. 1998; Zalevsky et al. 2001). In more detail, two arginine residues within the 146-KKRRK-150 sequence of ActA are crucial for the binding of Arp2/3 (Pistor et al. 2000). The Arp2/3 affinity for binding to ActA is dependent on the eukaryotic serine/threonine kinase CK2, which phosphorylates ActA on serine 155 and 157 (Chong et al. 2009). In addition, the formation of the so-called actin cloud by the Arp2/3 complex is promoted by collapsin response mediator protein-1 (CRMP-1), which binds both actin as well as ActA (Yu-Kemp and Brieher 2016).

Binding of monomeric actin molecules also occurs via two regions: amino acids 85–104 and 121–138 (Skoble et al. 2000; Zalevsky et al. 2001). Actin-monomer binding is crucial for the nucleation activity with the Arp2/3 complex (Skoble et al. 2000).

Ena/VASP proteins, Mena, VASP and Ena/VASP-like (EVL), regulate actin dynamics. They contain three structural domains: the N-terminal Ena/VASP homology 1 (EVH1), the proline-rich domain and the C-terminal EVH2. EVH1 binds to a motif with the consensus sequence (D/E)FPPPPX(D/E)(D/E). The proline-rich region allows the recruitment of profilin, an actin-monomer-binding protein. The EVH2 exhibits a G-actin-binding site (GAB), an F-actin-binding site (FAB) and a coiled-coil mediating tetramerization (Bear and Gertler 2009). Binding

of Ena/VASP is mediated by its EVH1 domain to four E/DFPPPPXD/E motifs located in the proline-rich region of ActA (Niebuhr et al. 1997). The amino acids F335, P336, P339, E343 and L344 are essential for the high affinity binding of EVH1 to ActA (Ball et al. 2000). Interaction of ActA with Ena/VASP enables the recruitment of profilin via the proline-rich domain of VASP (Kang et al. 1997; Smith et al. 1996). The subsequent interaction of profilin with actin monomers, the presence of the G-binding site of Ena/VASP as well as the phosphorylation of Ena/VASP are essential for efficient filament elongation (Geese et al. 2002; Grenklo et al. 2003). Direct interaction of Ena/VASP to F-actin is dispensable for intracellular *L. monocytogenes* movement (Geese et al. 2002). However, bacteria lacking the EVH1 binding sites in ActA generally form only actin clouds or have very short actin tails suggesting defective intracellular motility. Subsequent studies have shown that Ena/VASP proteins are required for increasing motility of intracellular bacteria as demonstrated by dispersed bacteria harboring long actin tails (Niebuhr et al. 1997). The so formed actin tails achieve a length of up to 40 microns and allow the pathogen to move with a speed of 0.12–1.46 microns/sec within the host cell (Dabiri et al. 1990). The speed of *L. monocytogenes* is enhanced when the degree of ActA polarity and the amount of ActA is increased (Rafelski et al. 2009).

4.2 ActA-Triggered Spread to Neighboring Cells

Infection of macrophages by *L. monocytogenes* in vivo enables its spread to distant organs including the liver, spleen and brain (Disson and Lecuit 2013). Tissue culture models employing both phagocytic and non-phagocytic cell lines have been successfully employed to demonstrate spread of *L. monocytogenes* by cell-to-cell spread (Dramsi et al. 1998; Bakardjiev et al. 2004). For cell-to-cell spread, contact between epithelial structures is required to support the formation of membrane protrusions that extend into the adjacent cell allowing the bacteria to extrude into the neighboring cell (Tilney and Portnoy 1989). *L. monocytogenes* cell–cell contact is facilitated by InlC, which specifically interacts with the scaffold protein Tuba (DNMBP; dynamin binding protein), a protein that links dynamin to actin regulatory proteins such as N-WASP and Cdc42. Disruption of the interaction of Tuba with the N-WASP protein weakens the cortical actin tension required to maintain the integrity of the junctional membrane (Rajabian et al. 2009). Ultrastructural studies of *L. monocytogenes* infected cells revealed that unlike the branched network of the cytosolic tails found in the cytoplasm, actin filaments in the protrusions are short and branched only proximal to the bacteria (Sechi et al. 1997). Further studies have confirmed this finding indicating that actin is present throughout the protrusions, whereas the Arp2/3 complex is almost exclusively detected at the bacterial pole (Fattouh et al. 2015).

Accessory factors, apart from those involved in actin assembly, are also found in these membrane protrusions. Thus ezrin, a member of the ERM (ezrin-radixin-moesin) family of cytoskeletal proteins, is specifically found in *L. monocytogenes* protrusions,

where it participates in determining length and shape of the protrusion (Sechi et al. 1997; Temm-Grove et al. 1994). The ERM proteins are a class of highly homologous proteins involved in linking the plasma membrane to the cortical actin cytoskeleton such as those found in membrane protrusions (Ponuwei 2016). Additionally it was shown that myosin X also locates to these protrusions and is required for cell-to-cell spread (Bishai et al. 2013). Lamellipodin (Lpd), a host cell Ena/VASP binding protein, which is involved in lamellipodial protrusion, interacts with *L. monocytogenes* at late stages of infection via EVH1 domain with ActA-bound VASP (Krause et al. 2004; Wang et al. 2015). This interaction determines the protrusion formation and therefore the cell-to-cell spread of the pathogen (Wang et al. 2015).

One of the consequences of the recruitment of host cell actin cytoskeletal components is its effect on bacterial intracellular growth and division. Polymerization of host cell actin extends the non-dividing elongation period of the bacteria and forces cell division to occur within a relatively short period. Thus the bacteria appear to be able to operate a positive feedback loop that efficiently regulates the transition between cell division and optimal actin tail formation (Siegrist et al. 2015). The sensory cues governing this process are presently unknown.

5 Interaction of *L. monocytogenes* with Stathmin via ActA

The proline-rich region of ActA is also involved in binding of retinoblastoma 1 inducible coiled-coil 1 (RB1CC1, also known as CC1 or LaXp180). However, interaction with LaXp180 is only efficient when the proline-rich domain is co-expressed with either the N-terminal domain or the C-terminal domain (Pfeuffer et al. 2000). LaXp180 regulates cell growth, cell proliferation, cell survival, and cell spreading/migration via binding to several proteins (Gan and Guan 2008). One LaXp180 interaction partner is stathmin (Maucuer et al. 1995). Stathmin is a cytosolic phosphoprotein involved the regulation of microtubule dynamics by preventing assembly and promoting disassembly of microtubules (Curmi et al. 1999). *L. monocytogenes* binds stathmin via LaXp180 in an ActA-dependent manner. Interestingly, LaXp180 binding to ActA occurs only on one pole of the pathogen and this interaction is mutually exclusive with actin polymerization (Pfeuffer et al. 2000). This is consistent with a report showing that the amino acids E334 and E343 of the proline-rich region are also involved in LaXp180 binding (Bauer et al. 2003). VASP binds to the amino acids E336 and E337 and this interaction is enhanced by E343 and E344 (Ball et al. 2000). Thus, both proteins compete for the same amino acid, namely E343, in ActA for their binding explaining the mutually exclusive localization of LaXp180 and actin polymerization at opposite poles of the cell (Bauer et al. 2003). Presently it is not known whether LaXp180 and its interaction with actin regulatory proteins are also involved in cell-to-cell spread.

6 ActA and Autophagy

6.1 The Autophagic Machinery

Autophagy is an evolutionarily conserved eukaryotic cellular degradation mechanism whereby cargo is cloistered in a double-membrane vesicle known as autophagosome and subsequently degraded by lysosomal hydrolases. Damaged cellular organelles, protein aggregates, lipid droplets and intracellular pathogens constitute autophagy cargo (Wen and Klionsky 2016). Several factors can trigger autophagy in cells, viz. low cellular energy levels, nutrient starvation, hypoxia, oxidative stress, endoplasmic reticulum (ER) stress, withdrawal of growth factors, damage to cellular organelles and pathogen infection (Burman and Ktistakis 2010). The autophagy of damaged organelles and protein aggregates maintains cellular homeostasis, whereas its actions against pathogens act as a crucial cellular defense mechanism (Winchell et al. 2016). Autophagic control of bacterial multiplication promotes bacterial clearance during infection (Castrejón-Jiménez et al. 2015). However, many intracellular bacteria manipulate autophagy for their survival (Winchell et al. 2016).

The process of autophagy is mediated by autophagy-related proteins (Atg), and involves three steps: initiation, expansion and maturation (Burman and Ktistakis 2010). (a) Initiation: Unc-51 like autophagy activating kinase ULK1 (mammalian homolog of Atg1) is activated by dephosphorylation and, in turn, phosphorylates Beclin-1 (mammalian homolog of Atg6) resulting in activation of the Atg14L-containing phosphatidylinositol 3-kinase catalytic subunit type 3 (Vps34) complex (Russell et al. 2013). ULK1 phosphorylates also autophagy/Beclin-1 regulator 1 (Ambra1) which interacts with Beclin-1-Vsp34 leading to a subsequent translocation of the complex to the ER where it induces the formation of autophagosomes (Di Bartolomeo et al. 2010). (b) Elongation: the active Vps34 phosphorylates phosphatidylinositol to produce phosphatidylinositol 3-phosphate (PI3P) that serves as a docking site for WD-repeat protein interacting with phosphoinositides 1 and 2 (WIPI-1 and WIPI-2; mammalian homologs of Atg18), which then promote the formation of autophagy isolation membranes (Proikas-Cezanne et al. 2015). The elongation and closure of autophagosomes involves microtubule-associated protein 1 light chain 3 (MAP1LC3 or simply LC3; mammalian homolog of Atg8) which is lipidated at the C-terminal region by phosphatidylethanolamine in a process mediated by two ubiquitin-like conjugation systems. The first conjugation system involves Atg5 and Atg12, and the resultant complex associates with Atg16L1 to form the second conjugation system (Fujita et al. 2008b; Hanada et al. 2007). Lipidated LC3 contributes to autophagosome closure (Fujita et al. 2008a). (c) Maturation: closed autophagosomes fuse with lysosomes to degrade the cargo, which is mediated by the UV radiation resistance associated (UVRAG) protein (Liang et al. 2008).

Cargo that is targeted for degradation by autophagy is marked by ubiquitin. This modification is recognized by so-called autophagy adaptors, which bind the cargo and LC3 delivering it to the autophagosomal membrane (Deretic et al. 2013).

6.2 *ActA-Mediated Avoidance of Autophagy by* L. monocytogenes

Intracellular *L. monocytogenes* is targeted to autophagosomes where it is degraded (Birmingham et al. 2007; Py et al. 2007; Rich et al. 2003). However, *L. monocytogenes* has evolved a number of strategies to avoid or escape autophagic recognition. One such strategy, mediated by ActA, has attracted significant research interest over the past few years (Birmingham et al. 2007; Cemma et al. 2015; Yoshikawa et al. 2009). A pioneering study by Yoshikawa et al. (2009) revealed the mechanism of autophagy evasion by *L. monocytogenes* during primary infection. It was shown that it was not bacterial motility that is necessary for evasion of autophagy, but it is rather the ability of ActA to engage the host cell actin machinery that is required for escape from autophagic recognition. The Δ*act*A mutant of *L. monocytogenes,* which is deficient in recruiting host proteins involved in actin polymerization, co-localizes relatively more frequently with LC3, and has a lower rate of intracellular survival as compared to the wild-type strain in Madin-Darby Canine Kidney (MDCK) epithelial cells. *L. monocytogenes* strains which lack ActA undergo ubiquitination, autophagy adaptor sequestosome 1 (SQSTM1, also known as p62) recruitment and are subsequently degraded by autophagy. Presumably the ActA protein engages host cell actin machinery (including the Arp2/3 complex and Ena/VASP) to cover the entire bacterial surface protecting *L. monocytogenes* from autophagic recognition (Yoshikawa et al. 2009). In addition to p62, the autophagy adaptor calcium binding and coiled-coil domain 2 (CALCOCO2, also known as NDP52) is also recruited to ActA-negative *L. monocytogenes* (Mostowy et al. 2011).

6.3 *Strain-Specific Evasion of Autophagy by* L. monocytogenes

A recent study by Cemma et al. (2015) has shed light on the differences in evasion from autophagy by the commonly used *L. monocytogenes* strains, 10403S and EGD-e. This study suggests that *L. monocytogenes* may possess ActA-dependent and -independent mechanisms to evade autophagy confirming previous findings by Yoshikawa et al. (2009). Accumulation of ubiquitinated proteins and the autophagy adaptor protein p62 was observed on the surface of Δ*act*A mutants of either strain, whereas recruitment of the autophagy marker LC3 was observed at 8 h post

infection for the EGD-e strain. In contrast, LC3 was only visible for the 10403S strain when the bacteriostatic antibiotic chloramphenicol was added. However, the presence of chloramphenicol was observed to have no effect on the recruitment of ubiquitin or p62 by the wild-type or Δ*act*A mutant of 10403S and EGD-e. The Δ*act*A mutants of 10403S and EGD-e are capable of replicating at rates similar to their wild-type counterparts (Cemma et al. 2015). These studies suggest that additional factors/mechanisms act downstream of the process of ubiquitination to promote autophagy evasion.

7 ActA-Triggered Activation of NF-κB

Infection with *L. monocytogenes* results in activation of the nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB), a transcription factor that is involved in immune response (Hauf et al. 1994; Hoesel and Schmid 2013). NF-κB activation following infection with *L. monocytogenes* exhibits a biphasic response. There is a first transient induction which is probably related to a bacteria–host interaction. The second NF-κB activation phase is persistent and occurs only when the pathogen enters the cytoplasm. The persistent phase of NF-κB activation is mediated by PlcA, PlcB as well as ActA (Hauf et al. 1997). Intriguingly, the biphasic NF-κB activation is only seen in macrophages (P388D1 cells) and not in epithelial cells (Caco-2) where only the transient phase was observed (Hauf et al. 1997, 1999). These effects may be related to the localization of the bacteria in different compartments in the host cell following infection.

8 Involvement of ActA in Aggregation and Biofilm Formation

Biofilms are matrix-enclosed bacterial populations which adhere to each other and/or to a surface. The advantage of bacteria to form biofilms is related to resistance to antibacterial agents (Costerton et al. 1995). It was shown that different strains of *L. monocytogenes* form biofilms allowing their survival in food processing facilities (Borucki et al. 2003; Doijad et al. 2015; Lundén et al. 2000). Flagella-mediated motility of *L. monocytogenes* is one factor that is required for biofilm formation on abiotic surfaces (Lemon et al. 2007). ActA is another virulence factor of *L. monocytogenes*, which was attributed to biofilm formation (Travier et al. 2013).

A pivotal step in bacterial biofilm formation is bacterial aggregation (Costerton et al. 1995). It was recently demonstrated that ActA promotes aggregation of *L. monocytogenes* by ActA–ActA interaction in a pH window between pH 6.5 to pH 9, a pH where ActA is globally negatively charged (isoelectric point of

ActA = 4.95). The region required for ActA aggregation encompasses amino acids 126–231. However, mutants harboring deletions in other regions within the ActA protein indicate that the full ActA molecule is required for aggregation. This property has been postulated to allow the bacteria to persist within the gut lumen leading to long-term fecal shedding (Travier et al. 2013).

9 Invasion and Phagosomal Disruption

Within the extracellular space, ActA mediates invasion into host cells (Alvarez-Domínguez et al. 1997; Suárez et al. 2001). ActA-triggered entry is more pronounced in epithelial cells compared to macrophages, fibroblasts or hepatocytes (Suárez et al. 2001). Several positively charged amino acids located within the N-terminal region of ActA were proposed to constitute a heparan sulfate (HS) binding motif where these positively charged amino acids (lysine and arginine) are clustered and in close proximity to hydrophobic residues. Indeed, *L. monocytogenes* was shown to adhere and enter the host cell in a HSPG-dependent manner (Alvarez-Domínguez et al. 1997). HSPGs are located both at the cell surface as well as in the extracellular matrix (Simon David and Parish 2013). HPSGs are proteins with one or more covalently bound HS chains and the so-called HS binding motif (XBBXBX or XBBBXXBX; B being the basic amino acids arginine, lysine or histidine and X an aliphatic/aromatic amino acid), which is responsible for mediating binding of HSPGs to several ligands, for instance growth factors, cytokines, chemokines, enzymes, enzyme inhibitors, and extracellular matrix proteins (Simon Davis and Parish 2013; Sarrazin et al. 2011). However, it is not clear whether there is indeed a direct interaction between ActA and HSPGs. Therefore, more studies are required to clarify these observations.

ActA has also been implicated in phagosomal disruption via the amino acid region 31–58. It was postulated that actin monomers that accumulate around the phagosomes enter the vacuoles through the LLO-formed pores and either stabilize or widen these pores (Poussin and Goldfine 2010).

10 Conclusions

The ActA protein remains structurally and functionally a unique protein present exclusively in *Listeria* spp. Despite its discovery nearly 25 years ago, there is no known homologue in any other bacterial species. A distinguishing property is the presence of multiple sequence motifs generally only present in proteins of the vertebrate host cell, which are primarily interacting sites for proteins comprising the actin-based cytoskeleton. Indeed, many of the modern approaches employed to study the composition and regulation of actin-based networks can trace their inspiration back to ActA. ActA has now been implicated in multiple pathways that

enable the bacterium to successfully navigate intracellularly (Fig. 2). It promotes autophagy evasion and appears to be part of the repertoire of proteins required for invasion and internalization of *L. monocytogenes*. Other properties include promoting vacuolar escape, induction of NF-κB and an unexpected role extracellularly in the formation of protein-based biofilms. These properties provide further facets to the role of ActA as a major virulence factor in *L. monocytogenes*. In this review we summarized the evidence for the existence of other bacterially evolved factors able to embellish and modulate ActA´s primary activity such as InlC, which promotes cell-to-cell spread by usurping actin regulatory factors. It is tempting to speculate that the very fundamental property of intracellular motility led to the evolution of these other fine-tuning mechanisms enabling *L. monocytogenes* to navigate through the host cell during sublethal and acute infections.

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Actin-Dependent Regulation of *Borrelia burgdorferi* Phagocytosis by Macrophages

Xenia Naj and Stefan Linder

Abstract The spirochete *Borrelia burgdorferi* is the causative agent of Lyme disease, a multisystemic disorder affecting primarily skin, nervous system, and joints. If an infection with *Borrelia* proceeds unchecked, the disease can also enter a chronic stage, leading to the development of neuroborreliosis or cardiac arrhythmia. Successful elimination of *B. burgdorferi* by the host immune system is thus decisive for the positive outcome of a respective infection. Accordingly, host immune cells such as macrophages and dendritic cells have to be able to efficiently internalize and degrade infecting spirochetes. These processes are based on closely controlled rearrangements of the actin cytoskeleton, which enables the spatiotemporally fine-tuned formation of cellular protrusions and compartments that assist in the capturing, immobilization, and uptake of borreliae, as well as their further intracellular processing. Here, we discuss actin-based structures, in particular filopodia and coiling pseudopods that are involved in phagocytosis of *B. burgdorferi* by macrophages, their regulation by actin-associated proteins such as formins and Arp2/3 complex, as well as the subsequent intracellular processing of borreliae.

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1 Introduction

Borrelia burgdorferi is the causative agent of Lyme disease, a multisystemic disorder affecting primarily skin, nervous system, and joints. *Borrelia* belongs to the phylum of spirochetes, is characterized by a double membrane and an elongated helical morphology (Li et al. 2000), and can grow to lengths of 10–40 μm (Aberer and Duray 1991). Typical for spirochetes, borreliae feature a set of flagellae that run lengthwise along the cell body, in the periplasmic space between the inner and outer membrane, and enable considerable motility of the bacterium, with velocities of up to 4 μm/sec (Goldstein et al. 1994; Moriarty et al. 2008).

Borrelia burgdorferi sensu stricto is part of the *B. burgdorferi* sensu lato complex, which also encompasses further genospecies such as *Borrelia afzelii*, *Borrelia garinii* and others, many of which are pathogenic to humans. Borreliae typically propagate in rodents, deer, or birds and are transmitted by ticks of the *Ixodidae* family, with humans being inadvertent hosts (Lane et al. 1991). Once borreliae are transmitted by a blood meal, they can disseminate throughout the skin, which is often accompanied by the formation of *Erythema migrans*, a prominent rash that spreads from the center of infection and is enriched in neutrophils, dendritic cells, and macrophages (Salazar et al. 2003). These cells represent the first line of the host innate immune system, and their interaction with infecting borreliae is thus decisive for the outcome of a respective infection. In particular, uptake and elimination of borreliae by macrophages has been shown to be crucial to prevent dissemination of borreliae (Carrasco et al. 2015). Conversely, if an infection with *B. burgdorferi* proceeds unchecked, Lyme disease can also enter a chronic stage, leading to the development of neuroborreliosis or cardiac arrhythmia.

Successful uptake and elimination of infecting borreliae by macrophages involves a succession of tightly choreographed steps that are based on fine-tuned restructuring of the cytoskeleton, in particular of actin microfilaments. Macrophages form several actin-based structures during capturing and uptake of borreliae (Figs. 1 and 2). Molecular regulators of these structures, and particularly actin-regulatory factors such as formins and Arp2/3 complex, have been shown to critically influence effective phagocytosis of borreliae (Linder et al. 2001;

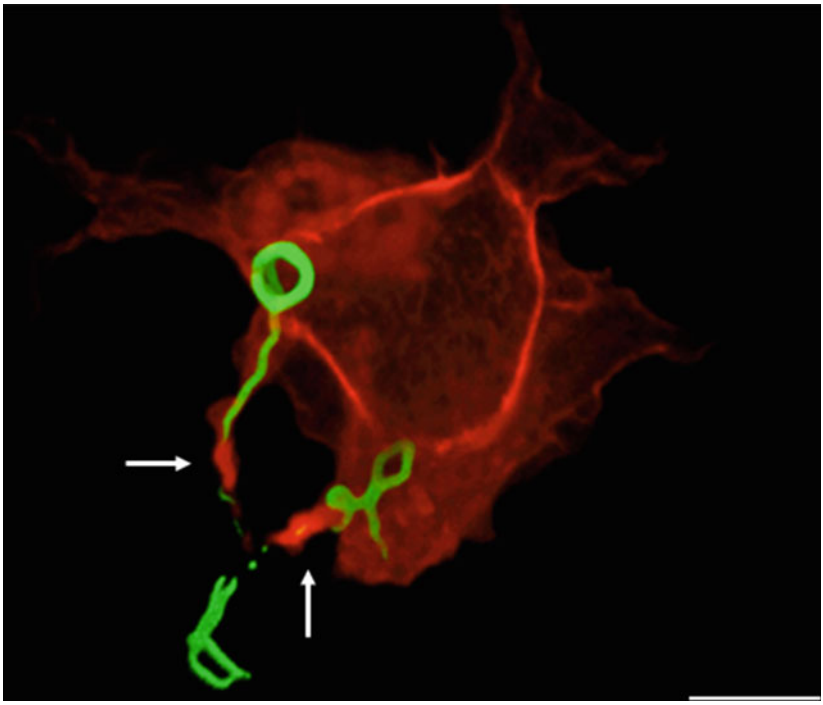

Fig. 1 Uptake of borreliae by human macrophages involves dynamic restructuring of the host actin cytoskeleton. Still image from confocal time-lapse video showing a primary human macrophage expressing RFP-Lifeact (*red*) internalizing several GFP-expressing spirochetes (*green*) with actin-rich cell protrusions (*white arrows*). Video can be openly accessed at http://www.linderlab.de/movies. Scale bar: 10 μm

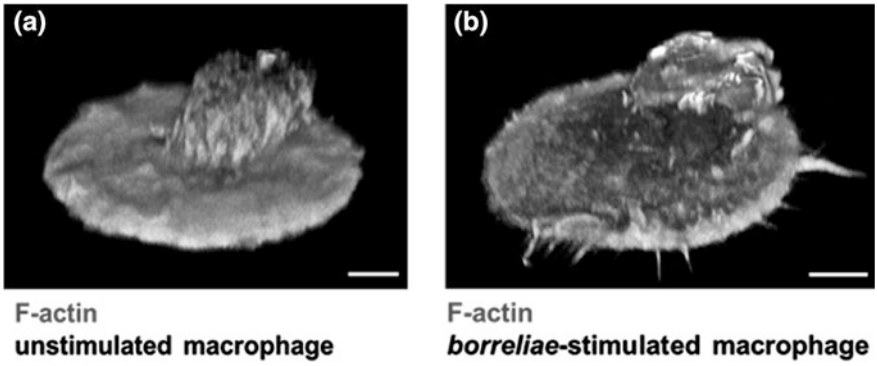

Fig. 2 *Borrelia* induces filopodia formation in human macrophages. Z-stacks of primary macrophages (stained for F-actin) without stimulation (**a**) or after 1 h of coincubation with *Borrelia burgdorferi* (**b**). Note formation of several filopodial protrusions after coincubation of macrophage with borreliae in (**b**). Scale bar: 5 μm

Hoffmann et al. 2014; Naj et al. 2013). Subsequently, regulators of vesicular trafficking such as small GTPases of the Rab family, steer the internalized spirochetes towards a degradative compartment (Naj and Linder 2015). Here, we discuss the current knowledge about actin-based uptake structures formed by macrophages during phagocytosis of borreliae, the intracellular processing of internalized spirochetes, as well as the respective molecular regulators of these processes.

2 *Borrelia* and the Stages of Lyme Disease

Lyme disease, also known as Lyme borreliosis, was first described in 1976 in Lyme, Connecticut, where an epidemic of juvenile rheumatoid arthritis occurred (Steere et al. 1977). In 1982, Willy Burgdorfer isolated spirochete bacteria from ticks of the *Ixodes* complex, which were abundant in this area. After the successful cultivation of the same spirochetes from patients, the causative agent of Lyme disease was called, based on its discoverer, *B. burgdorferi* (Burgdorfer et al. 1982).

To date, 18 different *Borrelia* genospecies are known (Margos et al. 2011). They are commonly referred to as the *B. burgdorferi* sensu lato complex. Among these 18 genospecies, seven have been identified to be infectious for humans, with three species being most frequently detected in patients. While *B. burgdorferi* sensu stricto is the most prevalent genospecies causing Lyme disease in North America, *Borrelia afzelii* and *Borrelia garinii* are the most commonly isolated human pathogenic species in Europe and Asia.

Lyme disease is a multisystemic disease that is considered to mainly originate from inflammatory responses to the *Borrelia* infection. The progression of Lyme disease is divided into three stages: (i) the early localized infection, (ii) the early disseminated infection, and (iii) the persistent infection (Zajkowska et al. 2012). However, not all three stages become necessarily apparent during the course of an infection.

The early, localized infection typically starts one to four weeks after transmission of borreliae with a painless skin rash spreading from the tick bite in a characteristic double ring shaped morphology called *Erythema migrans* (EM). EM develops through the response of immune cells, such as macrophages, neutrophils, and dendritic cells, which secrete inflammatory cytokines concomitantly with the spreading of the bacteria within the skin (Steere et al. 1983). Early infection with borreliae is often accompanied by flu-like symptoms, such as fever, malaise, and headache. If Lyme disease is diagnosed during that stage, successful treatment with antibiotics such as doxycycline or amoxicillin has a good prognosis (Jares et al. 2014). However, considering that EM occurs only in $\sim$60–80 % of all cases, infection with borreliae remains often unrecognized and can thus progress into the stage of the early disseminated infection.

During early disseminated infection, borreliae transmigrate from the skin into blood vessels, from where they spread through the blood stream to various organs (Kumar et al. 2015; Petzke and Schwartz 2015). Depending on the site of *Borrelia*

dissemination, the infection results in different symptoms. Therefore, beyond the originally identified manifestation in joints in the form of rheumatoid arthritis (Steere et al. 1977), patients can also develop cardiac symptoms like arrhythmia, skin lesions known as *Acrodermatitis atrophicans*, or Neuroborreliosis, which is accompanied by symptoms like facial palsy, meningitis, and encephalitis (Zajkowska et al. 2012; Steere et al. 2004).

Also at this later stage, patients can be cured by a prolonged treatment with antibiotics. However, in many cases symptoms persist even beyond such a regime. This stage is referred to as post-Lyme disease. It is under debate whether the symptoms are based on the presence of bacteria that persist despite the antibiotics therapy necessitating further or additional courses of antibiotics treatment (Aguero-Rosenfeld and Wormser 2015). The more widely accepted assumption, however, is that these posttreatment symptoms occur even in the absence of any remaining borreliae and are rather a consequence of damaged tissue, or of ongoing inflammation processes and autoimmune disorders (Berende et al. 2010; Pearson 2014).

To clearly answer the question whether disseminated borreliae are able to persist despite prolonged antibiotics treatment, successful cultivation of spirochetes from patient samples would be necessary. However, this is very inefficient and thus not reliable. Therefore, post-Lyme disease is still not well understood, and a clear definition of the causes as well as specific diagnostic tools are missing. The published guidelines of the "Infectious Diseases Society of America" (IDSA) advise against repeating courses of antibiotics treatment if symptoms reappear after a first antibiotic course (Wormser et al. 2006). Still, some uncertainty remains, as several studies detected persistent bacteria despite a long-term treatment with antibiotics (Stricker and Johnson 2013; Berndtson 2013). In rhesus macaques, which were infected with borreliae, low numbers of intact spirochetes were successfully recovered, despite an aggressive long-term antibiotics treatment (Embers et al. 2012). Due to these controversies, the existence of a post-Lyme syndrome, whether it results from a persistent infection, as well as its potential treatment, are still points of debate (Borgermans et al. 2014; Aguero-Rosenfeld and Wormser 2015).

3 Phagocytic Uptake of *Borrelia* by Macrophages

At the stage of the early localized infection, the immune system of the host can still prevent the spreading of bacteria. Thus, interaction of *Borrelia* with cells of the immune system, and especially with phagocytes, is critical for the outcome of the infection. EM biopsies have shown that T cells, macrophages and dendritic cells locally infiltrate the skin (Ziuzia Iu et al. 1999; Salazar et al. 2003; Duray 1989). In this review, we focus in particular on the interactions of *Borrelia* with macrophages, which are professional phagocytes and capable to efficiently eliminate bacteria from infected tissue.

Macrophages are part of the innate immune system, the first line of defense against infecting pathogens. At the same time, they also play a role as activators of the adaptive immune system. As professional phagocytes, macrophages are able to take up and efficiently eradicate a large number of bacteria per individual cell, through a process called phagocytosis. Phagocytosis is defined as the uptake of a particle >0.5 μm in diameter. It is a multistep process that involves detection of a phagocytic target, its capturing or immobilization, with subsequent internalization, followed by intracellular degradation.

Usually, recognition of bacteria as phagocytic targets takes place through either deposited opsonins, including factors of the complement system and antigen-targeting antibodies, or through conserved surface exposed proteins, so-called pathogen-associated molecular patterns (PAMPs). Opsonins and PAMPs are recognized by several cell surface receptors. Once a ligand–receptor interaction is established and the bacteria immobilized, macrophages develop localized protrusions, which engulf and help to internalize the pathogen. These steps of immobilization and internalization require a highly fine-tuned and localized regulation of the actin cytoskeleton. In the case of *Borrelia,* it is known that their phagocytosis is mediated by several different receptors, including opsonic receptors like FcγR (Benach et al. 1984; Montgomery et al. 1994) and the complement receptor 3 (CR3) (Garcia et al. 2005; Hawley et al. 2012; Cinco et al. 1997). Furthermore, internalization of borreliae can also be mediated by the non-opsonic toll like receptor 2 (TLR2) (Salazar et al. 2009), with downstream signaling involving both myeloid differentiation factor 88 (MyD88)-dependent but also -independent pathways. (Shin et al. 2009). It has been shown that knock-out mice, which are not able to express either TLR2, Fc receptor common gamma chain (FcεRγ) or CR3 develop higher *Borrelia* burdens and more pronounced symptoms (Wang et al. 2004; Lawrenz et al. 2003; Liu et al. 2004). In vitro, both opsonized and unopsonized borreliae were shown to attach to macrophages. However, opsonization of the spirochetes by serum containing factors of the complement system, or by *Borrelia*-targeting antibodies, enhances their attachment to macrophages 4–5 fold (Linder et al. 2001). Collectively, *Borrelia* phagocytosis is mediated by several receptors, which act in concert to allow efficient clearance of spirochetes.

Internalization of pathogens is accompanied by their uptake into a specific intracellular compartment, the phagosome, whose coat is derived from the pathogen-engulfing membrane, which is closed upon final internalization and pinched off from the plasma membrane (Fairn and Grinstein 2012). Phagosomes then undergo a process of maturation, which is based on their fusion with endosomes and lysosomes, resulting in their acidification and the acquisition of lytic enzymes (Vieira et al. 2002). Collectively, these processes result in the degradation of the internalized pathogen. After macrophages internalize and degrade bacteria, they present antigenic peptides on their major histocompatibility complex II (MHCII) and present it to T helper cells, which in turn activate B-cells to produce antigen-targeting antibodies (Hsieh et al. 1993a, b; Kahlert et al. 2000; Unanue and Askonas 1968; Hoffman et al. 2016).

4 Actin-Rich Uptake Structures: Filopodia and Coiling Pseudopods

To initiate phagocytosis, macrophages need to establish close physical contact with pathogens. Until recently, contact formation between an immune cell and its phagocytic target was seen as a more passive event, constituting a direct consequence of chemotactic immune cell migration and being followed by surface receptor clustering (Michl et al. 1983).

However, more recent work demonstrated that immune cells also actively enhance the probability of securing a target at their surface, by probing their environment with filopodia, receptor-containing cellular protrusions (Flannagan et al. 2010). Filopodia are elongated, finger-like protrusions of cells that contain bundles of linear actin filaments and can extend from the cell surface up to several tens of microns (Svitkina et al. 2003; Mallavarapu and Mitchison 1999). They are highly dynamic and constantly extend and retract, which is based on actin filament dynamics. Considering that macrophages mostly migrate in a three-dimensional environment and are embedded in the network of the extracellular matrix, an array of actively probing filopodial protrusions allows these cells to scan a much larger volume of space, compared to their actual cell body.

Filopodia dynamics are tightly regulated. Accordingly, receptor–ligand interactions established upon capturing a target are believed to induce a signaling cascade that leads to reduction of filopodia elongation, and instead favoring their retraction (Romero et al. 2011). However, clear evidence for the existence of such a signaling cascade is missing, and potentially involved molecular regulators remain to be identified.

Importantly, filopodia are also able to exert forces in the range of several hundreds of piconewtons (pN). This allows them to pull on attached particles, thereby bringing them into close contact with the surface of the host cell (Heidemann et al. 1990; Vonna et al. 2007; Kress et al. 2007). It was demonstrated that filopodia are able to pull persistently on objects during their retraction. Accordingly, filopodia of RAW (Abelson leukemia virus-transformed murine macrophage-like) cells that pulled on IgG-coated beads (Kress et al. 2007) or sheep red blood cells (Flannagan et al. 2010) were able to resist applied counterforce by an optical trap. Interestingly, the retraction speed of the bead-pulling filopodia slowed down in relation to the force applied by the optical trap (Kress et al. 2007). In contrast, macrophages that were treated with the F-actin stabilizing agent jasplakinolide, thus inhibiting actin turnover (Cramer 1999), failed to maintain this interaction (Flannagan et al. 2010). This latter experiment emphasizes the essential role of actin cytoskeleton dynamics for filopodia-mediated capturing of phagocytic targets.

The ability to maintain the attachment to objects despite applied counterforce becomes especially important in situations when phagocytes have to capture highly motile pathogens at their surface and prevent them from detaching. *B. burgdorferi*, equipped with periplasmic flagella, is an excellent example for such a highly motile pathogen. Intra-vital imaging showed the spirochetes are able to move at a speed of

4 μm/sec in murine ear tissue (Moriarty et al. 2008), similar to the speed of 4.25 μm/sec measured in vitro (Goldstein et al. 1994).

Intriguingly, coincubation of primary human macrophages with borreliae strongly enhanced filopodia formation per cell as compared to control cells (3.0 ± 0.2 in borrelia stimulated cells compared to 1.2 ± 0.2 in control cells) (Naj et al. 2013) (Fig. 2). Also, *Borrelia*-induced filopodia were longer compared to those of unstimulated cells [6.2 μm ± 0.4 μm for filopodia in borreliae stimulated macrophages, 3.2 μm ± 0.3 μm for unstimulated macrophages (Naj et al. 2013)]. Moreover, quantification of filopodia formation in cells stimulated solely with *Borrelia* culture supernatant revealed no difference compared to unstimulated cells, supporting the hypothesis that induction of filopodia is based on direct interaction of macrophages with pathogens, and not on soluble factors (Hoffmann et al. 2014; Naj et al. 2013). However, the respective molecular mechanism responsible for *Borrelia*-induced upregulation of filopodia formation is currently unclear.

Filopodia-dependent capturing by host cells has also been demonstrated for other bacteria such as enteroinvasive *Shigella flexneri* (Romero et al. 2011). In this case, entry of *Shigella* triggers opening of connexin-dependent hemichannels, resulting in enhanced levels of extracellular ATP, which in turn increased filopodia-mediated attachment of *Shigella* to Hela cells. This was accompanied by enhanced Erk1/2 activity, which was shown to be important for efficient filopodia retraction. It is an intriguing speculation that similar events might also play a role in *Borrelia* capturing by macrophage filopodia.

Comparable to the described experiments using latex beads, motile borreliae that were contacted by macrophage filopodia often remained in direct contact with the protrusions, reinforcing the notion that filopodia indeed constitute cellular organelles for capturing of pathogens, which are able to adhere continuously to a contacted bacterium. Moreover, multiple filopodia were often observed to surround captured borreliae at the cell surface of macrophages (Fig. 3), which might also point to a role for filopodia as a physical barrier that further hinders the escape of surface-attached spirochetes.

After borreliae are captured and brought close to the cell surface, they are preferentially internalized by a specific mechanism called coiling phagocytosis (Rittig et al. 1992) During this process, spirochetes are progressively enwrapped by a long actin-rich cell protrusion that arises from the cell surface at the *Borrelia*-contact site, surrounding the spirochete in multiple whorls (Fig. 1). Experiments using live or heat-inactivated borreliae (Rittig et al. 1998b; Rechnitzer and Blom 1989) and also other spirochetes showed that coiling phagocytosis is a host cell-driven process, which is probably based on the specific morphology of spirochetes and does not depend on the viability of bacteria.

Indeed, the phenomenon of coiling phagocytosis has been known for decades and was described for the uptake of several pathogens, including *Legionella pneumophila* (Horwitz 1984), *Trypanosoma cruzi*, *Leishmania spp*. as well as several fungal cells (Rittig et al. 1998c). It was found that coiling pseudopods are induced to the same extent by live as by killed pathogens (Rittig et al. 1998b; Rechnitzer and Blom 1989). Furthermore, neither supernatant of bacteria culture

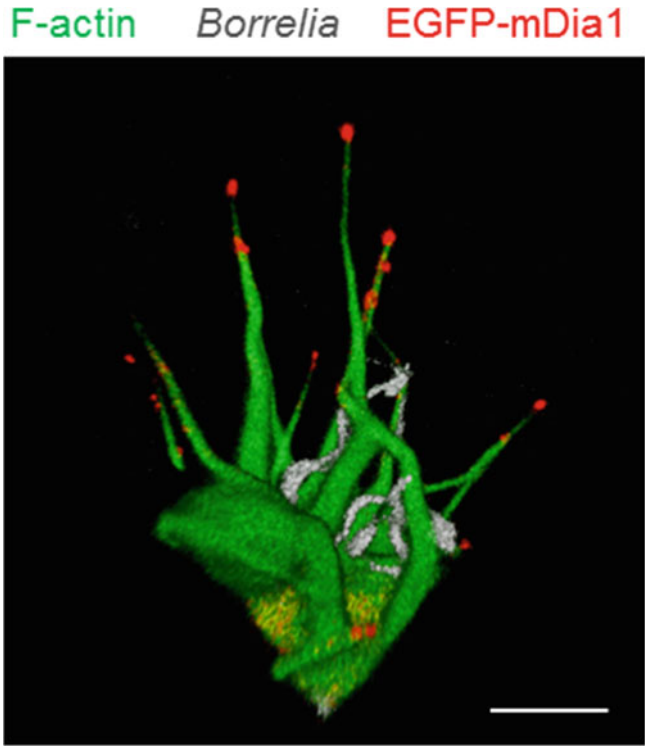

Fig. 3 Macrophage filopodia facilitate contact between borreliae and the host cell. 3D reconstruction using confocal Z-stacks of a macrophage stained for F-actin using Alexa 568 phalloidin (*green*) and expressing EGFP-mDia1 (*red*), with DNA of borreliae stained by Hoechst 33258 (*gray*). 3D reconstruction shows part of the macrophage surface that is in contact with several spirochetes, which are encaged by actin-rich filopodia. Note punctate enrichment of EGFP-mDia1 at the tips of filopodia. Scale bar: 5 μm

nor by sonification fragmented bacteria triggered pseudopod formation (Rittig et al. 1998b). Moreover, it has not been possible to relate this mechanism to any specific type of phagocytic receptor (Rittig et al. 1992), though coiling phagocytosis of borreliae shows characteristics of both CR3- and Fcγ-dependent phagocytosis (Linder et al. 2001). This again suggests that the helix-like structure of the coiling pseudopod is most probably a result of the spirochete morphology rather than being based on a particular ligand–receptor interaction (Rittig et al. 1998a, c).

This coiling pseudopod is highly flexible and contains multiple bending nodes, and is thus clearly distinct from the rather stiff filopodia (Figs. 1, 2, 3 and 4). Its flexible morphology allows the pseudopod to closely align along the spirally shaped body of the *Borrelia* cell (Naj et al. 2013; Hoffmann et al. 2014). Individual observations by live-cell imaging have shown that full internalization can require longer than 40 min (Naj and Linder 2015).

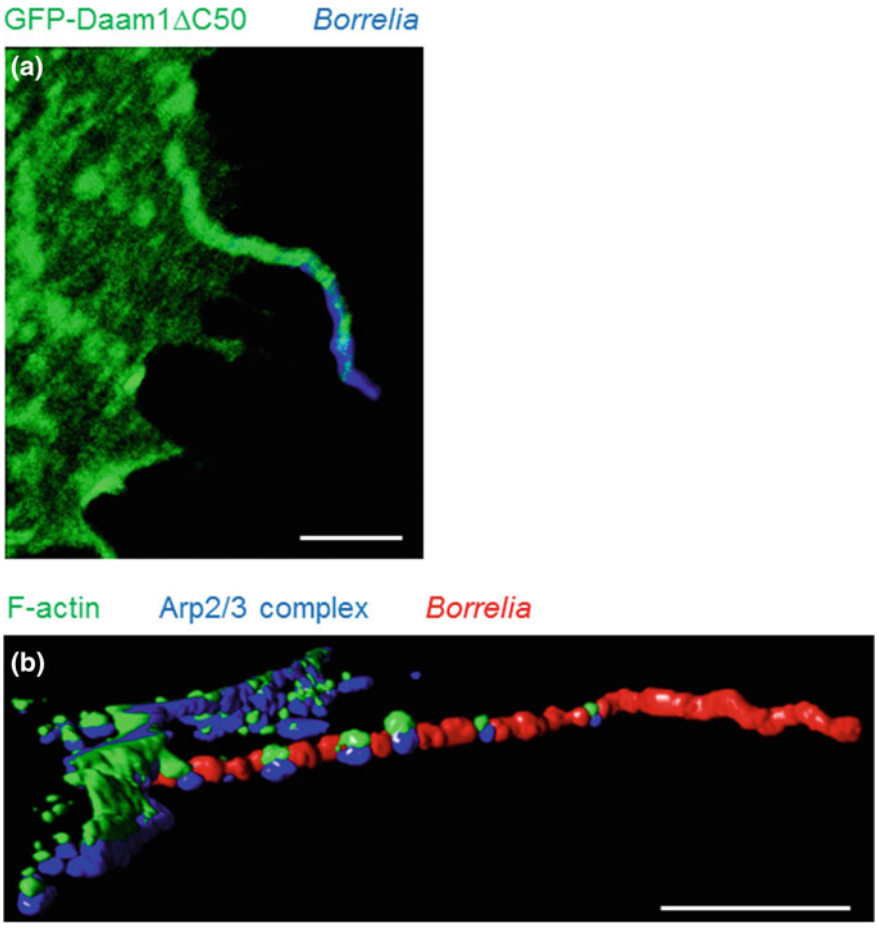

Fig. 4 Macrophage coiling pseudopods enwrapping borreliae are enriched in actin regulators. (**a**) Enrichment of GFP-Daam1ΔC50 during phagocytosis of borreliae. Confocal micrographs of primary human macrophage expressing GFP-Daam1ΔC50, a non-autoinactivated mutant (*green*) which is accumulated at the uptake structure of a *Borrelia* cell visualized by Hoechst 33342 staining of DNA (*blue*). (b) Isosurface reconstruction of confocal Z-stack, using Volocity software. *Borrelia* cell stained using antibody specific for OspA surface antigen (*red*), macrophage protrusion stained for F-actin (*green*) and Arp2/3 complex (*blue*). Macrophage cell body not shown. Note dot-like enrichment of Arp2/3 complex along the *Borrelia* cell, typical for coiling phagocytosis. Scale bars: 5 μm

Considering the more recently discovered involvement of actin-based filopodia prior to the formation of the coiling pseudopod during *Borrelia* phagocytosis, it was unclear whether both structures are formed independently or whether the coiling pseudopod arises from enhanced lateral growth of the already existing filopodia. Importantly, live-cell imaging experiments showed that coiling pseudopods indeed constitute separate structures that are formed de novo after borreliae are captured by

filopodia, and are not developed by further growth of filopodia that are already in contact with spirochetes This is also reflected by the distinct requirement for different actin regulators during the formation of either structure.

5 Actin Dynamics During *Borrelia* Phagocytosis: The Roles of Formins and Arp2/3 Complex

Formation and restructuring of actin filaments in cells is exquisitely controlled on many levels, to ensure exact formation of the required structures in time and space. Filaments can be formed de novo by nucleation or through fragmentation of existing ones. Elongation of filaments can be promoted or stopped by respective regulators, and dissolution is driven by processive disassembly or by severing into smaller filaments. Finally, individual filaments can be associated into higher ordered structures by bundling or crosslinking factors (Mellor 2010; Mattila and Lappalainen 2008).

Formation of filopodia involves the nucleation and elongation of unbranched actin filaments, as well as their connection in higher ordered bundles, to achieve the required stiffness. Of note, the formins FMNL1 and mDia1 have been localized to borreliae-induced filopodia, and their activity was shown to be required for filopodia formation in response to macrophage contact with borreliae, and also for subsequent internalization (Naj et al. 2013). Proteins of the formin family, 15 of which are expressed in human tissues (Schonichen and Geyer 2010), are important regulators of unbranched filaments. In principle, they are able to regulate all of the actin-related activities described above, including nucleation, elongation, capping, depolymerization, severing, and bundling with the individual set of abilities varying widely between the different isoforms (Schonichen and Geyer 2010; Grikscheit and Grosse 2016; Bohnert et al. 2013). In vitro, FMNL1 displays actin severing activity, thus giving rise to free barbed ends that can be used for the growth of new actin filaments (Harris et al. 2004), while mDia1 shows actin elongation and crosslinking activity (Li and Higgs 2003; Esue et al. 2008).

Furthermore, FMNL1 was localized along the whole shaft of borreliae-induced filopodia, whereas mDia1 showed a dot-like accumulation at the tips of filopdia (Naj et al. 2013) (Fig. 3). Combining both sets of observations, it is thus likely that mDia1 at the tips of filopodia regulates the growth of these structures by elongation of actin filaments, while FMNL1 might be involved in filopodia growth through the generation of free barbed ends along the shaft of the structure (Fig. 5). Structural stability of filopodia is supplied by bundling of actin filaments through fascin, which, comparable to FMNL1, localizes at the filopodial shaft (Naj et al. 2013). Of note, filament bundling is probably further supported through the activity of yet another formin, Daam1 (Hoffmann et al. 2014). Like FMNL1, endogenous and overexpressed forms of Daam1 were found to localize along the whole shaft of borreliae-induced and fascin-positive filopodia (Fig. 5).

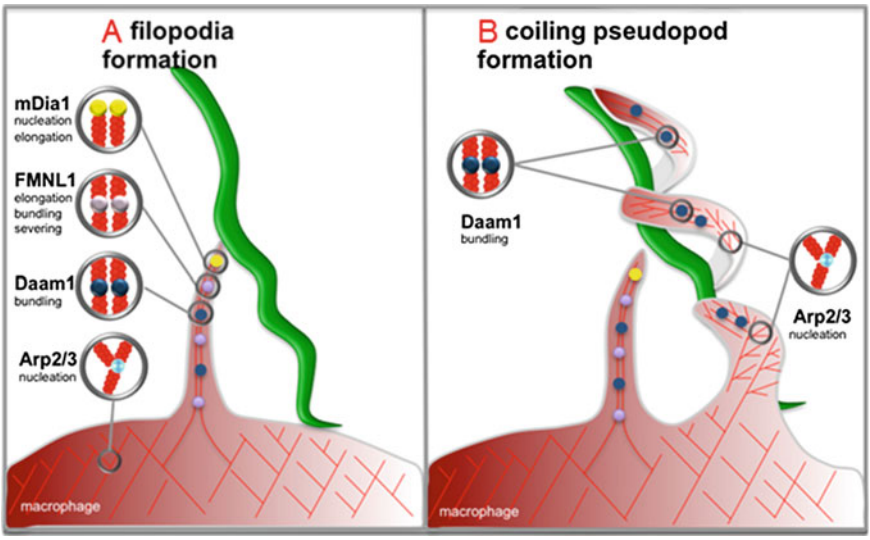

Fig. 5 Model of formin- and Arp2/3 complex dependent actin regulation in coiling phagocytosis of *Borrelia*. (**a**) Upon stimulation with borreliae, macrophages form filopodial protrusions that arise from the cortical network. Filopodia are enriched in the formins mDia1 (localized at tips) and FMNL1 (localized at tips and shaft), which probably contribute to longitudinal growth of filopodia, and Daam1 (localized in filopodial shaft), which is probably involved through its actin-bundling activity. (**b**) Upon capturing of a *Borrelia* cell by filopodia, the spirochete is enwrapped by a coiling pseudopod. Until recently, it was unclear whether coiling pseudopods develop from filopodia or constitute independent structures. Live-cell experiments showed that Daam1-positive coiling pseudopods arise as a second independent structure from the macrophage surface and enwrap borreliae. The flexibility of coiling pseudopods that enables them to enwrap the spiral-shaped borreliae is probably due to dot-like accumulations of Arp2/3 complex, which lead to formation of small branched actin networks and probably act as "hinges" at coiling nodes

This requirement for more than one bundling factor is surprising, but fascin has been shown to stabilize Daam1 at filopodia in B16F1 mouse melanoma cells, and silencing of Daam1 in these cells led to a decrease in the number of filopodia and also defects in their architecture (Jaiswal et al. 2013), pointing to a cooperative role of Daam1 and fascin in both formation and stabilization of filopodia. Similarly, siRNA-mediated knockdown of Daam1 in human macrophages resulted in a two-third reduction of filopodia formed upon contact with borreliae (Hoffmann et al. 2014), comparable to the effect of a fascin knockdown in these cells (Hoffmann et al. 2014). Interestingly, knockdown of Daam1 had a more pronounced effect on the number of filopodia than knockdown of either FMNL1 or mDia1 (40–50 % reduction each), which may point to the relative importance of Daam1 in filopodia formation or stabilization. Combined knockdown of all three formins, however, had no additive effect, showing that these formins work in the same pathway that ensures efficient filopodia formation (Hoffmann et al. 2014). Of note, the requirement for specific formins in filopodia regulation has been shown to

vary between cell types, and especially between adherent and suspension cells (Young et al. 2015). Thus, also the relative importance for FMNL1, mDia1, and Daam1 for formation of *Borrelia*-capturing filopodia may vary, depending on the type of immune cell involved, and also on the two- or three-dimensional context in which borreliae are encountered by immune cells in the body.

Inside–outside stainings of formin-depleted macrophages also showed that reduction of filopodia resulted in a ~50 % decrease of internalized borreliae, demonstrating that capturing of spirochetes by filopodia is an important step for efficient internalization by macrophages (Hoffmann et al. 2014). This is probably based on the effects that 1) filopodia allow cells to scan a larger volume of space and 2) immobilization of the highly motile spirochetes allows more time for the development of the coiling pseudopod, which is in most cases the decisive surface structure mediating phagocytosis of borreliae (Rittig et al. 1992; Naj et al. 2013).

Interestingly, live-cell imaging revealed that Daam1 apparently plays a dual role during uptake of borreliae by macrophages: not only through stabilization of borreliae-capturing filopodia, but also through formation of the coiling pseudopod itself (Hoffmann et al. 2014) (Fig. 4). This observation also resolved the question whether the coiling pseudopod forms independently from filopodia or through lateral growth of these structures. As the primary biochemical function of Daam1 is bundling of actin filaments, it is likely that this formin also works as an actin-bundling factor in the coiling pseudopod (Fig. 5).

This leaves the question how actin filaments in coiling pseudopods are nucleated or elongated. A partial answer to this lies in the localization of actin-nucleating Arp2/3 complex and its activator WASP (Wiskott-Aldrich Syndrome protein) at coiling pseudopods of macrophages (Linder et al. 2001). Interestingly, Arp2/3 complex has been shown to form dot-like accumulations along the coiling pseudopod, which often coincide with helical turns of the spirochete body (Fig. 4). It is thus tempting to speculate that Arp2/3 complex, as a generator of branched actin filament networks (Amann and Pollard 2001) provides nodes of branched actin that may alternate with sections of unbranched actin filaments, thus bringing the necessary flexibility to the coiling pseudopod structure that has to closely follow the helical spirochete morphology (Fig. 5). Of note, Arp2/3 complex has also been shown to be important for a subset of filopodia, by providing localized actin networks as a structural basis for their longitudinal extension (Young et al. 2015). However, the potential impact of Arp2/3 complex on borreliae-induced filopodia has not been tested yet.

An important upstream activator of WASP is Cdc42, a small GTPase of the Rho family (Mullins 2000). Accordingly, microinjection of dominant negative Cdc42 strongly reduced coiling pseudopod formation (Linder et al. 2001). It is thus very likely that an activation cascade Cdc42-WASP-Arp2/3 complex regulates formation of borreliae-induced coiling pseudopods. Furthermore, Cdc42 and other RhoGTPases, most notably RhoA or Rac1, may also be involved in the regulation of formin-dependent activities during *Borrelia* capturing and internalization. Formins are usually autoinhibited by backfolding of an inhibitory DID domain (diaphanous inhibitory domain) to a regulatory DAD domain (diaphanous

autoregulatory domain), and only binding of RhoGTPases and other factors leads to release of this autoinhibition and to full activity of formins (Kuhn and Geyer 2014; Higgs 2005). Accordingly, many of the experiments regarding the involvement of formins in borreliae-induced filopodia formation were performed using non-autoinhibited constructs such as Daam1ΔC50 that lack the respective DID domains and thus circumvent the need for RhoGTPase-dependent activation. An important role for RhoGTPases in borreliae-capturing filopodia is thus highly likely, although the involvement of specific RhoGTPases in activation of respective formins has not been tested yet.

Collectively, these data lead to the following multistep model of *Borrelia* phagocytosis by macrophages: (1) physical contact of borreliae with macrophages leads to increased formation of filopodia that are able to contact and bind borreliae, thus leading to immobilization of the highly motile spirochets on the bacterial surface. Filopodia formation depends on the concerted activity of three formins, FMNL1, mDia1, and Daam1, which respectively regulate actin filament formation, elongation, and bundling, with further bundling activity provided by fascin. In a second phase, a filopodia-independent structure, the coiling pseudopod, arises from the macrophage surface. It closely follows the helical spirochete morphology and thus tightly enwraps captured borreliae (Fig. 5). Actin within coiling pseudopods is probably present alternatingly as elongated unbranched filaments that are bundled by Daam1 and as nodes of branched actin networks formed by Cdc42-WASP-Arp2/3-dependent actin nucleation. This architecture would allow the necessary flexibility that is required for this structure that enwraps the helical spirochete. Finally, the coiling pseudopod has to contract, to be brought in close contact with the macrophage surface, and the captured borreliae have to be internalized. These steps likely involve regulators of actin-based contractility such as myosin II and also disassembly of actin filaments, possibly necessitating further formin activity. This should prove to be a fertile field for future research. Of note, due to their elongated morphology, intracellular processing of captured borreliae can already be in progress, as outlined below, even when extracellular parts of the spirochetes are still being enwrapped by actin-driven coiling pseudopods.

6 Intracellular Processing of *Borrelia*—A Central Role for RabGTPases

During phagocytosis, bacteria enter the cell in a membrane-delimited compartment termed the phagosome. Apart from the internalized target, the phagosome is initially filled with fluids that derive from the extracellular space. Subsequent alteration in phagosome composition proceeds through highly coordinated exchange of material with vesicles of the endomembrane system (Fairn and Grinstein 2012). Ultimately, these steps lead to the maturation of the phagosome into an acidic, oxidative compartment that is enriched in hydrolytic enzymes. According to the

enrichment and/or loss of respective marker proteins and also to the progressive drop of the intraluminal pH, phagosomes are classified into distinct stages: (i) early phagosome, (ii) late phagosome, and (iii) phagolysosome. The fully matured phagolysosome is able to digest lipids, proteins, and carbohydrates and thus neutralizes infecting bacteria.

Key regulators of this maturation process include members of the Rab (Ras-related proteins in brain) GTPase family, which are molecular switches cycling between their active GTP-bound and inactive GDP-bound state (Vieira et al. 2002; Stenmark et al. 1994). In their active state, RabGTPases interact with their respective effector proteins that control various processes including phagosomal membrane fusion and fission events and motor-dependent transport (Gautreau et al. 2014; Hutagalung and Novick 2011).

In this respect, it could be shown that borreliae are initially internalized into a Rab22a positive phagosome. Subsequently, the phagosome is contacted by Rab5a positive vesicles. Quickly after internalization, the elongated spirochetes are compacted into globular, dense structures. This striking compaction of borreliae is accompanied by repeated tubulation of membrane from phagosomes, suggesting that reduction of the phagosomal surface could be a driving force for spirochete compaction. Interestingly, fission of membrane tubules occurs preferentially at sites where Rab5a positive vesicles contact the Rab22a positive phagosomal membrane, indicating that the concerted activity of both RabGTPases is necessary for this process (Naj and Linder 2015) (Fig. 6).

Previous reports showed that the endoplasmic reticulum (ER) contacts Rab5 positive early endosomes at subdomains just before fission occurs at those sites (Rowland et al. 2014). Consistent with the notion that similar processes could be involved in the membrane fission events from the phagosomes during *Borrelia* compaction, it was observed that the ER forms a network around *Borrelia*-containing phagosomes and that Rab5a vesicles contact the Rab22a-positive phagosomal membrane along the ER (Naj and Linder 2015) (Fig. 6).

Moreover, an F-actin coat was detected to surround the early, *Borrelia*-containing phagosome (Naj and Linder, unpublished). It is therefore conceivable that F-actin plays a role during *Borrelia*-compaction and the concomitant membrane tubule fission events during this process. Indeed, previous studies showed that WASH, a vesicle-localized Arp2/3 complex activator of the WASP family (Gautreau et al. 2014) regulates membrane fission from Rab5-positive early endosomes (Duleh and Welch 2010). It is thus tempting to speculate that WASH-mediated actin polymerization at phagosomes could contribute to the observed membrane fission events that occur during *Borrelia* compaction. However, this concept requires further evaluation.

Furthermore, Rab22a and Rab5a knockdown not only inhibited the compaction of the spirochetes, but also the maturation of *Borrelia*-containing phagosomes. This was demonstrated by decreased ($\sim$20–40 %) lysosomal associated membrane protein1 (LAMP1) acquisition at the phagosomal membrane (Naj and Linder 2015) and also by decreased ($\sim$15–45 %) phagosomal colocalization with DQ-BSA (Naj and Linder 2015), a marker for proteolytic activity (Fig. 6). Moreover, knockdown

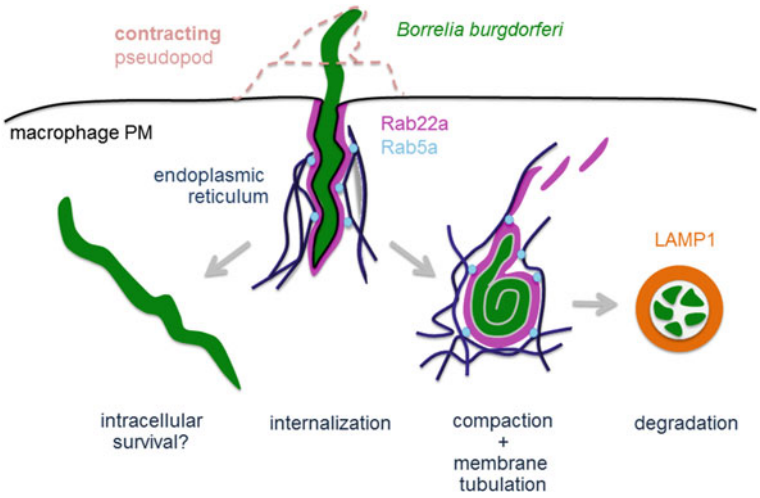

Fig. 6 Model of intracellular processing of borreliae by macrophages. Borreliae captured by macrophages via coiling pseudopods are internalized through uptake into Rab22a-positive phagosomes. Phagosomes are subsequently contacted by Rab5a positive vesicles mediated by the endoplasmic reticulum. Subsequent membrane tubulation causes reduction of the phagosome surface, leading to visible compaction of borreliae. Further maturation of this compartment leads to its development into a degradative phagolysosome, indicated by the presence of the lyosomal marker protein LAMP1, and resulting in elimination of spirochetes. In contrast, escape from Rab22a-/Rab5a-dependent processing can lead to enhanced intracellular survival of borreliae. Modified from Naj and Linder (2015), with permission

of Rab22a and Rab5a in macrophages led to enhanced (∼6 fold) intracellular survival of the spirochetes (Naj and Linder 2015).

Of note, the specific subset of RabGTPases can vary between phagosomes that contain different bacteria. Moreover, several bacteria have evolved strategies to influence RabGTPase recruitment and/or activity as part of an escape strategy to influence phagosome maturation and thus avoid being degraded (Smith and May 2013). It is thus noteworthy that a small subpopulation of borreliae (∼5 %) colocalized only transiently with Rab22a and Rab5a, did not undergo compaction and retained their elongated morphology (Naj and Linder 2015). Indeed, former electron microscopy studies demonstrated the presence of elongated borreliae localized within the cell cytoplasm without any clearly detectable phagosomal membrane (Filgueira et al. 1996; Ionescu et al. 1997; Hechemy et al. 1992). It is thus conceivable that this could be a subpopulation, which escapes the phagosome. Supporting this notion, heat killed borreliae were detected consistently surrounded by a phagosomal membrane in Vero cells (Hechemy et al. 1992). In contrast, borreliae are not known to harbor any secretion system or to express any virulence factors (Fraser et al. 1997). Closer investigation of this hypothetical subset of borreliae, their potential to persist in human immune cells, as well as the possible molecular mechanisms involved, should thus be an interesting challenge for the future.

7 Conclusions

Efficient uptake and elimination of borreliae by immune cells is crucial for countering the development of Lyme disease. In particular, macrophages form an important part of the initial defense line that prevents dissemination of *B. burgdorferi* within the host through a carefully orchestrated succession of capturing, internalization, and degradation of spirochetes.

In this context, recent research has highlighted the role of local restructuring of the macrophage actin cytoskeleton, which enables the formation of specific surface structures that interact with infecting spirochetes. First, macrophages respond to the presence of borreliae by forming filopodia, long, rigid protrusions that contain a core of linear, bundled actin filaments. Filopodia enable the capturing and immobilization of the highly motile spirochetes. Second, a filopodia-independent structure is formed, the coiling pseudopod, which enwraps captured borreliae and promotes their internalization. Closely following the helical morphology of the spirochete, this structure requires a more flexible arrangement of actin filaments.

Accordingly, these different structures have been shown to depend on different subsets of actin regulators, with *Borrelia*-induced filopodia depending the formins FMNL1 and mDia1, regulators of unbranched actin filaments, while the coiling pseudopod apparently contains nodes of Arp2/3 complex-generated branched actin networks. In addition, both structures depend on the activity of the formin Daam1.

Due to the elongated morphology of the *Borrelia* cell, intracellular processing of internalized parts of the spirochete can happen concomitantly with the uptake of still extracellular parts of the spirochete. Internalized borreliae have been shown to enter phagosomes, which is accompanied by successive compaction of borreliae. Further maturation of phagosomes into degradative lysosomes involves the RabGTPases Rab22a and Rab5a. The activities of both RabGTPases are coordinated by the endoplasmic reticulum, which closely enwraps the internalized parts of the spirochete, thus forming an intracellular counterpart to the coiling pseudopod that enwraps the extracellular parts of the spirochete.

Collectively, these novel insights into the subcellular and molecular regulation of *Borrelia* capturing, uptake, and degradation illustrate the highly efficient mechanisms that macrophages have developed to counter respective infections. On the other hand, it will be highly interesting to determine if and to which extent *B. burgdorferi* is able to counter these mechanisms and to thus support its dissemination in the human host.

Finally, the unique spirochete morphology of *Borrelia* has enabled the detection of subcellular mechanisms during uptake and processing by macrophages that would be difficult to visualize using more globular bacteria. *B. burgdorferi* is thus also emerging as a useful tool for the detailed study of organelle interactions during phagocytosis in general.

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New Aspects on Bacterial Effectors Targeting Rho GTPases

Emmanuel Lemichez

Abstract The virulence of highly pathogenic bacteria such as *Salmonella*, *Yersinia*, *Staphylococci*, *Clostridia,* and pathogenic strains of *Escherichia coli* involves intimate cross-talks with the host actin cytoskeleton and its upstream regulators. A large number of virulence factors expressed by these pathogens modulate Rho GTPase activities either by mimicking cellular regulators or by catalyzing posttranslational modifications of these small proteins. This impressive convergence of virulence toward Rho GTPases and actin indeed offers pathogens the capacity to breach host defenses and invade their host, while it promotes inflammatory reactions. In return, the study of this targeting of Rho GTPases in infection has been an invaluable source of information in cell signaling, cell biology, and biomechanics, as well as in immunology. Through selected examples, I highlight the importance of recent studies on this crosstalk, which have unveiled new mechanisms of regulation of Rho GTPases; the relationship between cell shape and actin cytoskeleton organization; and the relationship between Rho GTPases and innate immune signaling.

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1 Introduction

The expression of virulence during infection is intimately linked to the capacity of a bacterium to corrupt, either directly or indirectly, the actin cytoskeleton organization and dynamics. One of the most direct examples of this intimate crosstalk is represented by the C2 toxin from *Clostridium botulinum*, which ADP-ribosylates actin monomers, thereby promoting actin filament disassembly (Aktories et al. 1986). Following this seminal work, the last three decades have witnessed the discovery of an unprecedented convergence of virulence factors targeting not only actin monomers but also a subset of small Rho GTPases (Chardin et al. 1989; Paterson et al. 1990; Aktories 2015). These small Rho GTPases that belong to the superfamily of p21-Ras GTPases are critical upstream regulators of the actin cytoskeleton and several cell signaling pathways controlling gene expression. The targeting of Rho GTPases by this family of virulence factors confers major virulence traits upon microbial pathogens of humans, animals, and crops, thereby having a major impact on public health and the economy (Lemichez and Aktories 2013).

The pathogenic potential of bacteria is largely defined by their production of specific virulence factors targeting key host factors (Lemichez and Barbieri 2013). Their contribution to virulence, on the other hand, depends on the expression of host factors, such as adequate toxin receptors and cellular targets. For example, unlike humans, mice are resistant to diphtheria toxin due to a few variations in the amino acid sequence of the toxin receptor. Among these virulence factors, one can distinguish enzymes that affect the regulation of host factors that are crucial to the maintenance of cell homeostasis (Ribet and Cossart 2010). Examples of such virulence factors include the so-called bacterial AB toxins, such as the glucosylating toxins (TcdA and TcdB) from *C. botulinum* (Just et al. 1995) or the CNF1 toxin from pathogenic strains of *Escherichia coli* (Flatau et al. 1997; Schmidt et al. 1997). These toxins function as molecular syringes composed of an enzyme (the A-polypeptide) and one to several functional peptides (B-polypeptides) for host cell binding and the translocation of the A-domain into the cytosol. This mode of intoxication, which is independent of the presence of bacteria, is particularly interesting for studying the impact of intoxication on innate immune signaling in the absence of conserved bacterial components, such as lipopolysaccharide, and for developing tools in medicine. The other mode of injection of virulence enzymes that has been developed by bacteria consists of a direct injection of these enzymes

by cell-bound bacteria through needle-like appendages or injectisomes, such as the type-III secretion system from *Salmonella* (Erhardt et al. 2010). Here, an assortment of different types of virulence effectors is injected into host cells (Galan 2009). These virulence effectors target several host proteins, acting cooperatively, synergistically, antagonistically or/and redundantly.

Studying the crosstalk between bacterial virulence factors and Rho GTPases continues to provide us with an invaluable source of information on the function and the regulation of Rho proteins, as well as their importance at the different stages of infection. Ultimately, these studies may provide us with a more comprehensive view on how evolutionary pressures might have operated to select such a remarkable diversity of virulence factors acting on small Rho GTPases and actin (Boquet and Lemichez 2003).

2 Switching On and Off the Rho GTPases with Virulence Factors

2.1 General Aspects of Rho GTPases

The small GTPases of the Rho family are essential regulatory switches that belong to the p21-Ras superfamily of GTPases (Heasman and Ridley 2008). Primarily, Rho proteins fulfill the critical function of controlling the architecture and dynamics of the actin cytoskeleton via their association with multiple effectors (Heasman and Ridley 2008). RhoA, Rac1, and Cdc42, the most studied Rho proteins, were discovered for their capacity to promote the formation of actin stress fibers, membrane ruffles and filopodia, respectively (Ridley and Hall 1992; Ridley et al. 1992; Nobes and Hall 1995) (Fig. 1). These Rho proteins are encoded by essential genes (Heasman and Ridley 2008). These GTPases also interfere with the signaling

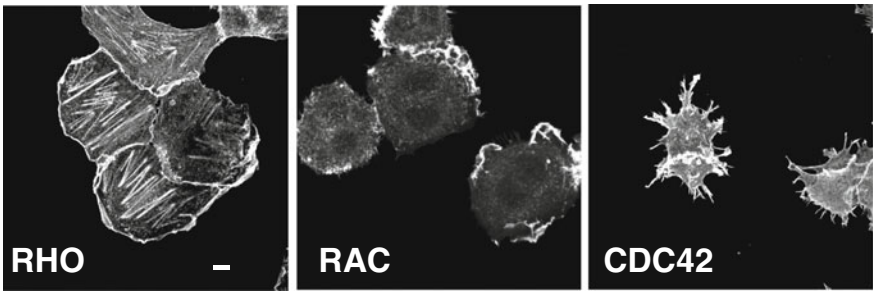

Fig. 1 Rho-, Rac-, and Cdc42-mediated actin cytoskeleton organization. Typical F-actin structures observed in different cell types treated with CNF1 toxin. From *left to right*: Vero cells displaying stress fibers (RhoA-like phenotype), HEp-2 cells displaying membrane ruffles (Rac1-like phenotype) and HEK293 cells displaying filopodial extensions (Cdc42-like phenotype). The cells were treated with 10^{-9}M CNF1 for 24 h, and the actin cytoskeleton was labeled with FITC-modified phalloidin. Scale bar, 5 μm

pathways controlling gene expression, notably those involved in the regulation of NF-κB-driven inflammatory and apoptotic processes (Jaffe and Hall 2005). The switch of Rho GTPases between an active and inactive form is under stringent spatiotemporal control via multiple interactions with a wide range of regulators. These regulators can be divided into three main categories (Fig. 2). Briefly, the G-domains of small GTPases form a canonical motif of highly conserved amino acids involved in the binding and hydrolysis of guanosine triphosphate (GTP) into guanosine diphosphate (GDP) (Wittinghofer and Vetter 2011). The guanine nucleotide-based conformational switch of these proteins involves the hydrolysis of GTP that is stimulated by GTPase-activating proteins (GAPs) (Moon and Zheng 2003) and GDP/GTP exchange by guanine nucleotide exchange factors (GEFs) (Rossman et al. 2005). The hydrolysis of the gamma-phosphate in GTP is catalyzed by a conserved glutamine residue in the switch-II region of small GTPases (Q61 for

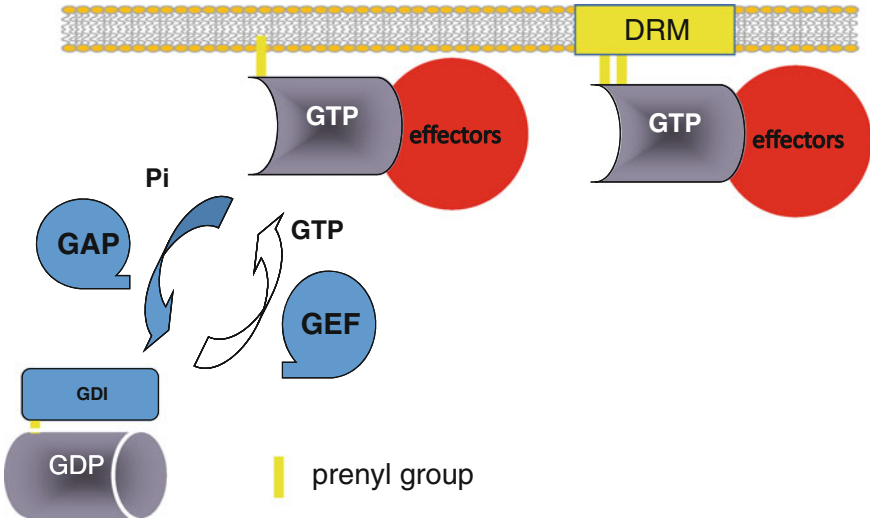

Fig. 2 GTPase-based spatiotemporal cycle. Rho proteins orchestrate the timely local organization of complex arrays of protein-protein interactions. This involves the binding and hydrolysis of guanosine 5′-triphosphate (GTP) into guanosine 5′-diphosphate (GDP). Several residues, including glutamine-61 of Rac1 (Q63 in RhoA), are essential for the hydrolysis of the gamma-phosphate of GTP. Transitions between the guanine nucleotide-bound forms of the Rho proteins produce conformational changes in two flexible regions referred to as the switch-I and II regions. Binding to GTP enables the switch-I region to bind to and activate downstream effector proteins. The transition between the GTP/GDP forms of Rho is catalyzed by a large number of proteins containing GEF (guanine nucleotide exchange factors) or GAP (GTPase-activating protein) domains. The targeting of Rho proteins to cellular membranes for their activation is regulated by guanine nucleotide dissociation inhibitor (GDI) factors that retain the GDP-bound form in the cytosol. Following geranylation, Rac1 can incorporate palmitate at cysteine-178, which likely stabilizes its GTP-bound form and its association with detergent-resistant (liquid ordered) membrane domains (DRMs) (Navarro-Lérida et al. 2012)

Rac1/Cdc42 and 63 for RhoA) that is strictly conserved among canonical small GTPases (Wittinghofer and Vetter 2011). In addition, contacts between the gamma-phosphate of GTP and threonine-35 in Rac1 (37 in RhoA) stabilize the switch-I region, which adopts a conformation prone to bind to CRIB-like domain-containing effectors for activation (Burbelo et al. 1995; Manser et al. 1998). In contrast to these highly conserved switch regions, the divergent carboxy-terminal portions of small GTPases contribute to confer specificity of interactions with effectors by promoting additional interactions and promote the interaction of the small GTPases with negatively charged lipids (Yeung et al. 2006; Lam and Hordijk 2013). Finally, small Rho GTPases are prenylated at their carboxy-termini. Therefore, Rho proteins remain cytosolic due to their interaction with guanine dissociation inhibitors (GDIs), which mask the hydrophobic prenyl group (DerMardirossian and Bokoch 2005; Navarro-Lérida et al. 2012). GDI also protects the Rho proteins from being targeted to proteasomal degradation (Boulter et al. 2010). Rac1 is palmitoylated after its geranylation for partitioning into membrane micro-domains, where it controls actin reorganization (Navarro-Lérida et al. 2012) (Fig. 2). Small Rho GTPases are thus switch proteins that oscillate between the cytosolic GDP-bound "off" form and a GTP-bound active form that is engaged in a series of intermolecular interactions at specific membrane locations to transduce specific signals.

2.2 Bacterial Factors Targeting Rho GTPases

There is an astonishing convergence of virulence factors toward small Rho GTPases (Aktories 2011, 2015) (see examples in Fig. 3). These factors primarily target RhoA and Rac GTPases (Aktories 2011, 2015). Strikingly, this convergence on a group of proteins of small surface contrasts with the high degree of diversity of structures, activities, and modes of action of these virulence factors (Aktories 2011, 2015). We distinguished factors that mimic regulators of Rho proteins, i.e., GEFs, GAPs, and GDIs, from those that catalyze irreversible posttranslational modifications (Galan 2009; Aktories 2011, 2015). In terms of irreversible posttranslational modifications, we distinguished the factors that target the switch-I region of Rho from those targeting the switch-II (Fig. 3). These two different types of post-translational modifications of switch domains inhibit or activate Rho proteins, respectively. All known factors that target the switch-II domain of Rho modify a glutamine residue (61 in Rac1 or 63 in RhoA), which catalyzes the hydrolysis of the gamma-phosphate (Wittinghofer and Vetter 2011). The different types of toxin or virulence factors-mediated posttranslational modifications targeting this glutamine residue encompass deamidation, transglutamination and ADP-ribosylation (Aktories 2011). This is of broad interest considering that Rho GTPases are also controlled by posttranslational modifications in cells (Visvikis et al. 2010).

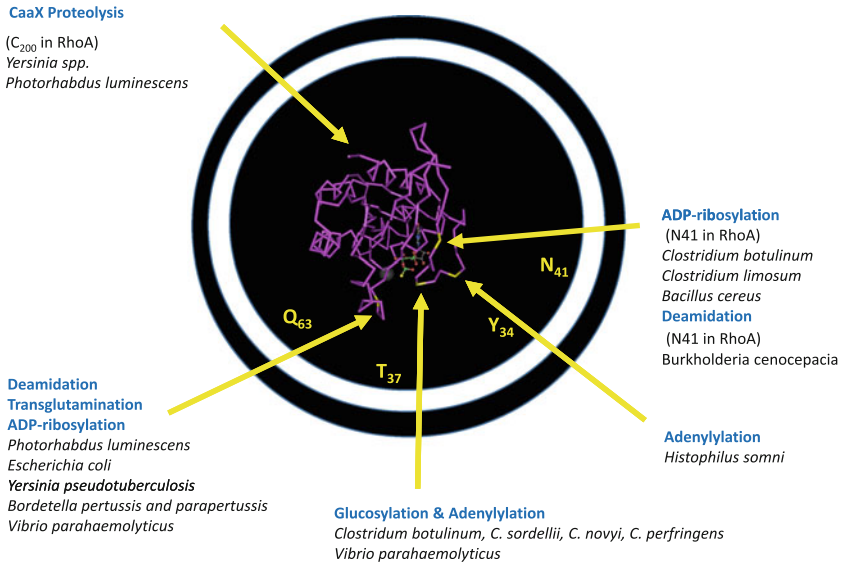

Fig. 3 Convergence of posttranslational modifications toward Rho GTPases. The target represents the structure of the GTPase RhoA. This diagram shows key amino acids (*yellow*) that are targeted by bacterial virulence factors. Examples of pathogens targeting Rho GTPases and the different types of biochemical modifications at each amino acid position are indicated. For exhaustive information see (Aktories 2011)

Posttranslational modifications or direct mutations of this key glutamine residue provoke a permanent activation state of the GTPases. In contrast, when several amino acids of the switch-I effector-binding region are modified, the GTPase signaling capacities are interrupted by blocking the interactions with effector proteins. These residues include RhoA threonine 37 (35 in Rac1/Cdc42), tyrosine 34 (32 in Rac1 and Cdc42) or asparagine 41 in RhoA (39 in Rac1/Cdc42) (Aktories 2015) (Fig. 3). A variety of posttranslational modifications affect the function of these amino acids, comprising ADP-ribosylation, several types of glycosylations, adenylylation, and deamidation (Aktories 2015; Aubert et al. 2016). In addition, the carboxy-terminal portion of Rho proteins can be proteolyzed by YopT from *Yersinia* spp. and LopT from *Photorhabdus luminescens* (Aktories 2011; Shao et al. 2003). In conclusion, these families of virulence factors are extremely diverse in terms of structures, enzymatic activities, and target different amino acids residues on Rho proteins for activation or inactivation. Recent studies on the glucosyltransferase domain of the lethal toxin (TcsL) of *Clostridium sordellii* revealed that efficient modification of small GTPases occurs at the interface with membranes, for example enriched in phosphatidylserine (Mesmin et al. 2004). Deciphering how toxic reactions occur at the interface with membranes is likely key to better understand many aspects of toxin specificity and mode of action.

2.3 Targeting Rho GTPases in Infection

It is still unclear when and how this remarkable diversity of factors corrupting Rho GTPases has been selected to benefit the bacteria. Indeed, they are produced by a large diversity of bacteria that are pathogenic in hosts from insects to mammals. This unprecedented convergence of virulence factors toward such small host proteins probably reflects the remarkable primary sequence conservation among these GTPases in all eukaryotes. The selective pressure may be related to the critical functions of Rho GTPases, notably during phagocytosis, which is conserved in amoebas that are natural predators of bacteria in the soil (Vlahou et al. 2009). A striking example of potential positive selective pressure is provided by the TccC3 toxin component from *P. luminescens*, which ADP-ribosylates the Rho GTPases (Lang et al. 2010). These toxin-producing bacteria can colonize the gastrointestinal tract of infective entomopathogenic nematodes to serve a symbiotic function. *P. luminescens* are released by the worm once it reaches the gastrointestinal tract of insect larvae, thereby offering a way to compromise the insect immune system and promote the development of worms in the insects. TccC3 is also toxic for human cells and thus represents a potential danger if transferred horizontally to human pathogens (Lang et al. 2010).

3 Ubiquitin and Proteasomal System Control of Rho GTPases

3.1 Ubiquitin and Proteasomal Machineries

Degradation of cellular proteins by the ubiquitin and proteasomal system (UPS) is implicated in a large number of biological functions, comprising signaling and immunity, and is deregulated in a large number of human diseases (Ashida et al. 2014). Ubiquitin is a protein of small size (8.5 kDa) that is ubiquitously expressed in tissues of eukaryotes. Ubiquitin can be cross-linked to lysine residues on the target after a series of transfer reactions between ubiquitin carrier proteins (Swatek and Komander 2016). Among these factors, the E3 ubiquitin-ligases (E3L) confer the specificity to the reaction by binding specifically to target proteins. Of note, the degradation of host cell factors can also be triggered by bacterial E3L-like toxins completing the arsenal of pathogens (Ashida et al. 2014). Additional molecules of ubiquitin can be subsequently attached to one of the seven lysines of the previously cross-linked ubiquitin molecule, leading to the formation of various types of poly-ubiquitin chains, such as Lysine-48 (K48) poly-ubiquitylation for substrate targeting to proteasomal destruction (Swatek and Komander 2016; Grabbe et al. 2011). Posttranslational modifications of proteins by mono-, multi-, and poly-ubiquitylation form a repertoire of modifications, allowing specific interactions with multiple families of ubiquitin-binding domain-containing proteins

(Swatek and Komander 2016; Grabbe et al. 2011). If not degradative, protein ubiquitylation can be reverted by the action of de-ubiquitylating enzymes (Reyes-Turcu et al. 2009). Ubiquitylation is now viewed as a system of molecular barcodes controlling protein sorting at the membrane, as well as, local and/or temporal activation of proteins and specific inhibition by degradation.

3.2 *Control of Rho GTPases by Ubiquitin and Proteasomal Machineries*

Targeted proteolysis of proteins is likely the most efficient way to halt or attenuate the transduction of signals, and it has been exploited by potent toxins to corrupt host cells, such as the lethal factor from *Bacillus anthracis*, which cleaves MAPK kinases; AIP56 from *Photobacterium damselae piscicida*, which cleaves NF-κB; or YopT from *Yersinia* spp., which cleaves the carboxy-terminal portion of Rho proteins (Duesbery et al. 1998; Silva et al. 2013; Shao et al. 2003). The cellular ubiquitylation machinery is a direct target of bacterial virulence factors also restraining the activation of Rho GTPases triggered by several bacterial toxins (Doye et al. 2002; Visvikis et al. 2010) (Table 1). For example, once they are injected into the host cell cytosol, CHBP from *Burkholderia pseudomaleimi* and Cif from *enteropathogenic E. coli* preferentially catalyze the selective deamidation of the glutamine residue Q40 in ubiquitin and in Nedd8 ubiquitin-like molecule (Cui et al. 2010). The crosslinking of Nedd8 to the Cullin subunits of Cullin-RING E3 ubiquitin-ligases (CRLs) is critical for their activation (Cui et al. 2010). Therefore, the posttranslational modification of Nedd8 has a blocking effect on CRLs, in particular the Cullin3/Roc1/BACURD complex, which controls the level of total RhoA and therefore its activity, thereby promoting the formation of actin cables (Chen et al. 2009; Jubelin et al. 2010). In addition, the local activation of RhoA and the duration of its signaling activity is controlled by the ubiquitin-mediated degradation of the GTP-bound form of RhoA, which is catalyzed by the HECT-domain containing E3L Smurf1 (Wang et al. 2003; Ozdamar et al. 2005; Boyer et al. 2006b). The Cytotoxic Necrotizing Factor-1 (CNF1) from *E. coli* provides us with a second example of interference of UPS with bacterial virulence. Here, the study of CNF1 has been instrumental in unraveling the control of Rho GTPases by UPS and in unveiling the E3L targeting Rac1 (Doye et al. 2002; Visvikis et al. 2010). CNF1 belongs to a group of dermonecrotic toxins and virulence factors injected by the Type-3 secretion system (TSS3) found in *E. coli*, *Yersinia pseudotuberculosis*, *Bordetella,* and *Vibrio*. CNF1 toxin catalyzes the deamidation of the glutamine-61 residue in Rac1 or Cdc42 and the equivalent glutamine-63 in RhoA, thereby inhibiting their intrinsic and GAP-stimulated GTPase activity (Lerm et al. 1999; Flatau et al. 1997; Schmidt et al. 1997). Once Rac1 is activated, it is sensitized to ubiquitin-mediated proteasomal degradation (Doye et al. 2002). This downregulation of Rho signaling by UPS was initially

Table 1 Rho GTPase ubiquitylation machinery

E3 ligases	Rho GTPases	Targeted form	Known lysine(s) targeted	References
Smurf1[a]	RhoA	Nucleotide-free and/or GTP-bound	K6, K7	Wang et al. (2003), Ozdamar et al. (2005), Boyer et al. (2006b)
Smurf2	RhoA	Likely membrane associated RhoA (blocked by the C2 domain interacting with HECT)		Lu et al. (2011), Wiesner et al. (2007)
Cul3/ROC1/BACURD[a]	RhoA	GDP-bound	Unknown	Chen et al. (2009)
Hace1[a]	Rac1	GTP-bound	K147	Torrino et al. (2011), Castillo-Lluva et al. (2013), Goka and Lippman (2015)
XIAP, c-IAP1	Rac1	GTP-bound	K147	Oberoi et al. (2012)
SCF-FBXL19	RhoA	GTP/GDP-bound	K135	Wei et al. (2013)
SCF-FBXL19	Phospho-Ser71-RhoA	GTP/GDP-bound	K166	Zhao et al. (2013)
NEDD4L	RhoB			Zhang et al. (2016)

[a]E3L proteins known to interfere with bacterial toxins

recognized as a means for cells to control the duration and extent of G-protein signaling. In support of this hypothesis is the finding that activation of Rac1 by the GEF-domain of Dbl sensitizes this GTPase to ubiquitylation. Again, the activity of CNF1 was essential to screen for the E3 ubiquitin-ligase responsible for the degradation of Rac1 (Torrino et al. 2011). The regulation of Rac1 by HACE1 was confirmed by independent groups, which extended these findings to show the importance of this regulation in the control of cell migration and reactive oxygen species production (Castillo-Lluva et al. 2013; Goka and Lippman 2015; Daugaard et al. 2013). The activated form of Rac1 binds to the HECT-domain and ankyrin-repeat containing E3 ubiquitin protein ligase 1 (HACE1) and is prone to poly-ubiquitylation by HACE1 E3 ubiquitin-ligase activity (Torrino et al. 2011). In vitro experiments have established the high level of specificity of ubiquitylation of the GTP-bound form of Rac1 (Torrino et al. 2011). Depletion of HACE1 leads to an increase in the Rac1-dependent migration of mouse embryonic fibroblasts and

increase of NADPH-mediated production of ROS (Castillo-Lluva et al. 2013; Daugaard et al. 2013). The importance of the regulation of UPS in the control of Rho GTPases continues to be unveiled with recent findings implicating the involvement of Nedd4L E3L in the proteasomal degradation of RhoB (Zhang et al. 2016).

4 Corruption of the Endothelial Barrier

4.1 Induction of Cellular Dewetting by Toxins

The endothelium forms a critical semipermeable barrier that prevents the dissemination of bacteria once they reach the bloodstream (Lemichez et al. 2010). The actin-myosin cytoskeleton and its upstream regulators largely control the barrier function of the endothelium (Bazzoni and Dejana 2004). RhoA signaling controls non-muscle myosin-II (NMII)-dependent F-actin fasciculation into stress fibers and contractility. In addition, RhoA establishes a coherent actomyosin network that transmits forces across the cytoplasm to equilibrate centripetal forces exerted at the edges of cells (Cai et al. 2010). A series of recent studies established that the breaching of the coherent actomyosin network in endothelial cells via direct inactivation of RhoA or inhibition of the Rho kinase ROCK with Y-27632 promotes the spreading of cells and, as a consequence, triggers the opening of transendothelial cell macroaperture (TEM) tunnels (Boyer et al. 2006a; Lemichez et al. 2012) (Fig. 4). Opening and widening of TEMs occur via a newly identified process, referred to as cellular dewetting (Gonzalez-Rodriguez et al. 2012; Lemichez et al. 2012). They open following disruption of actin cytoskeleton cohesion, due for example to an increase of the flux of cyclic AMP and consequently the downstream activation of the PKA and the exchange protein directly activated by cAMP (Epac)/Rap pathways (Maddugoda et al. 2011). This phenomenon is exacerbated by bacterial adenylate-cyclase toxins from *B. anthracis* and *Bordetella* spp. (Maddugoda et al. 2011). TEMs open transiently. Major progress has been made in identifying the physical principles governing the widening of TEMs and the molecular machinery that drives their closure (Maddugoda et al. 2011; Gonzalez-Rodriguez et al. 2012). Indeed, the opening of TEMs is a newly described cellular form of liquid dewetting. This common phenomenon occurs when a viscous liquid is forced to spread on a hydrophobic substrate, similar to heated oil on a nonstick pan (de Gennes et al. 2004). With regard to liquid dewetting, the aperture enlarges up to the formation of droplets as TEMs reach a maximal size in cells. Therefore, the cellular dewetting theory allows us to postulate the existence of a linear tension at the edge of TEMs, which equilibrates the membrane tension that pulls on the edges (Gonzalez-Rodriguez et al. 2012). TEMs represent not only a challenging model of direct crossing of the endothelium by pathogenic bacteria and/or their toxins but also a remarkable model system to investigate the

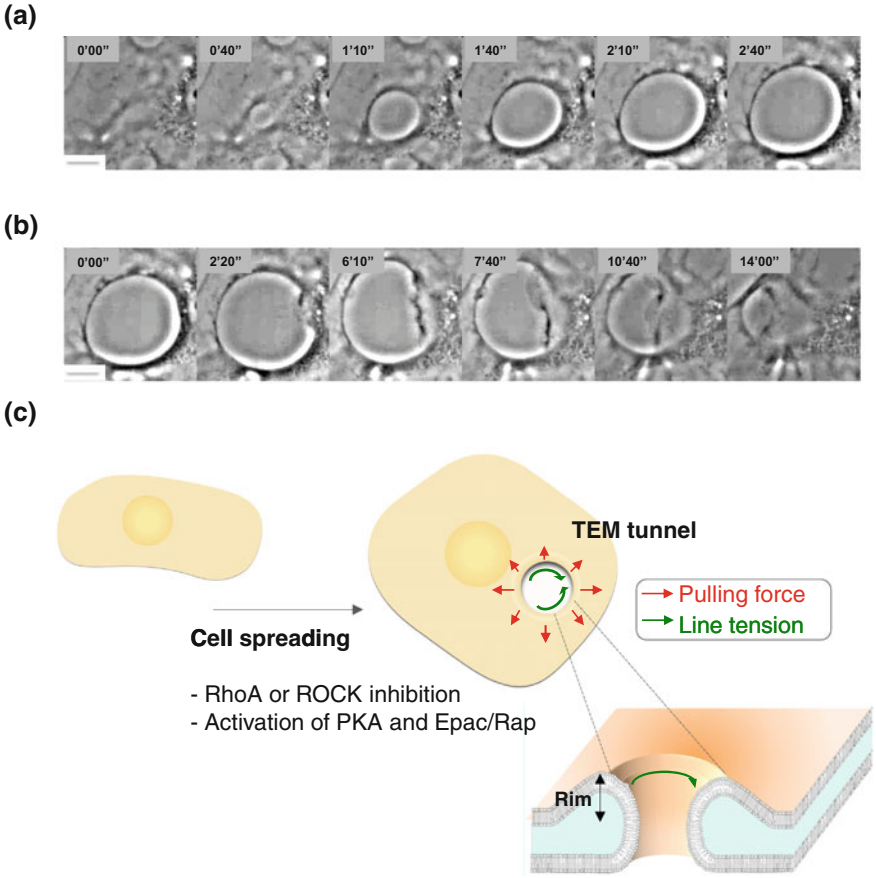

Fig. 4 Opening of transendothelial cell macroaperture (TEM) tunnels. **a**, **b** Series of video images of the opening (**a**) and closure (**b**) of a transendothelial cell macroaperture (TEM) tunnel in a HUVEC intoxicated with the EDIN homologue C3-exoenzyme from *Clostridium botulinum* 24 h. Bar = 10 μm. **c** Schematic representation of TEM tunnel opening that results from the spreading of cells due to a reduction of contractility of the actomyosin cytoskeleton that promotes an increase of membrane tension. *Green* and *red arrows* represent forces at play around the edge of transcellular tunnels together with the distance between membranes determined by atomic force microscopy

relationships between cell architecture and cytoskeletal organization. This idea is supported by the finding that the inverse-Bin, Amphiphysin and Rvs, or (I-BAR) domain-containing protein missing in metastasis (MIM) senses via its I-BAR domain the curved edge of TEMs, where it accumulates soon after the opening (Maddugoda et al. 2011; Saarikangas et al. 2009). The recruitment of MIM in part controls the linear tension, as well as the later recruitment and activation of Arp2/3 complex that promotes the extension of membrane waves that close the TEMs (Maddugoda et al. 2011; Gonzalez-Rodriguez et al. 2012).

4.2 Invasive Properties of Virulence Factors Targeting RhoA

Most bacterial toxins target various Rho GTPases, with the exception of the group of C3-like ADP-ribosyltransferases, which catalyze the ADP-ribosylation of RhoA (Wilde et al. 2003). C3-like exoenzymes consist of a single polypeptide chain that penetrates into the host cells via an undefined molecular mechanism. C3-like factors are isolated from different strains of *Clostridia*, as well as from *Staphylococcus aureus*, among which there are three isoforms, EDIN-A, EDIN-B and EDIN-C (Yamaguchi et al. 2001). ADP-ribosylation of RhoA promotes its tight association with RhoGDI, leading to the sequestration of an inactive form of the GTPase in the cytosol (Fujihara et al. 1997; Genth et al. 2003). Most recently, genetic evidence demonstrated the invasive properties conferred by *edin*B to *S. aureus* in a model of pneumonia-induced bacteremia (Courjon et al. 2015). Indeed, the deletion of *edin*B reduced the risk of bacteremia by 50 % in animals suffering from pneumonia triggered by the European lineage community-acquired methicillin resistant *S. aureus* (CA-MRAS) strain ST80-MRSA-IV. This property was restored upon the re-expression of wild type (WT) EDIN-B but not by the expression of a catalytically inactive mutant. The presence of *edin*B does not improve the capacity of *S. aureus* to persist in the bloodstream, reinforcing the idea that this factor acts by promoting bacterial translocation through the successive pulmonary alveolar epithelial and endothelial barriers rather than by promoting an anergy of phagocytes in the context of the bloodstream (Courjon et al. 2015; Munro et al. 2010). Of note, EDIN-like factors such as C3 from *C. botulinum* may interfere with immune cells in other context (Barth et al. 2015). This dual role of breaching the epithelial and endothelial barriers through the inhibition of RhoA is consistent with previous cell biology studies that had implicated RhoA in the control of tight junction stability by studying C3 from *C. botulinum* (Nusrat et al. 1995). All these findings are in accordance with a combination of epidemiological surveys that point to a critical role for EDIN factors in the dissemination of *S. aureus* and the induction of septic metastasis (Lemichez et al. 2012).

5 Corruption of Innate Immunity

5.1 Virulence Factors Activating Rho GTPases and the Inflammasome

We must address rather conflicting relationships with the bacterial world. On one hand, we have learned how to confine and tolerate bacteria of the gut flora, which contribute to essential functions in digestion and help maintain our body homeostasis (Sansonetti 2011; Brodsky and Medzhitov 2009). On the other hand, our bodies must discriminate bacteria that are pathogenic and therefore express distinct

combinations of toxins that corrupt the host cells homeostasis (Brodsky and Medzhitov 2009; Stuart et al. 2013; Lemichez and Barbieri 2013). Several studies have recently revealed that toxins activating Rho GTPases found in extra-intestinal pathogenic strains of *E. coli* and in some strains of *Yersinia* promote acute inflammatory responses (Diabate et al. 2015; Munro et al. 2004; Schweer et al. 2013). The importance of Rho-activating factors such as SopE, SopE2 and SopB in *Salmonella*-induced inflammatory responses has also been demonstrated (Bruno et al. 2009). Acute inflammatory responses involve a combination of (i) a priming-phase of gene expression of inflammatory cytokines and chemokines, also comprising pro-inflammatory IL-1β/IL18 in immune cells, as well as (ii) the establishment of an execution-phase that involves the organization of the inflammasome complex for IL-1β/IL18 processing and secretion (Zhao and Shao 2016; Broz and Monack 2011). The induction of expression of inflammatory mediators triggered by Rho-activating factors, in absence of other bacterial factors, is consistent with initial studies that have determined the capacity of Rho GTPases, notably Rac1 and Cdc42, in promoting c-Jun, p38 MAP kinase and NF-κB signaling pathway activation (Minden et al. 1995; Coso et al. 1995; Perona et al. 1997; Munro et al. 2004). More recent studies have revealed the importance of Rho GTPase activity combined with that of Nucleotide-binding Oligomerization Domain (NOD) and the RIP-kinases components for efficient NF-κB-dependent transcription of inflammatory mediators in response to virulence factors and conserved bacterial components (Arbibe et al. 2000; Keestra et al. 2013; Boyer et al. 2011; Stuart et al. 2013; Kawano et al. 2010). Variations of hierarchy and/or interplay between these factors have been analyzed in different model organisms and contexts of infection (Stuart et al. 2013). Importantly, this capacity of cells to sense the imbalance of cell signaling triggered by virulence factors activating Rac1/Cdc42 GTPases has been proposed as a general means to discriminate between the presence of commensal bacteria versus pathogen attack (Stuart et al. 2013; Keestra et al. 2013; Xu et al. 2014). Paralleling their effects on gene expression, the activation of Rac1 and Cdc42 by virulence factors can directly engage inflammasome signaling to promote IL-1β secretion, which plays a major role in the execution of efficient programs of pathogen eradication (Stuart et al. 2013). For example, the inflammation of the gut epithelium that is elicited by *Salmonella typhimurium* infection is triggered by the activation of caspase-1 signaling, which is mediated by SopE at the level of enterocyte mainly (Muller et al. 2009). The SopE-mediated activation of caspase-1 in vitro is mediated by Rac1 and Cdc42. We have begun to better appreciate how some bacteria deal with these anti-bacterial responses, although this has to be better characterized mechanistically. In the context of the bacteremia induced by CNF1-producing extra-intestinal strains of *E. coli*, IL-1β-mediated host responses promote an efficient destruction of bacteria and favors host survival (Diabate et al. 2015). Therefore, in this context, the CNF1 toxin acts as an anti-virulence factor. These detrimental responses for bacteria are specifically blunted by the concurring action of the pore-forming toxin

α-hemolysin from *E. coli* (Diabate et al. 2015). This α-hemolysin acts downstream of Rac1 activation to prevent IL-1β secretion (Diabate et al. 2015). In conclusion, the combined sensing of conserved products of bacteria and the specific virulence factor activity, notably toward Rho GTPases, synergistically modulate the level of inflammasome-mediated anti-microbial responses to efficiently fight pathogen attack. The precise consequences of the activation or inhibition of Rho GTPases remain to be fully deciphered, as does the importance of actin cytoskeleton remodeling in this context. Some pathogens respond to these host countermeasures by the production of a second set of factors that blunt virulence factor sensing.

5.2 *RhoA-Inactivating Toxins and the Inflammasome*

Normally in our body the bacterial flora is confined by the joint action of physical mucoepithelial barriers, the immune system and growth limitation promoted by inter-species competition (Sansonetti 2011; Brodsky and Medzhitov 2009). The induction of bacterial imbalance or dysbiosis triggers deadly inflammatory bowel diseases (DuPont and DuPont 2011). One such example is provided by *Clostridium difficile*, which can overgrow following antibiotic treatment and promote diseases ranging from diarrhea to pseudomembranous colitis, which is potentially fatal. Several strains of *C. difficile* produce two potent toxins (TcdA and TcdB), which glucosylate and inactivate RhoA, Rac1, and Cdc42 (Just et al. 1995). Cell biology studies have established the capacity of these toxins to disrupt the function of the epithelial barrier, establishing the importance of RhoA and Rac1 in the control of adherent and tight junctions (Boquet and Lemichez 2003; Lemichez and Aktories 2013). Following toxin action, the diffusion of bacterial conserved factors across the epithelial barrier likely contributes to stimulate the gut immune system. Recent new exciting findings highlighted how these large clostridial toxins targeting RhoA promote directly inflammatory responses. Indeed, the glucosyltransferase activity of TcdB in bone marrow-derived macrophages (BMDM) activates the nucleotide-binding oligomerization domain receptors (NLR) protein Pyrin, which in turn mediates the activation of the ASC/Caspase-1 inflammasome complex for interleukin-1 beta processing and secretion (Xu et al. 2014). Study of this mode of activation of pyrin and the inflammasome is of high importance considering that activating mutations in the pyrin-encoding gene *MEFV* also cause an auto-inflammatory disease known as Mediterranean fever (French 1997). Other NLR factors, including NOD1/2, Nlrp3, Nlrc4 and AIM2 are not involved. Toxins inhibiting small GTPases other than RhoA do not stimulate pyrin/inflammasome signaling (Xu et al. 2014). Activation of the inflammasome by RhoA inhibition results from the activity of several toxins targeting the switch-I domain at different positions and by different biochemical mechanisms (Xu et al. 2014). This holds true for the virulence factor TecA from *Burkholderia*

cenocepacia that inhibits RhoA by deamidation of its asparagine 41 and an equivalent in Rac1 (Aubert et al. 2016). The toxic activity of TecA is perceived by host cells and converted into efficient activation of the pyrin inflammasome thereby restricting the infection and promoting animal survival (Xu et al. 2014; Aubert et al. 2016). This phenomenon of inflammasome activation does not involve the sensing of actin depolymerization as shown by cytochalasin-D treatment. Instead it appears to involve an uncharacterized indirect sensing of the consequences of RhoA inactivation (Xu et al. 2014).

6 Conclusions

In conclusion, during these last years, we have witnessed the discovery of an incredible variety of examples of virulence factors of pathogens targeting Rho GTPases. The coming years will probably see an enrichment of the repertoire of modifications of Rho and other targeted GTPases by newly described toxins, and the identification of host factors critical for posttranslational-based regulation of the activity of Rho GTPases, a so far less known area. Deregulations of the small Rho GTPases are implicated in a large variety of human diseases, comprising immunological disorders, mental retardations, and cancer. Much remains to be elucidated on this remarkable convergence on a few targets during infection. It is certainly important to determine in greater detail the action of factors targeting Rho GTPases at the different steps of infection. Ultimately, we might be able to use this knowledge to develop innovative therapeutic molecules and diagnostic tools in infectiology and vaccinology, as well as in degenerative diseases and oncology.

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Type III Secreted Virulence Factors Manipulating Signaling to Actin Dynamics

Theresia E.B. Stradal and Sonia C.P. Costa

Abstract A key aspect of bacterial pathogenesis is the colonization and persistence within the host and, later on, its dissemination to new niches. During evolution, bacteria developed a myriad of virulence mechanisms to usurp the host's sophisticated defense mechanisms in order to establish their colonization niche. Elucidation of the highly dynamic and complex interactions between host and pathogens remains an important field of study. Here, we highlight the conserved manipulation of the actin cytoskeleton by some Gram-negative gastrointestinal pathogens, addressing the role of type III secreted bacterial GEFs at the different steps of pathogenesis. As a final topic, we review cytoskeleton dynamics induced by EPEC/EHEC strains for pedestal formation.

Contents

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1 Introduction

Signaling pathways regulated by proteins of the Rho GTPase family are involved in many cell functions, ranging from cell polarity, migration, cell division and vesicle trafficking to transcription and inflammatory reactions, just to name a few (Hall 2012). Rho GTPases have the distinguishing feature of cycling between an inactive GDP-bound state and an active GTP-bound state. Two flexible domains, switch I and switch II, are involved in nucleotide binding and undergo conformational changes, required for cycling between states. Furthermore, the inactive/active cycling is controlled by many GTPase binding proteins (Corbett and Alber 2001). For instance, guanine nucleotide exchange factors (GEFs) regulate the activation of GTPases by facilitating the exchange of GDP for GTP. In the GTP-bound state, the GTPase then binds to downstream effector proteins, thus activating signaling pathways (Worthylake et al. 2000). Inversely, guanine nucleotide dissociation inhibitors (GDIs) function to maintain the Rho GTPases in an inactive GDP-bound state (Scheffzek et al. 2000) and/or protect it from degradation (Boulter et al. 2010). Finally, inactivation of Rho GTPases is further regulated by GTPase-activating proteins (GAPs), enhancing their intrinsic hydrolysis rate (Vetter and Wittinghofer 2001).

The best characterized members of the Rho GTPases family, comprising 20 members (Heasman and Ridley 2008), are the proteins RhoA, Rac1, and Cdc42. RhoA has been shown to be involved in the formation of stress fibers, while Rac is responsible for actin-rich lamellipodia protrusions and Cdc42 for finger-like filopodia structures (Jaffe and Hall 2005). Most likely due to their wide distribution in eukaryotes and owing to their conserved key roles in controlling cytoskeletal reorganization, Rho GTPases are a preferred target of a myriad of bacterial pathogens (Finlay 2005; Popoff 2014).

Gram-negative gastrointestinal bacterial pathogens such as several pathogenic *E. coli* (EPEC and EHEC), *Shigella*, or *Salmonella* are responsible for millions of infections and >1 million deaths of children under age of 5 years each year worldwide (Miller et al. 2008). Remarkably, a common infection feature of these pathogens is the refined delivery of proteins directly into the host cell. These bacterial virulence factors, also known as effectors, are injected by sophisticated syringe-like nanomachines designated Type III secretion system (T3SS). While T3SSs are conserved among different species, each bacterium secretes a relatively distinctive set of effectors (Galan et al. 2014). Notably, the respective effector cocktail seems to define the specific colonization niche and typical lifestyle of a given bacterium. *Salmonella* and *Shigella* species are intracellular pathogens that trigger their uptake into non-phagocytic gut epithelial cells (Dunn and Valdivia 2010). Invasion by these bacteria depends on the activation of Rho GTPases by a concerted action of a set of T3 effectors that ignite strong actin rearrangements leading to their engulfment by a distinctive, strong membrane ruffling (Rottner et al. 2005). On the other hand, members of the EPEC/EHEC group (also known as A/E lesion pathogens) are primarily extracellular pathogens that adhere to the surface of gut epithelial cells, causing effacement of microvilli and formation of the so-called

actin-rich pedestals underneath bacteria. To do so, these bacteria also deliver T3 effectors, manipulating the actin cytoskeleton and causing the designated attachment/effacement (A/E) lesions (Campellone 2010a).

Only few conserved families of T3 effectors have been defined despite the common targets they affect in the host. In the last decade, work by Alto and colleagues was instrumental for the identification of the WxxxE family of bacterial GEF mimics, a novel T3SS effector family. Moreover, crystal structures of *Salmonella* T3 effectors SopE/SopE2, also harboring GEF activity, brought to light the elegant mimic of bacteria proteins that hijack Rho GTPases (Buchwald et al. 2002; Huang et al. 2009; Klink et al. 2010). Together, WxxxE and SopE/SopE2 effectors define a family of bacterial GEFs whose catalytic activity plays a pivotal role in the lifestyle and pathogenicity of enteric bacteria of the genus *Salmonella* and *E. coli* including *Shigella*.

2 WxxxE Effectors

Effectors delivered by T3SSs are present in several distantly related bacteria and have been extensively studied in the past 20 years. These bacterial proteins are very diverse, with little overall sequence homology, and co-opt a broad range of host pathways, challenging target identification and characterization. Generally, T3 effectors have at least two well-defined regions, such as an N-terminal secretion/translocation signal and a C-terminal catalytic region (Buttner 2012). Bioinformatic BLAST database analyzes using the EHEC effector Map as bait led to the identification of a group of effectors harboring two invariant amino acids, a tryptophan (W) and glutamic acid (E) spaced by three variable residues, termed, accordingly, WxxxE motif, in their C-terminus. Alanine replacement of either conserved residue abolished the cytoskeletal phenotype as induced by expression of the respective ectopic effector in eukaryotic cells. Thus, the WxxxE motif provided the first identified domain with a molecular signature of functionality present in 24 T3 effectors of enteric pathogens, namely MAP, EspM, and EspT of EHEC/EPEC and the related *Citrobacter rodentium*, IpgB1, IpgB2 of *Shigella* sp. and SifA, SifB of *Salmonella* sp. (Alto et al. 2006). While it was initially reported that this newly discovered WxxxE family embodies GTPase mimics, subsequent structural data revealed that WxxxE effectors rather display GEF activity (Ohlson et al. 2008).

This and other structural studies (Buchwald et al. 2002; Huang et al. 2009; Klink et al. 2010; Ohlson et al. 2008) also revealed that the WxxxE family GEFs share a common fold with *Salmonella* SopE and SopE2, the first bacterial GEF mimics for Rho GTPases that were identified almost a decade earlier. The lack of sequence conservation between SopE-type GEFs and WxxxE family GEFs indicates that Gram-negative bacteria harboring a T3SS invented this type of enzyme twice, highlighting the advantage, which is provided by proteins with GEF activity to these bacteria.

Proteins of the WxxxE family and SopE-type GEFs are a hallmark in the manipulation of Rho GTPase signaling, and their study led to the illumination of virulence mechanisms at a new level of complexity and refinement. Understanding how different pathogens manipulate host–defense mechanisms is fundamental to develop new therapeutic approaches, in particular in view of the advent of multidrug-resistant strains.

3 *Salmonella*

The discovery of *Salmonella* T3 effectors with GEF activity was instrumental in the field of host–pathogen interaction. SopE and SopE2 share >70 % amino acid sequence homology and are responsible for the formation of actin-rich ruffles at the site of *Salmonella* invasion. Bacterial mutants for SopE/SopE2 show reduced invasion rates and consequently attenuated virulence (Bakshi et al. 2000; Hardt et al. 1998; Wood et al. 1996). SopE activates Cdc42 and Rac equally well, while SopE2 has a preference for Cdc42 (Friebel et al. 2001). Interestingly, despite the absence of sequence similarity with eukaryotic GEFs, SopE/SopE2 regulates the activation of GTPases by a similar mechanism: Many eukaryotic Rho GEFs harbor a common Dbl homology (DH) domain that forms the catalytic region, responsible for binding to switch I and II regions of Rho GTPases (Milburn et al. 1990). Notably, structural studies revealed that SopE interacts with GDP-bound Cdc42 between the switch I and II in a lock-and-key mechanism as described for eukaryotic Dbl (Buchwald et al. 2002). Furthermore, superimposed structures of SopE/Cdc42 and the eukaryotic Tiam1/Rac revealed, for the first time, a strikingly similar rearrangement in the switch I and II regions of GTPases induced by bacterial and eukaryotic GEFs (Buchwald et al. 2002; Williams et al. 2004). This highlights the fascinating, refined evolution of enteric bacteria, harboring proteins with dedicated GEF activity toward a foreign host target. In addition to SopE/E2 from *Salmonella* sp., two more related T3 effectors were identified in other Gram-negative bacteria, namely CopE from *Chromobacterium violaceum* (Miki et al. 2011) and BopE from *Burckholderia pseudomallei* (Stevens et al. 2003).

It is interesting to note that in addition to two GEFs for Rac and Cdc42, *Salmonella* invasion was recently elegantly described to depend also on RhoA (Hanisch et al. 2011, 2012). Inhibition of both the Rac-WAVE-Arp2/3 and Rho-ROCK-myosin II pathways of invasion was additive and abrogated invasiveness of bacteria. Likewise, the deletion of SopE/E2 (the two GEFs) and another factor, SopB, was additive and diminished invasion (Zhou et al. 2001). SopB is a phosphoinositide phosphatase (PIPase), and the presence of this T3 effector could clearly be correlated with myosin II-dependent invasion (Hanisch et al. 2011). The pathway from this enzyme to RhoA activation may well involve host cell signaling initiated by its products, such as PiP2, but the molecular details are currently ill-defined (Hanisch et al. 2012).

Lastly, the WxxxE effector SifA (*Salmonella*-induced filaments A) of *Salmonella* is crucial for the formation of tubular filament extensions from the phagosome, reminiscent of other forms of endosomal tubulation. These structures are necessary for the maintenance of the *Salmonella*-containing vacuole (SCV), an acidic compartment, in which *Salmonella* species survive intracellularly (Beuzon et al. 2000; Brumell et al. 2002; Garcia-del Portillo et al. 1993; Stein et al. 1996). In fact, *Salmonella* strains deleted of SifA display slower replication rates and are attenuated for virulence in mice (Brumell et al. 2001). SifA activity mainly affects Rab GTPase signaling, resulting in actin reorganization during endosomal tubulation (Guignot et al. 2004; McEwan et al. 2015). Unlike the other WxxxE members, SifA comprises an additional domain situated between the N-terminal secretion signal and the C-terminal WxxxE domain. This additional domain interacts with the PH domain of a kinesin-binding protein (known as SKIP, for SifA and kinesin-binding protein) (Diacovich et al. 2009), crucial for endosomal tubulation, as SKIP suppression, by siRNA, rendered infected cells unable to form SIFs (***S****almonella*-**i**nduced **f**ilaments) (Dumont et al. 2010). SifA, along with its homolog SifB, clearly belongs to the WxxxE family, and SifA apparently binds to RhoA (Ohlson et al. 2008), although both SIFs fail to induce the typical phenotypes of RhoA, Rac1, or Cdc42 activation when expressed in mammalian cells (Alto et al. 2006). However, ectopic expression of constitutively active RhoA, RhoB, and RhoC along with another *Salmonella* effector, SseJ, leads to the formation of SIFs. SseJ per se is not essential for endosomal tubulation, as a deletion strain still exhibits SIF formation (albeit with lower frequency) (Ruiz-Albert et al. 2002). It was thus suggested that a complex formed by SseJ, RhoA, SifA, and SKIP is sufficient to induce endosomal tubulation (Ohlson et al. 2008), but formal proof for the activity of such complex or exact knowledge on the molecular mechanisms involved is still missing.

4 *Shigella*

Shigella enters host cells in a fashion that is phenotypically reminiscent of *Salmonella*. Notwithstanding, no ortholog of SopE or SopE2 could be identified. Upon first description of the WxxxE family and followed by the elucidation of the activities of the *Shigella* representatives of this family, namely IpgB1 and IpgB2, it became clear that bacterial GEFs for Rho GTPases are a common theme in host invasion. The effector IpgB1 ignites membrane ruffling crucial for *Shigella* invasion (Ohya et al. 2005). IpgB1 has in vitro GEF activity toward Rac and, to a lesser extent, Cdc42 (Alto et al. 2006; Huang et al. 2009). Deletion of IpgB1 decreases *Shigella* invasion and, subsequently, virulence (Ohya et al. 2005). In eukaryotic cells however, activation of Rac can be regulated by complex processes in response to a plethora of intrinsic and extrinsic signaling stimuli. For instance, RhoG is an upstream stimulator of Rac involved in the activation of ELMO (engulfment and motility protein), which subsequently interacts with the atypical GEF Doc180. The

formed complex ELMO/Doc180 localizes to the membrane where it concomitantly activates Rac (Brugnera et al. 2002; Grimsley et al. 2004; Katoh and Negishi 2003). It was suggested that the bacterial GEF IpgB1 could also induce ruffling at the bacterial entry site through the activation of the ELMO/Dock188 complex (Handa et al. 2007). Finally, the subcellular localization of IpgB1 is conferred by a MLD-like domain within the N-terminus of the protein, presumably targeting it to the membrane through hydrophobic residues (Costa and Lesser 2014).

Regarding the second *Shigella* WxxxE member, IpgB2 is thought to be important for the efficient *Shigella* invasion of polarized epithelial cells in conjunction with IpgB1 (Hachani et al. 2008). IpgB2 induces stress fiber bundles through the activation of the GTPase RhoA. Stress fiber formation is dependent on the WxxxE motif, as a mutation in the invariant residues failed to induce the IpgB2 phenotype when transiently expressed in mammalian cells (Alto et al. 2006). Structural studies of IpgB2 and RhoA showed that this effector is a GEF for RhoA (Klink et al. 2010). Interestingly, binding of IpgB2 to RhoA occurs through charged or polar interactions. Furthermore, proper orientation of the catalytic loop of IpgB2 plays a role in RhoA activation. Moreover, structural analysis revealed that a double serine motif in the catalytic loop forms hydrogen bonds with the WxxxE motif, pivotal for RhoA activation. Additionally, microscopy studies confirmed that substitution of both serines by alanines resulted in an IpgB2 variant that can no longer induce stress fibers in mammalian cells (Klink et al. 2010).

It should be noted that *Shigella* harbors another virulence factor, which is homologous to a *Salmonella* SopB, namely IpgD (Niebuhr et al. 2002). The activity of IpgD was described to contribute to the escape from primary vacuoles formed upon invasion (Mellouk et al. 2014). Moreover, IpgD products activate PI 3 kinase (Pendaries et al. 2006) and stimulate Tiam-1 and Rac-dependent ruffling (Viaud et al. 2014). It remains to be shown whether this factor also triggers Rho activation and contraction like *Salmonella* SopB and—in the case of *Shigella*—in concert with IpgB2.

5 A/E Lesion Pathogens

Members of the EPEC/EHEC group, as well as the rodent pathogen *C. rodentium*, are grouped together as so-called A/E lesion pathogens (Kaper et al. 2004). They remain primarily extracellular, where they adhere to the plasma membrane of gut epithelial cells and cause effacement of microvilli and formation of actin-rich pedestals underneath bacteria. To do so, these enteric bacteria also deliver T3 effectors that manipulate the actin cytoskeleton, causing the so-called attachment/effacement (A/E) lesions (Campellone 2010a).

Among the many translocated effector proteins, the translocated intimin receptor Tir plays a critical role in intimate attachment through binding the bacterial outer membrane protein intimin. Signaling of Tir to the host cell cytoskeleton induces actin assembly and consequently pedestals, which will be discussed in detail below.

Nonetheless, these bacteria also secrete a number of additional T3 effectors, among them bacterial GEFs of the WxxxE family acting on host Rho GTPases. Whereas their activity is dispensable for pedestal formation, these are crucial for establishing the full infection phenotype, as their deletion significantly attenuates virulence in vivo.

Map (mitochondrial associated protein) (Kenny and Jepson 2000) has been shown to be involved in EPEC/EHEC-induced filopodia formation at very early stages of infection, i.e., upon first contact of bacteria with the host cell plasma membrane (Kenny et al. 2002). In fact, activation of Cdc42 during EPEC infection depends on Map (Berger et al. 2009; Huang et al. 2009). Ectopic expression of Map results in actin rearrangements characterized by the formation of filopodia and, to a lesser extent, lamellipodia in eukaryotic cells (Alto et al. 2006). Interestingly, the C-terminus of Map harbors a PDZ domain-binding motif (PSD-95/Disc Large/ZO-1) (Alto et al. 2006), which was demonstrated to be crucial for filopodia formation (Berger et al. 2009). The PDZ-binding motif of Map mediates interaction with the PDZ domain of the eukaryotic ezrin-binding protein 50 (Ebp50), and remarkably, deletion of this short motif (Map ΔTRL) abrogates filopodia formation. In this study, Map induction of filopodia depended on the proper intracellular targeting of this effector conferred by the PDZ ligand Ebp50. Structural studies of Map, in complex with its eukaryotic target Cdc42, demonstrated that this WxxxE effector again utilizes a mechanism similar to eukaryotic GEFs. Map binds to the switch I region of Cdc42, in a lock-and-key pair mechanism, leading to a V-shaped structure (Huang et al. 2009). Furthermore, structural and mathematical modeling, along with synthetic biology methods and experimental analyses, revealed that the activation of Cdc42 by Map links Map and F-actin through a scaffolding complex consisting of Ebp50 and ezrin. In addition and by a Map-dependent positive feedback, Cdc42 polarity and subsequently actin-rich clusters at the bacterial adhesion site are enhanced (Orchard et al. 2012). From early on, Map was also shown to contribute to epithelial barrier dysfunction in tissue culture cells (Dean and Kenny 2004) and more recently to pathogenicity in vivo (Simpson et al. 2006).

EspM effectors (EspM, EspM2, and EspM3) also belong to the WxxxE motif family and are involved in the induction of stress fibers in infected host cells (Arbeloa et al. 2008). Alanine substitution of the invariant residues within the WxxxE motif, as well as of residues within the catalytic serine–serine loop that forms hydrophobic and hydrogen bonds with the WxxxE motif, abolishes stress fiber formation upon infection (Alto et al. 2006). EspM is a bacterial GEF for the host GTPase RhoA. Furthermore, it was shown that EspM2 and EspM3 function to activate ROCK (Rho coiled-coil p160 serine/threonine kinase), a classical downstream effector of RhoA (Arbeloa et al. 2008, 2010). Curiously, EspM effectors, as well as Map, have been shown to counteract or delay pedestal biogenesis underneath attached A/E lesion pathogens, at least to a certain extent. Finally, EspM, through a RhoA-signaling cascade, induces tight junction alterations, resulting in a "bulging out" morphology of the polarized epithelium (Simovitch et al. 2010).

It is interesting to note that only few A/E lesion pathogens are enteroinvasive and it seems that the effector EspT plays a critical role in this outside of the box

lifestyle. EspT induces actin remodeling leading to lamellipodia formation and membrane ruffling, through activation of the GTPases Rac and, to a lesser extent, Cdc42. siRNA studies confirmed that ruffling is dependent on Wave2 (WASP family verprolin-homologous protein) (Bulgin et al. 2009), a downstream effector of Rac that regulates actin dynamics through the Arp2/3 complex (Stradal and Scita 2006). In addition to EspT, Map was also shown to mediate EPEC uptake into non-phagocytic cells (Jepson et al. 2003), aside from promoting filopodia formation.

Curiously, these enteroinvasive Tir-expressing bacteria are then capable of inducing pedestal-like structures from inside the phagosome (Bulgin et al. 2009), which is then somewhat reminiscent of actin tails on endomembrane vesicles leading to the so-called vesicle rocketing (Benesch et al. 2002; Rozelle et al. 2000).

5.1 Pedestal Formation

Pedestal formation essentially depends on the secretion of virulence factors via a T3SS, which in turn causes the so-called A/E lesions in the gut, accompanied by the loss of microvilli (A: attachment; E: effacement). Upon membrane contact and translocation of several virulence factors, bacteria firmly adhere to host cells and induce local assembly of actin filaments directly underneath the attached bacteria. One essential factor for this type of attachment is the translocated intimin receptor Tir (Deng et al. 2003; Donnenberg et al. 1993b), which inserts into the host cell membrane and serves as receptor for the bacterial surface protein intimin (Kenny et al. 1997). In the following 60–180 min, actin accumulations form beneath bacteria and mature into the so-called pedestals. Notably, at least in EPEC, both Tir and intimin are required for disease induction. Mutant strains lacking either protein are avirulent (Deng et al. 2003; Donnenberg et al. 1993b), and intimin is a key inflammatory mediator (Donnenberg et al. 1993a; Higgins et al. 1999). Finally, while the significance of pedestals, i.e., Tir-induced actin assembly, for the infectious process is not fully understood, the specific inability of bacteria to form these structures, e.g., due to carrying point mutations in Tir, is associated with diminished attachment and with reduced translocation of T3 effectors (Battle et al. 2014).

5.2 Phosphorylation of Tir

Within the host cell, bacterial Tir is extensively post-translationally modified and inserted into the plasma membrane: Upon translocation, Tir becomes phosphorylated by host cell kinases on serines (Warawa and Kenny 2001) and—in the case of EPEC—also on tyrosines (Gruenheid et al. 2001).

All tyrosine phosphorylation sites known to date are found in the C-terminal cytoplasmic tail of Tir, namely the region essential for pedestal formation

(Campellone 2010a; Campellone et al. 2004a). Interestingly, tyrosine phosphorylation is critical for EPEC but not EHEC pedestal formation (see below) and the identity of the host tyrosine kinases responsible for TirEPEC phosphorylation was difficult to define. Initially, the Src family kinase c-Fyn was reported to be necessary and sufficient for EPEC pedestal induction (Phillips et al. 2004) in a reconstituted system that made use of intimin-expressing bacteria interacting with Tir ectopically expressed by the host cell. However, when using wild-type strains of EPEC2348/69 with intact T3SS, inhibitors of Src family kinases could not block pedestal formation and phosphorylation was then achieved by Abl family tyrosine kinases, which were sufficient, though not necessary (Swimm et al. 2004b). In the following years, more specific functions for Fyn (Hayward et al. 2009) and for c-Abl (Manthey et al. 2014) in pedestal formation were identified. Hence, some redundancy seems to exist in the repertoire of kinases able to phosphorylate Tir (Schuller et al. 2007). Consequently, less specific small-molecule inhibitors of tyrosine kinases targeting all SH1-type kinase domains (Fes, Tec and Src and Abl families) effectively inhibit pedestals of WT EPEC 2348/69 (Bommarius et al. 2007).

Serine phosphorylation of the Tir molecule was first described in 2001 (Warawa and Kenny 2001) and found important for efficient pedestal elongation. Of note, the sites in Tir that are most likely phosphorylated lie within the same C-terminal region (e.g., in EPEC 2348/69 S434, Y454, S463, Y474). In the following years, Brand and colleagues found that during infection, activated PKA phosphorylates Tir and in addition to the host cell GTPase Rac on S71 resulting in its attenuation (Backert et al. 2010; Brandt et al. 2009).

Recently, association of the kinase STK16 with the C-termini of TirEPEC and TirEHEC was described (Blasche et al. 2014), but it is still unknown whether this adds to the redundancy concerning phosphorylation of residues S434 and S463, or alternatively, whether yet additional residues in Tir can be targeted by host kinases, for hitherto unknown reasons. Interestingly, STK16 is membrane-bound and involved in secretory vesicle trafficking (Berson et al. 1999; In et al. 2014). Therefore, it is of great interest to learn more about the underlying molecular mechanisms and their impact on disease.

5.3 Recruiting the Actin Machinery

Subsequent to insertion, the extracellular domain of Tir binds to the bacterial surface protein intimin leading to firm attachment (Liu et al. 1999b). C- and N-terminal parts of the Tir protein reach into the host cell cytoplasm and recruit protein complexes leading to local actin assembly. EPEC- and EHEC-induced actin polymerization largely depends on N-WASP and Arp2/3 complex (Lommel et al. 2001, 2004). Small C-terminal peptides of Tir, when fused to transmembrane receptors, are sufficient to induce N-WASP recruitment and formation of pedestal-like structures upon triggering of receptor clustering (Campellone et al. 2004a, 2006). Moreover, deletion of these sequences from Tir abolishes pedestals.

Therefore, it can be assumed that formation of canonical pedestals depends on N-WASP-Arp2/3-mediated actin assembly. It should be noted that in one cell line, Tir-mediated bacterial attachment was reported to induce actin accumulations despite the absence of N-WASP (Vingadassalom et al. 2010). However, a stimulus of the adhering bacterium elicited by other interactors of Tir (e.g., cortactin, see below) or even other T3-secreted virulence factors (Berger et al. 2009; Simpson et al. 2006) might explain the described changes in the host's cortical actin in the absence of N-WASP.

5.4 Deciphering the Different Pathways Leading to Pedestal Formation

5.4.1 The Tyrosine—474 Pathway in EPEC

EPEC-Tir is phosphorylated by host cell kinases on four tyrosines, switching on several cellular signaling cascades. Of these, four residues, Y454, Y474, Y483, and Y511, only the first two, Y454 and Y474, are essential for pedestal formation as demonstrated by point mutating the individual tyrosines to phenyl alanines (Y → F) (Campellone and Leong 2005; Gruenheid et al. 2001). Y483 and Y511 modulate pedestal formation by recruiting SHP2, a host inositol phosphatase that generates a PI(3,4)P2-enriched lipid platform for the recruitment of further cytoskeletal regulators (Smith et al. 2010).

The first signal for actin polymerization in pedestals is, however, the phosphorylation of EPEC-Tir tyrosine 474 (Gruenheid et al. 2001). Upon phosphorylation, pY474 specifically recruits the cellular SH2–SH3 adaptor Nck via the SH2 domain of Nck. Two Nck paralogs exist in the mammalian genome, Nck1 and Nck2. Characterization of the binding specificities of both SH2 domains was shown to be essentially indistinguishable, and crystal structures of both domains in complex with EPEC-Tir-derived phosphopeptides superimpose in the specific recognition and high-affinity binding sites of the respective phosphopeptide (Frese et al. 2006). In turn, the three SH3 domains of Nck recruit and activate the protein N-WASP in a concerted manner, which is released from its autoinhibitory bond (Rohatgi et al. 2001) and thus free to activate the F-actin-branching Arp2/3 complex (Fig. 1).

5.4.2 The Tyrosine—454 Pathway in EPEC

The above pathway, functioning via phosphorylation of Y474, is not the only one in EPEC-Tir, since mutation of tyrosine 474 to phenylalanine (Y474F) still leads to some pedestal formation albeit strongly reduced. The double mutant EPEC-Tir (Y474F/Y454F) can no longer induce pedestals (Campellone and Leong 2005; Campellone et al. 2004a). Therefore, it was suggested that pY454 recruits other

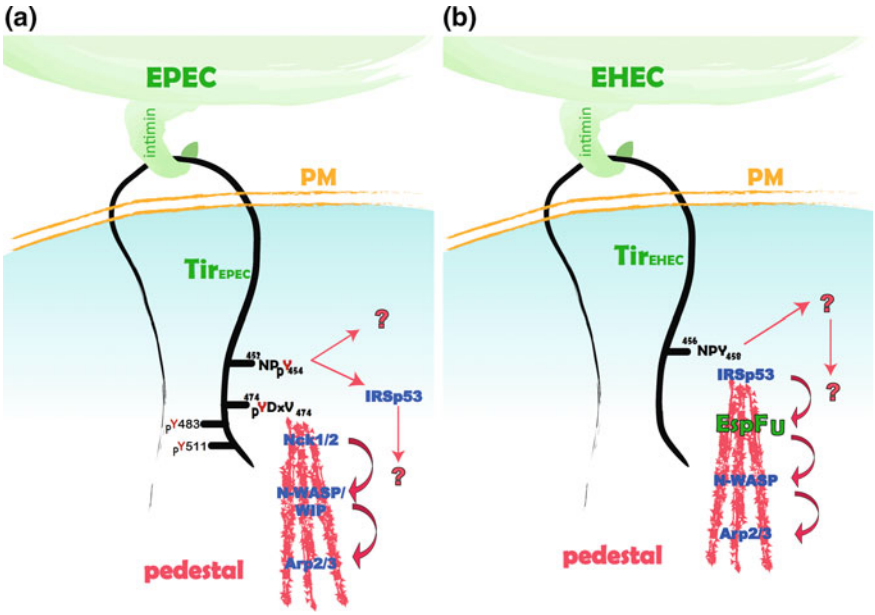

Fig. 1 Pathways to pedestal formation in EPEC and EHEC. The translocated intimin receptor Tir of either EPEC or EHEC is inserted into the host cell membrane and binds to the bacterial surface protein intimin. **a** Tir_{EPEC} is phosphorylated on four C-terminal tyrosine residues. Two (Y454, Y474) are required for pedestal formation. The pathway from pY474 to actin assembly is predominant and involves the host adaptor Nck, the nucleation-promoting factor N-WASP, and Arp2/3 complex as essential components. Molecular details of the second pathway from phosphotyrosine pY454 are ill-defined. The motif around Y454 recruits the host adaptor IRSp53, but it remained elusive whether this contributes to pY474-independent pedestal formation. **b** Tir_{EHEC} is not phosphorylated. The conserved NPY motif around Y458, which corresponds to pY454 in Tir_{EPEC}, recruits the host adaptor IRSp53 or its paralog IRTKS. This in turn binds to the second essential EHEC factor, $EspF_U$, which constitutes a strong recruiter and activator of N-WASP culminating in Arp2/3 complex activation and actin assembly. The same NPY motif—like NPY454 in TirEPEC—supports only inefficient actin accumulation, although the molecular details remain unclear. Bacterial factors are in black, host factors are in blue, and F-actin is red

proteins with SH2 domains, leading to N-WASP/Arp2/3 activation. However, such a connection has not been identified so far. Rather, pY454 recruits the regulatory subunit of PI3 (phosphoinositide-3) kinase (Sason et al. 2009), which by itself cannot induce N-WASP-mediated actin polymerization. Thus, there must be an alternative pathway in EPEC that accounts for the low-efficacy pedestal formation and that is somehow disturbed by the Y454F mutation (Allen-Vercoe et al. 2006b; Brady et al. 2007; Campellone and Leong 2005). The amino acid sequence of the region around Y454 is conserved between EPEC and EHEC (there Y458) and is essential for EHEC pedestal formation (Allen-Vercoe et al. 2006b). However, elucidation of the pathway leading to pedestal formation in EHEC (see below) cannot explain for the alternative low-efficacy pathway in EPEC, because it requires an

additional T3 effector, namely EspFu (Campellone et al. 2004b; Garmendia et al. 2004), which is absent from EPEC. The assumption that these regions are conserved between EPEC and EHEC-Tir is corroborated by the finding that expression of EspFu, the EHEC T3 effector missing in EPEC, greatly enhanced Nck-independent pedestal formation in EPEC via Y454 (Brady et al. 2007). Further research is required to uncover all aspects of actin assembly in EPEC pedestal formation.

5.4.3 The tyrosine—458 pathway in EHEC

Tir proteins are highly conserved between EHEC and EPEC, except for a short stretch around EPEC tyrosine Y474, which is lacking in EHEC. Therefore, it was inherently clear that the pY474/NCK signaling pathway cannot account for EHEC pedestal formation. Consequently, in Nck1/2 double-knockout cells, which do not support EPEC pedestal formation, the frequency of EHEC pedestal formation was unchanged (Gruenheid et al. 2001). Subsequent studies confirmed that a mutation of all candidate tyrosine residues in question to phenylalanines does not negatively affect pedestal formation in EHEC. Therefore, EHEC-Tir—unlike EPEC-Tir and despite harboring a conserved sequence around Y458—does not depend on phosphorylation by host kinases. Finally, in 2004, another EHEC-specific virulence factor was discovered (Campellone et al. 2004b; Garmendia et al. 2004), which is indispensable for effective pedestal formation, namely TCPP (Tir cytoskeleton coupling protein)/$EspF_U$ (EspF homolog from genomic prophage U). Remarkably, $EspF_U$ binds to and activates N-WASP (Campellone et al. 2008).

$EspF_U$ (TCCP) is, in fact, an EHEC-specific paralog of the *E. coli* virulence protein EspF and, like EspF, harbors multiple tandem repeats of 47 (or 38) amino acids, respectively (Alto et al. 2007). $EspF_U$ and EspF both harbor a conserved hydrophobic N-WASP-binding helix, followed in $EspF_U$ by a proline-rich region. $EspF_U$ directly binds to the autoinhibitory domain of N-WASP and thus is a strong activator of N-WASP- and Arp2/3-complex-mediated actin assembly. Nonetheless, $EspF_U$ alone could not explain for pedestal formation in EHEC, since it cannot directly bind to Tir and thus is unable to connect Tir to the actin polymerization machinery. Hence, a bridging factor is required. This missing link was finally identified in 2009, meeting all criteria: It must bind to the tripeptide NPY (amino acid residues 456–458) in Tir (Brady et al. 2007; Campellone et al. 2006) and has to bind to either $EspF_U$ or N-WASP. Two groups simultaneously identified this missing factor that strikingly turned out to be a host protein, bridging two bacterial virulence factors (Vingadassalom et al. 2009; Weiss et al. 2009). IRSp53 (insulin receptor substrate p53; BAIAP2) and its paralog IRTKS (insulin receptor tyrosine kinase substrates; BAIAP2L1) are adapter proteins of the BAIAP2 family (brain-associated angiogenesis inhibitor-associated protein 2) and are characterized by the presence of an I-BAR and a SH3 domain interacting with Tir and $EspF_U$, respectively (Fig. 2).

Thus, IRSp53/IRTKS with the N-terminal IMD (I-BAR domain) binds to the NPY motif in Tir and to the proline-rich sequences in TCPP/$EspF_U$ with its

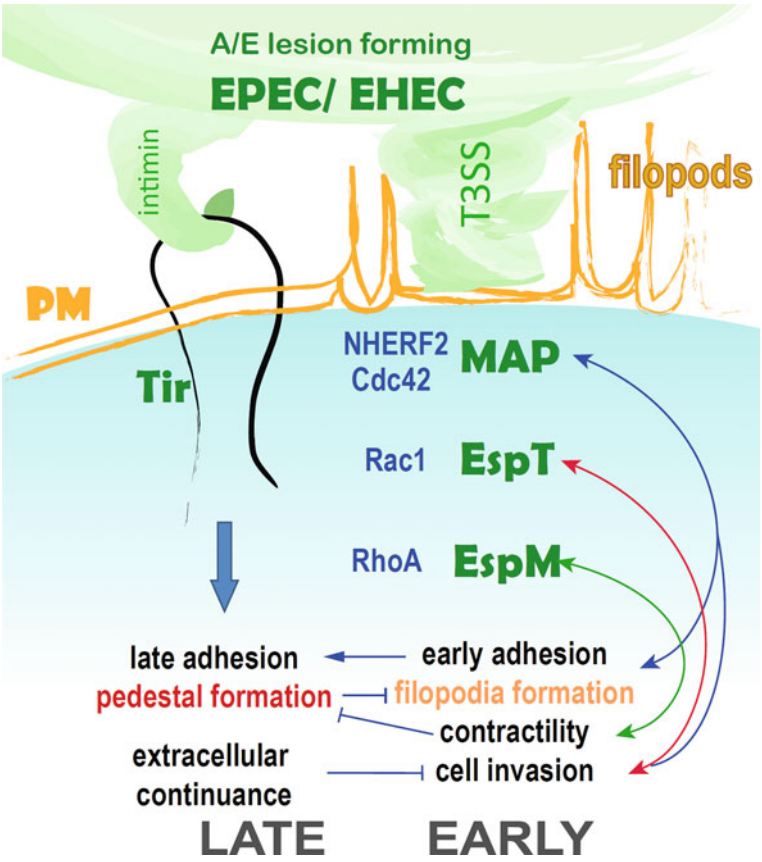

Fig. 2 Interplay of Rho GTPase regulation and pedestal formation during EPEC/EHEC infection. Upon initial contact, A/E lesion-forming *E. coli* secrete a cocktail of virulence factors into the host cell cytoplasm. Early effects of this first contact are, e.g., filopodia formation at the contact site in response to activation of the host GTPase Cdc42 through the bacterial GEF Map. The bacterial GEF EspM activates RhoA and leads to stress fiber formation in infected cells and damages the architecture of the epithelium. These effects occur within minutes upon bacterial contact. The bacterial GEF EspT acting on Rac1 seems to promote bacterial internalization, an unusual behavior of these bacteria. Map and EspM activities, unlike EspT, suppress pedestal formation at least in early stages of infection. Pedestal formation is a slow process and is observed not earlier than 1 h after the onset of infection and reaches its maximum several hours thereafter in tissue culture experiments. Note that the interplay between the different virulence factors and host responses exemplified here, which focuses on 4 factors and a selection of processes, is more complicated in real life. It is clear that these bacterial effectors drive in a concerted fashion intricate events leading, for instance, to evasion from autophagy or to feedback to type III secretion efficiency, but clarification of their exact individual contributions requires additional investigations

C-terminal SH3 domain. IRSp53/IRTKS seems the ideal choice, as its cellular role lies in promoting actin reorganization at the plasma membrane (Scita et al. 2008; Suetsugu et al. 2010). However, its SH3 domain is not an activator of N-WASP (as

opposed to that of Nck proteins), which is where the T3 effector $EspF_U$ comes into play. Importantly, the genetic ancestor of $EspF_U$, EspF, is already capable of N-WASP activation, albeit in a different context (Alto et al. 2007; Cheng et al. 2008).

Finally, it can be assumed that IRSp53, which binds to EPEC-, EHEC-, and C. rodentium Tir might also be involved in the EPEC Y454 pathway (Crepin et al. 2010) or in N-WASP-independent actin accumulations beneath EHEC (Vingadassalom et al. 2010) since it might be able to recruit other actin regulatory factors such as the WAVE complex (Miki et al. 2000; Suetsugu et al. 2006) or the Ena/VASP family member Mena (mammalian Ena) (Krugmann et al. 2001). However, the molecular details and precise consequences of these interactions still remain ill-defined.

5.5 *Other Factors Influencing Actin Pedestals*

In addition to the essential machineries discussed so far, numerous proteins binding actin and/or regulating endocytosis and other regulatory agents have been found in the context of pedestal formation. In the following section, we will briefly summarize these other factors, although this list is not at all exhaustive (Table 1).

Table 1 Host components that bind to Tir and/or contribute to pedestal formation

Tir-binding host components	Reference
Endocytic machinery	
Eps15, Epsin	Lin et al. (2011)
CD2AP	Guttman et al. (2010)
Clathrin	Bonazzi et al. (2011), Veiga et al. (2007).
Calcium-binding proteins	
Calmodulin, IQGAp, calcium	Brown et al. (2008)
HPCAL1, HPCAL4, NCALD	Blasche et al. (2014)
Calpactin	Goosney et al. (2001)
Membrane and lipid raft components	
SHP1	Yan et al. (2012)
SHIP2	Smith et al. (2010)
Annexin A2	Rescher et al. (2004), Zobiack et al. (2002)
Cholesterole	Allen-Vercoe et al. (2006a)
c-Fyn	Hayward et al. (2009)
PI3-kinase, PtdIns-4,5-bisphosphate	Sason et al. (2009)

(continued)

Table 1 (continued)

Tir-binding host components	Reference
Actin binding proteins	
Talin	Cantarelli et al. (2001), Goosney et al. (2001), Huang et al. (2002)
Cofilin, gelsolin, CD44, zyxin, vinculin	Goosney et al. (2001)
Cortactin	Cantarelli et al. (2000), Guttman et al. (2010)
Shank3	Huett et al. (2009)
Kinases	
c-Fyn	Hayward et al. (2009), Phillips et al. (2004)
c-Abl	Swimm et al. (2004a)
Serine threonine kinases	Warawa and Kenny (2001)
Stk16	Blasche et al. (2014)
Other	
14-3-3tau	Patel et al. (2006)
CrkII Grb2 LPP p130cas Shc CD44	Goosney et al. (2001)
Nck	Gruenheid et al. (2001)
IRSp53, IRTKS	Vingadassalom et al. (2009), Weiss et al. (2009)
N-WASP and Arp2/3	Lommel et al. (2001, 2004), Snapper et al. (2001)

Note that this table is not exhaustive. Moreover, not all of these components were shown to bind directly to Tir and may be co-recruited to the bacterial attachment site by other means

Host **endocytic proteins** that have been identified to be targeted by EPEC include clathrin, adaptor protein-2 (AP-2), dynamin-2, Eps15, and a family of epsin proteins (Veiga et al. 2007). The mechanism of EPEC-mediated usurpation of the host endocytosis-associated proteins and the role these proteins play during pedestal formation are poorly understood. Lin and colleagues (Lin et al. 2011) reported that in addition to clathrin, pedestal formation also involves Eps15 and epsin1 but not AP-2. It is possible that recruitment of the clathrin machinery is important for full activation of N-WASP-mediated actin assembly at the plasma membrane, since N-WASP is also at play at clathrin-coated pits prior to internalization (Benesch et al. 2005; Merrifield et al. 2002). It remains a fascinating question how A/E lesion-forming bacteria omit internalization, yet recruit the same machinery utilized for invasion by other pathogens (Veiga et al. 2007).

Calcium has been implicated in microvilli effacement and disruption (Ferrary et al. 1999; Potter et al. 2003). In addition, Ide and colleagues found that Ca(2+) is also involved in the regulation of type III secretion: While secretion of functional Tir was enhanced under calcium chelation conditions, Esp secretion was strongly reduced (Deng et al. 2005; Ide et al. 2003).

Recently, another group of Tir-EHEC interactors was discovered, including three calcium-binding proteins of the VILIP family, namely HPCAL1, HPCAL4, and NCALD (Blasche et al. 2014), with highly similar amino acid sequences

(62 % identity). Although mainly known as proteins involved in neuronal signaling (Burgoyne 2007), they also appear to be expressed in tissues of the digestive tract (Kapushesky et al. 2012). The interactions of TirEHEC with these three calcium-binding proteins should be kept in mind, since the mechanisms involved and the contribution of Ca^{2+} signaling to EPEC/EHEC infection are still mostly unclear (Goosney et al. 2000).

Some studies found evidence supporting a role for Ca(2+) directly in pedestal formation (Baldwin et al. 1991; Brown et al. 2008; Ide et al. 2003), while others did not (Bain et al. 1998).

Moreover, annexin-A2, a membrane- and calcium-binding protein, is recruited to the bacterial attachment sites upon infection. Murena and colleagues reported that annexin-A2 binds to both cytoplasmic tails of Tir through its C-terminal domain, which was independent of Tir-induced actin polymerization (Munera et al. 2012). Finally, recruitment of annexin-A2 was concomitant with the clustering of raft components at the bacterial attachment site (Zobiack et al. 2002). Together, Ca ions are involved in pedestal formation either directly or indirectly, perhaps on the level of type 3 secretion.

Moreover, several observations were made that support the idea of actin pedestals being **lipid raft**-enriched membrane domains, a notion that is supported by similar mechanisms utilized by other pathogens (Zaas et al. 2005). For instance, Tir translocation and clustering induce a concentration of cholesterol at the bacterial attachment site (Allen-Vercoe et al. 2006a; Zobiack et al. 2002), which may support recruitment and activation of c-Fyn for Tir phosphorylation (Hayward et al. 2006, 2009). In addition to raft components, other lipid-modifying enzymes were also described to be recruited to pedestals such as the cellular inositol phosphatase SHIP2 (Campellone 2010b; Sason et al. 2009; Smith et al. 2010).

Finally and not surprisingly, numerous **F-actin-binding proteins** such as cortactin (Cantarelli et al. 2000, 2007; Nieto-Pelegrin and Martinez-Quiles 2009; Selbach and Backert 2005), alpha-actinin (Allen-Vercoe et al. 2006b; Freeman et al. 2000; Huang et al. 2002; Shaner et al. 2005), VASP (Weiss et al. 2009), and several focal adhesion components (Cantarelli et al. 2001; Goosney et al. 2001) were identified in pedestals. Noteworthy, integrin is not participating in pedestal formation (Liu et al. 1999a), in spite of the well-established, major importance for attachment of the distantly related enteropathogen *Yersinia* (Isberg and Leong 1990). However, despite some controversial discussions, all aforementioned pedestal-associated proteins appear not to be essential for pedestal formation, but instead either modulate them or become passively co-recruited.

6 Concluding Remarks

Interactions of pathogens with their hosts confront the complexity of two worlds. At their interface, a large number of interactions have developed, which depend on intricate adaptations at the molecular level reflecting an impressive co-evolution.

First, establishment of a colonization niche is paramount to the survival of the pathogen. Additionally, escape from the equally evolved host–defense mechanisms is substantial. Development of the bacterial T3SS secretory complex and its effectors, exclusively dedicated to host interactions, highlights the imperative necessity to communicate with certain host mechanisms, namely Rho GTPase signaling with all its consequences including actin dynamics. Manipulation of these signaling pathways and that of other regulatory processes such as ubiquitination events (not concerned here) are crucial for the pathogen's fate. Understanding how different pathogens manipulate host signaling pathways and consequently defense mechanisms will pave the way for developing new therapeutic approaches that act by blocking pathogenicity mechanisms rather than just killing microbes.

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Acting on Actin: Rac and Rho Played by *Yersinia*

Martin Aepfelbacher and Manuel Wolters

Abstract Pathogenic bacteria of the genus *Yersinia* include *Y. pestis*—the agent of plaque—and two enteropathogens, *Y. enterocolitica,* and *Y. pseudotuberculosis*. These pathogens have developed an array of virulence factors aimed at manipulating Rho GTP-binding proteins and the actin cytoskeleton in host cells to cross the intestinal barrier and suppress the immune system. *Yersinia* virulence factors include outer membrane proteins triggering cell invasion by binding to integrins, effector proteins injected into host cells to manipulate Rho protein functions and a Rho protein-activating exotoxin. Here, we present an overview of how *Yersinia* and host factors are integrated in a regulatory network that orchestrates the subversion of host defense.

Contents

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1 Introduction

Yersinia pestis can cross the skin- or lung barrier of the human body in an instant through a flea bite or inhalation of infectious droplets, respectively. Rapidly thereafter, the bacteria are attacked by cells of the immune system. In comparison, the enteropathogenic *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are ingested with contaminated food and first move to the distal part of the small intestine without much known contact to the immune system (Putzker et al. 2001). Only upon arrival in the ileum the yersiniae invade and cross the intestinal epithelium through microfold (M)-cells and then come in contact with subepithelial phagocytes that reside in a basal pocket of the M-cells or in Peyer's patches (Jepson and Clark 1998). After breaching the epithelia of skin, lung, or intestine all pathogenic *Yersinia* spp. are supposed to display a mainly extracellular lifestyle which depends on the expression of effective antiphagocytic mechanisms (Chung and Bliska 2016). The pathogenic yersiniae proliferate in lymphatic tissues, i.e., in local lymph nodes or Peyer's patches, and likely from this location can further disseminate into liver or spleen of infected animals (Pepe and Miller 1993; Pepe et al. 1995). It is evident that the rapid succession of the invasive and antiphagocytic activities of *Yersinia* requires a well-orchestrated expression of virulence factors. Invasion and crossing of the intestinal epithelium is mainly executed by invasin, a protein expressed in the outer membrane of the bacteria at 27 °C. Invasin clusters and activates β1-integrins on the apical surface of M-cells and the ensuing intracellular signals lead to actin cytoskeleton rearrangements that drive bacterial internalization (Wong and Isberg 2005a). In comparison, the antiphagocytic part of the *Yersinia* infection cycle is mostly brought about by inhibition of actin regulators in professional phagocytes such as neutrophils or macrophages (Cornelis et al. 1998; Viboud and Bliska 2005; Aepfelbacher et al. 2007). It is well accepted that finely tuned actin rearrangements are essential for phagocytosis (Freeman and Grinstein 2014). Noteworthy, the same actin-regulatory proteins that are activated during *Yersinia* invasion are later inactivated by the bacteria for inhibiting phagocytosis. The strong antiphagocytic activities of pathogenic *Yersinia* spp. are mediated by toxins/effector proteins (termed Yops for *Yersinia* outer proteins), which become translocated into phagocytes via a bacterial injection machine/injectisome (termed type III secretion system; T3SS). Interestingly, effector translocation itself is governed by not well-defined rearrangements of the actin cytoskeleton in host cells (Viboud and Bliska 2001). This feature of the T3SS supports a negative feedback loop whereby the actin-disrupting effectors already present in the host cell dose-dependently diminish further effector translocation by inhibiting the activity of the T3SS (Aepfelbacher et al. 2011). Thus, both invasive and antiphagocytic virulence features of enteropathogenic *Yersinia* spp. as well as the control of effector translocation are associated with rearrangements of the host cell actin cytoskeleton. We provide below an overview of the intimate links between the cellular microbiology of *Yersinia*, the actin cytoskeleton and Rho GTP-binding proteins.

2 Invasion of Cells by Enteropathogenic *Yersinia* Spp

Intestinal cell invasion by enteropathogenic Yersiniae is thought to be mainly accomplished by the outer membrane protein invasin that binds to the β1-chain of integrins, including α3β1, α4β1, α5β1, α6β1, and αvβ1 (Isberg and Leong 1990). The structural biology of invasin and the cellular signals induced by invasin have been reviewed elsewhere (Dersch 2003; Wong and Isberg 2005a). Invasin is a strong activator of β1-chains of integrins due to its capability to induce integrin clustering and its high binding affinity that by far surpasses that of natural β1-integrin ligands like fibronectin (Tran Van Nhieu and Isberg 1993). When a carboxy-terminal construct of invasin is attached to latex beads or when invasin is heterologously expressed in *E. coli* laboratory strains, it provides these particles with strong cell invasion potential (Isberg and Leong 1990). The most important physiological role of β1-integrins is to mediate cell-extracellular matrix and cell–cell adhesion both under stationary and dynamic/migratory conditions. Upon binding to their ligands β1-integrins transmit signals to the cytoskeleton with multiple outcomes for cell behavior (Hynes 2002). Such signals can under not well-understood conditions result in uptake of the integrin-bound particulate ligands such as cell associated fibronectin fibrils (Sottile and Hocking 2002). It may be inferred that the strategy of invasin-expressing bacteria is to imitate extracellular matrix coated particles destined for uptake whereby invasin can outcompete the physiological integrin ligands.

The invasin/β1-integrin triggered signaling pathways to the actin cytoskeleton have been thoroughly investigated. RhoA, RhoG, Rac1, and Cdc42 belonging to the Rho-family of Ras-like GTP-binding proteins as well as Arf6 belonging to the Arf-family were shown to play central roles in invasin-stimulated actin rearrangements triggering cell invasion (Alrutz et al. 2001; Roppenser et al. 2009; Wiedemann et al. 2001; Wong and Isberg 2005b). The 15 members of the Rho-family are best known for regulating the actin cytoskeleton, but are also involved in inflammatory mediator production, vesicle transport, gene transcription, and cell cycle control (Jaffe and Hall 2005; Van Aelst and D'Souza-Schorey 1997). Most Rho proteins are molecular switches that interact with their downstream effectors in the GTP-bound, active but not in the GDP-bound, inactive state. Often the interaction of Rho proteins with their effectors takes place at cellular membranes. Conversion of the GTP- to the GDP-bound state and vice versa is orchestrated by distinct regulatory proteins. GTPase activating proteins (GAPs) accelerate the intrinsic GTPase activity of Rho proteins, which causes rapid hydrolysis of the bound GTP to GDP. Conversely, guanine nucleotide exchange factors (GEFs) release bound GDP and promote GTP binding. Other players in this orchestra are guanine nucleotide dissociation inhibitors (GDIs), which are capable of extracting Rho proteins from membranes by binding and neutralizing their C-terminal isoprenoid tail. In the inactive state Rho, Rac, and Cdc42 but not the other Rho-family proteins are for the most part associated with GDI in the cytosol of cells (Fig. 1).

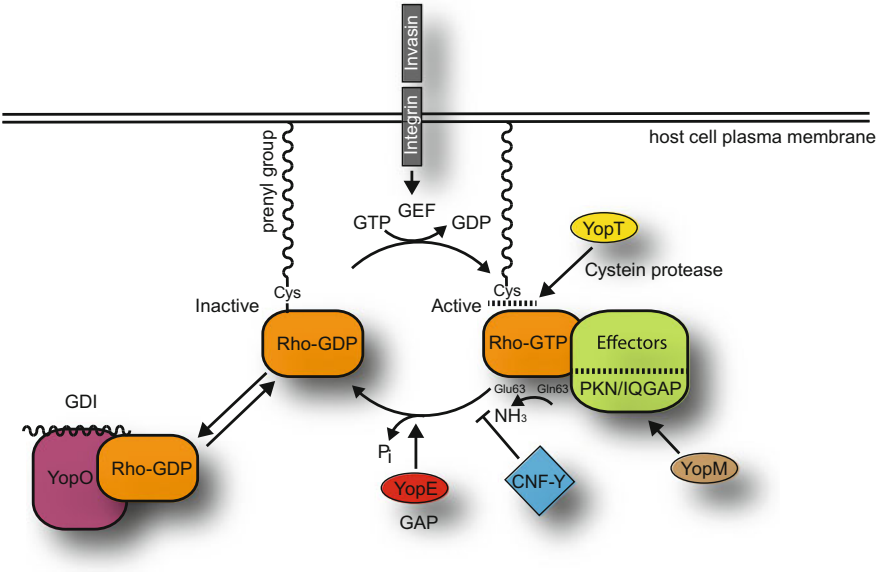

Fig. 1 Multifaceted manipulation of Rho GTP-binding proteins by *Yersinia* virulence factors. High-affinity β1-integrin binding by *Yersinia* outer membrane protein invasin stimulates signaling (i.e., activation of Rho GEFs) leading to GTP-binding and activation of Rho proteins. Rho proteins are inactivated by the GAP and GDI functions of YopE and YopO, respectively. The cysteine protease YopT extracts Rho from the plasma membrane by removing its prenyl group. Deamidation of Rho at glutamine-63 by CNF-Y abrogates the GTPase activity of Rho rendering it permanently GTP-bound. The functional consequences of YopM binding to the Rho effector proteins PKN and IQGAP are widely unknown

It became clear already in 2001 that Rac1 plays a central role in invasin-triggered invasion of epithelial cells (Alrutz et al. 2001; McGee et al. 2001; Wong and Isberg 2005a). Yet, in human macrophages, which employ different and more diverse mechanisms of actin regulation than epithelial cells and somewhat resemble M-cells, Rho and Cdc42 also are involved (Wiedemann et al. 2001). For stimulating actin polymerization in lamellipodial cell extensions and phagocytic cups Rac primarily signals through its downstream effector the actin nucleation-promoting WAVE regulatory complex (WRC), which in turn activates the seven-subunit Arp2/3 complex (Steffen et al. 2004). Yet, other downstream effectors of Rac are also known to contribute to actin organization (Campellone and Welch 2010). Whereas Arp2/3 complex was in fact implicated in invasin-induced uptake, a function for WAVE complex was not formally demonstrated and the contribution of the Cdc42 target N-WASP remained unclear (Alrutz et al. 2001; McGee et al. 2001; Wiedemann et al. 2001). In macrophages the WAVE- and N-WASP homolog WASP was shown to mediate uptake of invasin-coated beads, most likely through controlling Cdc42-dependent actin polymerization in phagocytic cups (Wiedemann et al. 2001). Notably, the more recently

described functions of ARF6 and RhoG in invasin-mediated cell invasion have also been linked to Rac1 (Mohammadi and Isberg 2009; Roppenser et al. 2009; Wong and Isberg 2003). Through activation of phosphoinositol-4-phosphate-5-kinase Arf6 stimulates a pathway that mediates invasin-induced uptake. Overexpression of phosphoinositol-4-phosphate-5-kinase even rescued uptake in cells in which Rac1 was inhibited (Wong and Isberg 2003). Thus, Rac1 and Arf6 appear to synergize in an aspect of integrin signaling that involves the synthesis and function of phosphatidylinositol-4,5-bisphosphate. Arf6 and Rac have been reported before to cooperate in actin organization, phosphatidylinositol-4,5-bisphosphate synthesis and phagocytosis (D'Souza-Schorey et al. 1997; Tolias et al. 2000; Zhang et al. 1998, 1999). It was also shown that RhoG becomes activated and is crucially involved in invasin-mediated cell invasion (Mohammadi and Isberg 2009; Roppenser et al. 2009). RhoG binds to a complex of the proteins Elmo and Dock180, which stimulates the GEF activity of Dock180 towards Rac1 thereby promoting GTP-loading and activation of Rac1 (deBakker et al. 2004; Katoh and Negishi 2003; Lu and Ravichandran 2006). Thus, RhoG acts as a major upstream regulator of Rac1 during invasin-stimulated cell invasion. A closer look on the specific roles of RhoG and Rac during cell invasion further indicated that RhoG functions through both, upstream control on Rac1 activity and individual Rac-independent functions (Fig. 2) (Mohammadi and Isberg 2009; Roppenser et al. 2009).

Although the outer membrane protein invasin is considered the decisive factor triggering cellular uptake and intestinal transcytosis of *Yersinia* in vitro and in vivo,

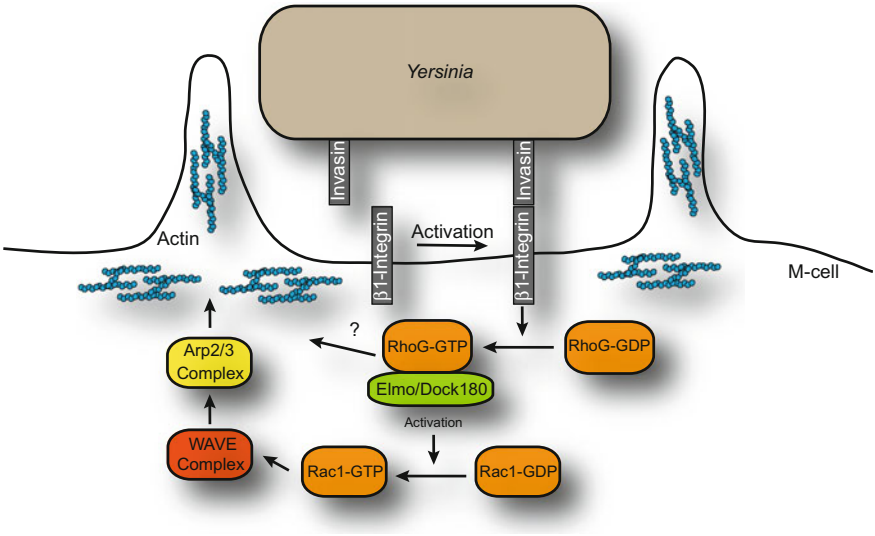

Fig. 2 Invasin/β1-integrin triggered cell invasion of *Yersinia*. During the invasive phase of infection, *Yersinia* binds to integrins via invasin, which triggers activation of RhoG and Rac1. RhoG can stimulate Rac1 through the Elmo/Dock180 GEF module. Signaling of RhoG and Rac1 to actin reorganization, i.e., via WRC and Arp2/3 complex, drives cell invasion

Yersinia spp. express numerous additional adhesins including Ail, YadA, -B and -C, Pla and pH 6 antigen, which have been reported to support cell invasion (Leo and Skurnik 2011; Marra and Isberg 1997; Miller and Falkow 1988). These adhesins bind to mucus, extracellular matrix proteins like collagen, laminin and fibronectin, complement factors or plasminogen, suggesting that they play a role in adherence to tissues and that they also may usurp integrin signaling for cell invasion (Bliska et al. 1993; Leo and Skurnik 2011; Mikula et al. 2012; Uliczka et al. 2009). However, the rather modest invasion potential of the non-invasin *Yersinia* adhesins in vitro and their not well-defined invasive activities in vivo have hitherto provoked little interest in elucidating their molecular mode of action. For detailed information about the biology of *Yersinia* adhesins we refer to excellent reviews published elsewhere (Leo and Skurnik 2011; Mikula et al. 2012).

In summary, the currently available data indicate that for crossing the intestinal barrier invasin of enteropathogenic *Yersinia* spp. stimulates integrin signaling to activate Rac1 in M-cells. Rac1 drives *Yersinia* internalization foremost through WAVE- and Arp2/3 complex mediated actin remodeling. RhoG and Arf6/phosphoinositol-4-phosphate-5-kinase synergize with Rac1 by directly regulating its activity or stimulating parallel signal pathways contributing to actin remodeling (Fig. 2). Although the mechanisms governing *Yersinia* cell invasion have been well defined, bacterial cell invasion notwithstanding is a highly complex event that is not only driven by actin rearrangements but also by vesicle trafficking and biomechanical processes. For instance, GAPs for Rho GTP-binding proteins have recently been shown to play a major role in integrin-mediated phagocytosis/cell invasion by switching off actin polymerization in phagocytic cups (Rauch et al. 2016; Schlam et al. 2015). Cdc42GAP thereby is transported towards the invading bacteria on recycling endocytic vesicles with the help of the exocyst complex (Rauch et al. 2016).

3 *Yersinia* Effector Proteins and an Exotoxin Manipulating Rho Proteins and the Actin Cytoskeleton

3.1 YopE

YopE is considered one of the most active and versatile T3SS effectors in pathogenic *Yersinia* species (Cornelis et al. 1998). Long before YopE was shown to be a GAP for Rho-family proteins (Black and Bliska 2000; Von Pawel-Rammingen et al. 2000) it was reported to disrupt actin filaments in HeLa cells and to strongly impede *Yersinia* phagocytosis by mouse macrophages (Rosqvist et al. 1990, 1991). In the test tube it displays GAP activity towards the Rho-family proteins Rho, Rac, Cdc42, and RhoG (Black and Bliska 2000; Roppenser et al. 2009; Von Pawel-Rammingen et al. 2000). When translocated into cells by the T3SS YopE induces a robust inhibitory activity towards Rac1 and RhoG (Fig. 1) (Aili et al.

2006; Andor et al. 2001; Mohammadi and Isberg 2009; Roppenser et al. 2009; Ruckdeschel et al. 2006). Interestingly, YopE can either directly bind and down-regulate Rac1 or inactivate Rac's upstream stimulator RhoG (Mohammadi and Isberg 2009; Roppenser et al. 2009). This underlines that the effect of YopE on Rac also depends on signal pathways through which Rac is activated, an idea already mentioned in a previous study (Andor et al. 2001). In cellular infection models, employing different cell types, *Yersinia* strains and infection procedures, RhoA, CDC42, and TC10 were found to be inactivated by YopE, yet with less efficiency and sometimes after a lag phase when compared to Rac1 (Aili et al. 2006; Roppenser et al. 2009). Because Rho proteins commonly activate or inactivate other Rho proteins as part of signaling cascades or feedback loops (Jaffe and Hall 2005; Van Aelst and D'Souza-Schorey 1997), it is generally difficult to determine whether inhibition of a given Rho protein in cells is due to a direct or indirect effect of the respective Rho protein inhibitor.

Altogether, the available data indicate that during ongoing *Yersinia* infection the RhoG-Rac1 signaling axis is efficiently down-regulated by YopE, which causes inhibition of actin-dependent immune cell functions such as phagocytosis or transendothelial migration (Nakaya et al. 2006; van Buul et al. 2007). It is notable that the function of YopE can be modified by a number of host and *Yersinia* factors. Some YopE isoforms get degraded by the host cell proteasome, which causes reactivation of Rac1 (Hentschke et al. 2007; Ruckdeschel et al. 2006). Further, a concerted action of overexpressed YopT and YopE was reported to produce an active nuclear pool and an inactive cytosolic pool of Rac1 in cells, with hitherto unknown consequences for cell behavior (Wong and Isberg 2005b). Finally, recent work showed that cytotoxic necrotizing factor-Y, an exotoxin produced by *Yersinia*, activates Rac1 in a way that it cannot be down-regulated by YopE anymore (Wolters et al. 2013). It has become evident that *Yersinia* YopE and its target Rac1 are subject of a sophisticated regulatory network involving numerous bacterial and host cell factors. Clearly, the role of this network in *Yersinia* pathogenesis is not entirely understood at present.

3.2 YopT

YopT belongs to the CA clan of cysteine proteases with invariant C/H/D amino acid residues and numerous homologs from diverse pathogens found in the databases (Aepfelbacher et al. 2005; Shao et al. 2002). Like in the case of YopE, the first cellular effect assigned to YopT was disruption the actin cytoskeleton (Iriarte and Cornelis 1998) and later the molecular basis for this YopT function was found to be inhibition of Rho proteins (Shao et al. 2002, 2003; Zumbihl et al. 1999). YopT was shown to inhibit phagocytosis of opsonized and non-opsonized yersiniae by neutrophils and macrophages (Grosdent et al. 2002). In human macrophages, YopT disrupted actin reorganization in phagocytic cups and in actin-rich adhesion structures required for chemotaxis (Aepfelbacher et al. 2003). YopT proteolytically

removes the lipid-modified C-terminal cysteine from Rho proteins (Shao et al. 2002; Aepfelbacher et al. 2003). In most Ras-like GTP-binding proteins, the C-terminal cysteine residue is linked to an isoprenoid moiety (in Rho proteins mostly a geranylgeranyl group) that mediates membrane– and protein–protein interactions, i.e., the interaction with GDIs (Fig. 1) (Glomset et al. 1992). Recombinant YopT in vitro or YopT overexpressed in cells most efficiently modified RhoA and to a lesser extent Rac and Cdc42, but H-Ras was not affected (Shao et al. 2002). Noteworthy, under physiological cell infection conditions YopT from *Y. enterocolitica* specifically modified endogenous RhoA but not endogenous Rac1 or Cdc42 and this was associated with release of RhoA, but not Rac1 or Cdc42, from cell membranes and Rho GDI (Aepfelbacher et al. 2003; Zumbihl et al. 1999). In a study mostly employing overexpression of YopT and/or Rac, YopT from *Y. pseudotuberculosis* was also shown to modify Rac1 (Wong and Isberg 2005b).

YopT is translocated into cells in much lower amounts than YopE (Aepfelbacher et al., unpublished) and Rho protein modification only develops over a time period of hours (Aepfelbacher et al. 2003; Zumbihl et al. 1999). Yet, the YopT effect is irreversible and does not require continuous binding of YopT to its target. In comparison, the YopE effect on Rac1 occurs rapidly after YopE translocation, depends on permanent direct YopE/Rac1 interaction and can be reversed by proteasomal degradation of YopE (Hentschke et al. 2007; Ruckdeschel et al. 2006). Thus, YopT and YopE not only act on different Rho proteins in cells, their effects also display different time courses and sustainability.

3.3 YopO/YpkA

YopO/YpkA (referring to the *Y. enterocolitica* and *Y. pseudotuberculosis* isoforms, respectively) comprises a number of activities and protein domains that could qualify it as integrator of the *Yersinia*-induced actin rearrangements and Rho protein modifications. It harbors a serine-/threonine kinase domain in the N-terminal half that autophosphorylates and phosphorylates external substrates upon binding to actin (Galyov et al. 1993; Juris et al. 2000; Pha et al. 2014; Trasak et al. 2007). The C-terminal half of YopO/YpkA consists of a Rho GDI-like domain implicated in binding and neutralizing Rho and Rac (Fig. 1) (Prehna et al. 2006). As in the case of YopE and YopT, YopO/YpkA was first shown to disrupt F-actin and produce cell rounding in host cells that were later held responsible for its rather modest antiphagocytic activity (Grosdent et al. 2002; Hakansson et al. 1996; Trasak et al. 2007). Actin was identified as the major factor in cell extracts stimulating the phosphokinase activity of YopO/YpkA, although additional factors may exist (Juris et al. 2000). In vitro experiments employing actin polymerization-, kinase- and pull down assays demonstrated that monomeric globular (G)-actin but not filamentous (F)-actin activated recombinant YopO/YpkA kinase and that activation required simultaneous G-actin binding to N- and C-terminal sites of the protein (Trasak et al.

2007). Sequestering of G-actin by YopO inhibited actin polymerization in vitro, revealing a dissociation constant (Kd) for the YopO/actin complex of around 2 μM (Trasak et al. 2007). A fascinating X-ray structure of YopO in complex with actin provided structural evidence that the N- and C-terminus of YopO sandwich actin subdomain 4. This structure explains in molecular terms how actin sequestration and kinase activation of YopO is brought about (Lee et al. 2015). The YopO/actin structure also suggested that the bound actin monomer is still capable of interacting with host actin-regulatory proteins (Lee et al. 2015). In YpkA, immunoprecipitated from cells, nineteen serine phosphorylation sites spread all over the protein were detected (Pha et al. 2014), two of which had previously been identified by in vitro autophosphorylation assays (Trasak et al. 2007). Systematic mutagenesis producing different sets of serine to alanine point mutants indicated that (auto)phosphorylation of multiple N-terminal sites regulates activation of the YopO/YpkA kinase (Pha et al. 2014; Trasak et al. 2007). Phosphorylation of the C-terminal sites did not affect kinase activity and one can speculate that some of the phosphorylation sites in YopO may be substrates for host cell kinases. Also, because many of the phosphorylated serines were in the N-terminal membrane localization domain and the C-terminal GDI domain of YopO, the function of these domains may be regulated by phosphorylation (Pha et al. 2014). The first reported cellular substrates of the YopO/YpkA kinase were actin, the deubiquitinating enzyme otubain-1 as well as the Gαq subunit of heterotrimeric G-proteins (Juris et al. 2000, 2006; Navarro et al. 2007). Whereas the consequences of Gαq phosphorylation for heterotrimeric G-protein signal transduction to the nucleus have been well worked out (Navarro et al. 2007), the physiological relevance of actin- and otubain-1 phosphorylation by YopO/YpkA has remained unclear (Edelmann et al. 2010; Juris et al. 2000). In line with its cellular effects YopO/YpkA was recently reported to associate with a number of actin regulators including nucleation-promoting factors, filament elongators, formins, severing proteins and depolymerizing proteins (Lee et al. 2015). Many of these regulators, i.e., VASP, EVL, WASP, gelsolin, and the formin diaphanous 1 were also phosphorylated by YopO (Ke et al. 2015; Lee et al. 2015). In an elegant series of experiments it was demonstrated that the actin regulators are sequestered and directly phosphorylated in ternary complexes consisting of a YopO molecule associated with an actin monomer and the respective actin regulator bound to a region of the actin molecule not occupied by YopO (Lee et al. 2015). Phosphorylation of VASP by YopO was shown to attenuate its actin polymerization capability but functional consequences of phosphorylation of the other actin regulators have not been reported (Lee et al. 2015).

Taken together, YopO/YpkA combines multiple activities that impinge on the actin cytoskeleton including G-actin-sequestration, phosphorylation of actin regulators and G-protein α-subunits as well as inhibition of Rho proteins. Although its significant role in *Yersinia* virulence has been unanimously demonstrated in mice, its function in cellular pathogenesis, i.e., how exactly it contributes to the antiphagocytic-, immunosuppressive- and/or T3SS-regulatory activities of *Yersinia* has not been well defined. One reason might be that the functions of the different YopO/YpkA domains cannot easily separated from each other either because they

elicit similar cellular effects or because they directly depend on each other, i.e., G-actin binding and kinase activation. Another reason might be that only relatively small amounts of YpkA/YopO are translocated into cells and great care must be taken not to over interpret results obtained by overexpressing YopO/YpkA or isolated domains of it in cells. Integrating the available data, it appears that the kinase- and GDI domain of YopO/YpkA synergistically contribute to F-actin disruption, cell rounding and antiphagocytosis (Grosdent et al. 2002; Groves et al. 2010; Wiley et al. 2006; Trasak et al. 2007). Nonetheless, only the kinase but not the other activities of YopO/YpkA were required to inhibit *Yersinia* uptake mediated by the surface protein YadA (Trasak et al. 2007).

To get a better understanding of the role of YopO/YpkA in *Yersinia* pathogenesis, specific strains must be constructed that translocate YopO/YpkA mutants deficient in one or more functions. Therefore, YopO/YpkA point mutants should be employed that abrogate membrane binding, actin binding, kinase- and/or GDI activity individually and in different combinations. Surprising insights can be expected, i.e., has it been reported that the GDI activity of YopO/YpkA is relevant for *Yersinia* pathogenesis only when the remaining functions of YopO/YpkA are intact but not when YopO/YpkA is missing altogether (Prehna et al. 2006).

3.4 Other Yops Affecting the Cytoskeleton

YopH is a highly active tyrosine phosphatase that dephosphorylates p130Cas, focal adhesion kinase (FAK), paxillin, Fyn-binding protein (FyB) and the scaffolding protein SKAP-HOM in different cell types (Black and Bliska 1997; Hamid et al. 1999; Persson et al. 1997). These YopH substrates are known to regulate the interaction of actin and the cytoplasmic tail of integrins in focal adhesion sites of many cell types (Brakebusch and Fassler 2003). Other YopH targets are linker for activation of T-cells (LAT) and SH2-domain containing leukocyte protein of 76 kDa (SLP-76) (Gerke et al. 2005). YopH has been made responsible for up to 50 % of the antiphagocytic activity of *Y. enterocolitica* and *Y. pseudotuberculosis* (Fallman et al. 1995; Ruckdeschel et al. 1996). Yet, it is presently not entirely clear through which of its known substrates YopH exerts its anti-phagocytic, anti-migratory, or anti-immune effects and whether unrecognized targets are also involved.

YopM is a leucin-rich repeat (LRR) protein that displays immunosuppressive activity by acting as a scaffold for numerous cellular signaling molecules (Berneking et al. 2016; Chung et al. 2014; Hofling et al. 2015). Although no effect of YopM on the cytoskeleton or on Rho GTP-binding proteins has been reported so far, it interacts with IQGAP and the protein kinase N/PRK family of kinases. Notably both of these proteins are effectors for Rho proteins and have been implicated in cytoskeletal regulation pointing to a potential impact also of YopM on actin reorganization (Fig. 1) (Chung et al. 2014; Hentschke et al. 2010).

3.5 *Cytotoxic Necrotizing Factor-Y*

Although inactivation of Rho GTP-binding proteins by the Yops has been regarded as a key feature in *Yersinia's* virulence strategy, some strains of *Yersinia pseudotuberculosis* also carry an exotoxin named CNF-Y that belongs to the family of cytotoxic necrotizing factors (CNFs), whereby Y stands for CNF from *Yersinia* (Hoffmann et al. 2004; Knust and Schmidt 2010). CNF exotoxins constitutively activate Rho GTP-binding proteins by deamidation of glutamine-63 (RhoA) or glutamine-61 (for Rac and Cdc42). Deamidation inhibits the intrinsic and GAP-stimulated GTPase activities of the Rho proteins rendering them permanently GTP-bound (Fig. 1) (Hoffmann et al. 2004). A newly discovered role of CNF-Y in regulation of effector translocation is described below.

4 Regulation of T3SS-Mediated Effector Delivery by Manipulation of Rho GTP-Binding Protein Activity and the Actin Cytoskeleton

Like in *Salmonella*, *Shigella* and *Pseudomonas*, expression, assembly and secretion of T3SS components in *Yersinia* are tightly controlled in response to environmental cues allowing the bacteria to accomplish their individual virulence strategies (Francis et al. 2002; Galan et al. 2014). After assembly of the functional T3SS, also called injectisome, the actual process of effector protein translocation/injection is triggered by host cell contact (Dewoody et al. 2013). Only then the T3SS inserts a bacterial translocation pore into the host cell membrane through which the effector proteins pass into the cell (Mueller et al. 2008). The importance of a precisely regulated translocation rate became apparent in studies of the *Yersinia* effector protein YopQ/K (named YopQ in *Y. enterocolitica* and YopK in *Y. pestis* and *Y. pseudotuberculosis*). YopQ/K mutants are characterized by increased delivery of Yops and an accelerated cytotoxic response in cells (Holmstrom et al. 1997). Despite this, YopQ/K mutants were essentially avirulent in mouse infection experiments (Straley and Cibull 1989; Holmstrom et al. 1995a, b). Thus not only a decrease, but also an unregulated increase of effector translocation, i.e., potentially any imbalance in effector activities may disturb the progression of *Yersinia* infection.

A series of excellent publications demonstrated that actin disruption by cytochalasin D, inhibition of Rho (A, B or C) by C3-transferase of *Clostridium botulinum* or translocated YopE itself can block both, effector translocation by *Y. pseudotuberculosis* and plasma membrane permeabilization by the translocation pore (Fig. 3) (Viboud and Bliska 2001; Mejia et al. 2008; Black and Bliska 2000). These and other reports (Aili et al. 2008; Schweer et al. 2013) employed *Y. pseudotuberculosis* and primarily implicated a Rho isoform (A, B or C) but not Rac in regulation of effector translocation and pore activity. Consistently, knockdown of

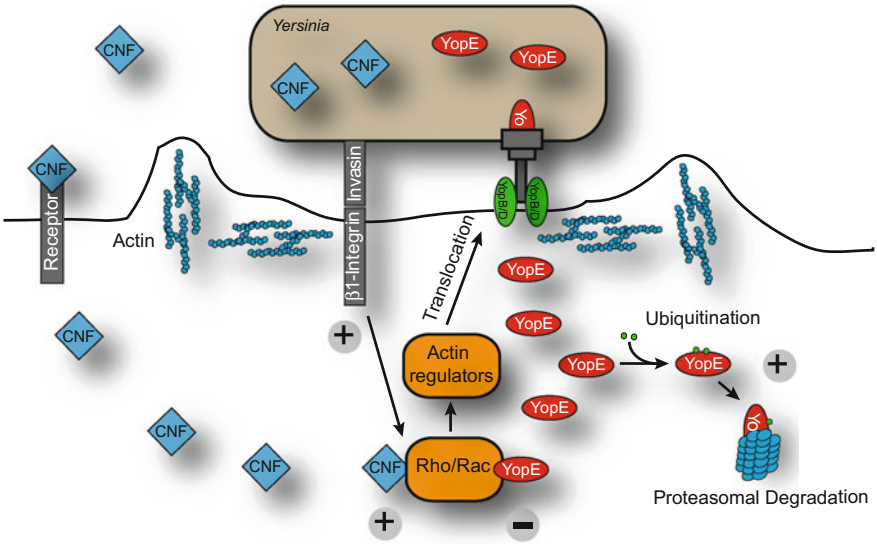

Fig. 3 A complex interplay of *Yersinia* and host factors regulates effector translocation. Upon adhesion to host cells *Yersinia* activates Rho and/or Rac, which in turn stimulate effector translocation by signaling to the actin cytoskeleton. CNF-Y released by the bacteria and taken up by the cells can further boost effector delivery by sustained activation of Rac and/or Rho. Translocated YopE downregulates Rac activity and thereby can inhibit further effector translocation in a negative feedback loop. This effect can in turn be relieved by proteasomal degradation of YopE. Effector translocation activating and inhibiting factors are indicated by plus and minus, respectively

the Rho effector protein Rock-II down-regulated Yop translocation by *Y. pseudotuberculosis* to a similar extent as knockdown of RhoA (Sheahan and Isberg 2015). However, other work performed with *Y. enterocolitica* suggested that Rac1 is the central Rho-family protein controlling these processes (Gaus et al. 2011; Koberle et al. 2009; Ruckdeschel et al. 2006; Wolters et al. 2013). To identify the Rac downstream effectors determining *Y. enterocolitica* Yop translocation the well established WAVE regulatory complex (WRC) acting through the Arp2/3 complex should be taken into account (Humphreys et al. 2012, 2013).

Because YopE was found to inhibit its own translocation and that of the other effector Yops as well as isolated translocation pore activity in host cells, it was proposed to be the main actor in a negative feedback loop of Yop translocation (Aepfelbacher et al. 2011; Aili et al. 2008; Dewoody et al. 2011; Gaus et al. 2011). The current view of how the feedback loop works is that the more YopE becomes translocated by the *Yersinia* injectisome and is active in target cells the more the injectisome becomes inhibited. Rho GAP activity is necessary for this intriguing YopE function, because the GAP activity-deficient YopE-R144A mutant and a YopE null mutant displayed a similar increase of Yop translocation and pore activity (Aili et al. 2008). The most likely YopE targets involved in feedback

control of Yop translocation are Rho (A, B, or C) for *Y. pseudotuberculosis* and Rac1 for *Y. enterocolitica*. On one hand these Rho proteins are inhibited by YopE and on the other hand their constitutive activation leads to enhanced Yop translocation in the, respectively, infected cells (Aepfelbacher et al. 2011; Roppenser et al. 2009; Schweer et al. 2013; Wolters et al. 2013). It was surprising to realize that the negative feedback loop of Yop translocation can even be further fine-tuned by a mechanism that in turn leads to removal of translocated YopE. YopE from the O8 serotype of *Yersinia enterocolitica* was shown to be modified by ubiquitination at the residues lysine-62 and lysine-75 and to be degraded by the host cell proteasome (Fig. 3) (Hentschke et al. 2007; Ruckdeschel et al. 2006). As expected, bacterial translocation of the degradable YopE was associated with reduced cytotoxicity, an increase of active Rac1 and increased cellular levels of Yops (even of YopE) when directly compared to translocation of a non-degradable YopE (in which the lysines serving as ubiquitination sites were replaced by other amino acids (Gaus et al. 2011). Interestingly, a *Y. enterocolitica* serotype O8 strain, which expresses the degradable YopE disseminated much better into liver and spleen of mice than an isogenic strain expressing the non-degradable YopE (Gaus et al. 2011). Thus, degradation of YopE can favor the pathogenicity of a specific *Yersinia* strain indicating that different mechanisms of fine-tuning Yop activities have evolved even among closely related *Yersinia* serotypes. These results also support the notion that any *Yersinia* strain is perfectly adapted to a unique play of Yop activities and any imbalances will negatively affect virulence.

Because YopT, YopE, and YopO inactivate Rho proteins supporting different aspects of *Yersinia* pathogenesis, it was unclear what role the Rho protein activator CNF-Y might play in the orchestra of Rho protein modulation. Recent work showed that when pretreated with CNF-Y *Yersinia*-infected cells display strongly increased levels of translocated Yops (Fig. 3) (Schweer et al. 2013; Wolters et al. 2013). A concordant CNF-Y effect could also be seen in mice where a higher number of cells displayed detectable levels of translocated Yops upon infection with a CNF-Y producing-versus a CNF-Y mutant *Y. pseudotuberculosis* strain (Schweer et al. 2013). Furthermore, the CNF-Y mutant strain was altogether attenuated in dissemination to mesenteric lymph nodes, spleen and liver (Schweer et al. 2013). Consistent with previous work (see above), the CNF-Y-stimulated increase in *Y. pseudotuberculosis* effector translocation could be abolished by Rho inhibition but not by Rac inhibition and the opposite was true for effector translocation by *Y. enterocolitica* (Schweer et al. 2013; Wolters et al. 2013). Also, the translocation activity of *Y. enterocolitica* was strongly stimulated by expression of constitutively active Rac1 (Rac1L161), while no such effect was observed when constitutively active RhoA (RhoAL63) was expressed in cells (Wolters et al. 2013). These data indicate that modulation of effector translocation by *Y. pseudotuberculosis* and *Y. enterocolitica* is accomplished by different Rho proteins. To understand the molecular background of this interesting difference in the biology of *Y. pseudotuberculosis* and *Y. enterocolitica* it will be required to first clarify the cellular mechanisms that are generally responsible for *Yersinia* effector protein translocation.

The currently available data allow to draw the following conclusions about the *Yersinia* host cell interplay regulating effector protein delivery. Upon cell infection Rho and/or Rac are activated presumably via β1-integrin signaling and then reorganize actin, which somehow stimulates the translocation process. Once Yops accumulate in the cell, the action of YopE and potentially also YopT and YopO terminate the further delivery of Yops. The system can be further steered by CNF-Y, which activates Rho proteins and translocation such that they cannot be down-regulated by YopE anymore. In addition, some YopE isoforms are ubiquitinated and degraded by the host cell proteasome, which reactivates translocation (Fig. 3). One major question in this process relates to the mechanisms by which Rho proteins and/or actin control effector translocation and pore activity. Actin rearrangements might cause a larger fraction of the bacterial surface to come into contact with the host plasma membrane leading to a higher number of injectisomes being engaged and activated. Also, a direct interaction with a specific actin structure might somehow control the activity of the translocation pore. Future experiments will have to distinguish between these possibilities.

5 Synopsis

As described in this overview, the effects of the *Yersinia* virulence factors invasin, YopE, YopT, YopO, and CNF-Y on Rho GTP-binding proteins and the actin cytoskeleton can be rapid or delayed, transient or sustainable, synergistic or antagonistic. These virulence factors also act in a host cell type specific manner and in distinct subcellular compartments. Clearly, the described *Yersinia* strategem must be perfectly organized to modulate phagocytosis, migration and other defense mechanisms of immune cells on one hand and effector protein delivery on the other hand. Already the known effects of *Yersinia* on actin and Rho protein signaling networks provide an idea of how perfectly orchestrated the bacteria have developed their infection strategy. Undoubtedly, many more features of this complex host pathogen encounter are still waiting to be uncovered. Finally, modulation of Rho proteins by Yersinia does not only impact the actin cytoskeleton, but also the pyrin inflammasome, which is formed in response to bacterial inhibition of Rho proteins and can be reversed by CNF-mediated stimulation of the Rho effector PKN (Chung et al. 2016).

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Bacterial Actins and Their Interactors

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Abstract Bacterial actins polymerize in the presence of nucleotide (preferably ATP), form a common arrangement of monomeric interfaces within a protofilament, and undergo ATP hydrolysis-dependent change in stability of the filament—all of which contribute to performing their respective functions. The relative stability of the filament in the ADP-bound form compared to that of ATP and the rate of addition of monomers at the two ends decide the filament dynamics. One of the major differences between eukaryotic actin and bacterial actins is the variety in protofilament arrangements and dynamics exhibited by the latter. The filament structure and the polymerization dynamics enable them to perform various functions such as shape determination in rod-shaped bacteria (MreB), cell division (FtsA), plasmid segregation (ParM family of actin-like proteins), and organelle positioning (MamK). Though the architecture and dynamics of a few representative filaments have been studied, information on the effect of interacting partners on bacterial actin filament dynamics is not very well known. The chapter reviews some of the structural and functional aspects of bacterial actins, with special focus on the effect that interacting partners exert on the dynamics of bacterial actins, and how these assist them to carry out the functions within the bacterial cell.

Contents

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1 Family of Bacterial Actins

Actin-like proteins that perform functions similar to the eukaryotic actin cytoskeleton began to be experimentally characterized about 15 years ago. For example, MreB, the chromosomally encoded bacterial actin, which is one of the major shape-determining factors in rod-shaped bacteria, was observed to form filaments within the bacterial cell, similar to actin (Jones et al. 2001). Structure determination of MreB indeed confirmed the striking similarities with the fold of the eukaryotic actin (van den Ent et al. 2002). Subsequently, many other bacterial actin-like proteins have been characterized both functionally and structurally. The prominent examples are those involved in shape determination (MreB; Jones et al. 2001; van den Ent et al. 2001), plasmid segregation (ParM; Møller-Jensen et al. 2002), organelle positioning (MamK; Komeili et al. 2006), and cell division (FtsA; van den Ent and Löwe 2000; Szwedziak et al. 2012). Furthermore, a large number of bacterial actins, termed as ALPs or actin-like proteins have been identified in a variety of bacterial genomes, most of which are predicted to be involved in plasmid segregation (Derman et al. 2009). Phylogenetic trees depicting representatives of actin family proteins show their sequence and functional divergence (Derman et al. 2009; Draper et al. 2011; Ingerson-Mahar and Gitai 2012; Ozyamak et al. 2013b).

This chapter describes the structure of bacterial actins, and their comparison with the structure of the eukaryotic actin, both as monomers and filaments. This is followed by how the variation in filament dynamics effects the cytoskeletal functions performed by the bacterial actins. Common features between the eukaryotic and bacterial actins in the context of interactors that affect filament dynamics are discussed.

1.1 Structure of Bacterial Actins

The members of the bacterial actin families share a common fold (Bork et al. 1992) and form polymeric assemblies that assist them in their function. This section describes a brief structural comparison between various actins with respect to their monomeric fold, arrangement in protofilaments and assembly of functional filaments. There are many recent reviews on the comparison of the various bacterial actins, and hence the readers are requested to refer to them for more details (Ingerson-Mahar and Gitai 2012; Ozyamak et al. 2013b; Eun et al. 2015). The

recent novel additions to the family of actins include the four-stranded tubular structure of *Bt*ParM (Jiang et al. 2016) and crenactin from the archaea *Pyrobaculum calidifontis* (Ettema et al. 2011; Lindås et al. 2014; Izoré et al. 2014; Braun et al. 2015). Though closer to eukaryotic actin in sequence and structure, crenactin possibly forms an interesting assembly of a single protofilament, hitherto not observed in any of the actin family filaments (Izoré et al. 2014; Braun et al. 2015). Since the chapter deals with bacterial actins and the function of crenactin is not known yet, further discussion on crenactin is not included here.

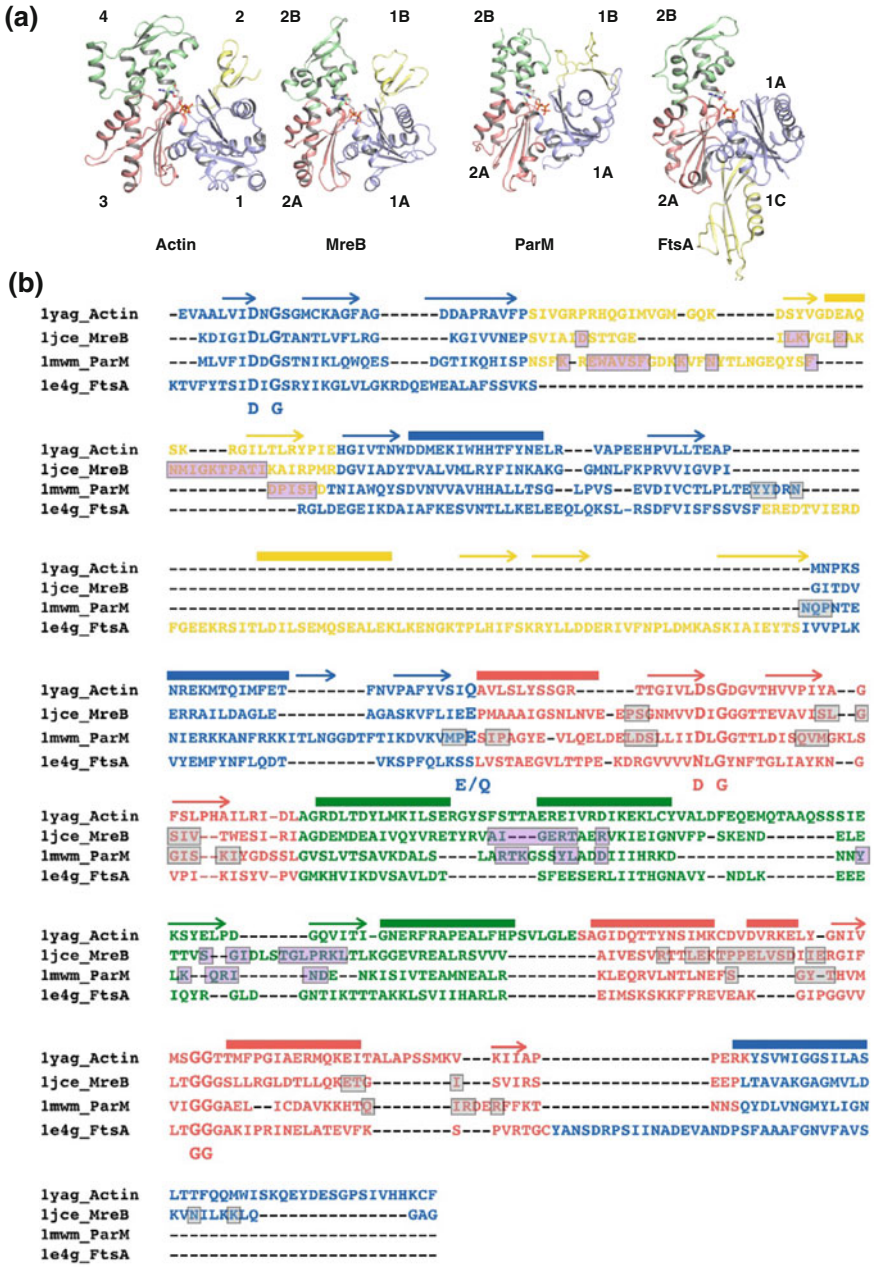
1.1.1 Monomer

All the major members of the bacterial actin families share the actin fold (Bork et al. 1992), comprising four subdomains (Fig. 1a), either termed as 1, 2, 3, and 4 (as in eukaryotic actin nomenclature) or as 1A, 1B, 2A, and 2B (according to the bacterial actin nomenclature) respectively. The largest variability among the four subdomains is observed in subdomain 2 (or 1B). Subdomain 2 (1B) is comparatively shorter in sequence in ParM. An exception is FtsA, in which subdomain 2 forms a domain-swapped arrangement (termed as subdomain 1C) and is present as an insertion within domain 1 (1A) in sequence (Fig. 1a, b; van den Ent and Löwe 2000; Szwedziak et al. 2012).

1.1.2 Protofilament

In addition to the actin fold, a common feature that unifies the bacterial actins is the assembly of their monomers into filamentous oligomeric structures. All the representative members of the bacterial actin families have an arrangement of monomers similar to that of actin within their protofilaments (Fig. 2a). In all protofilament architectures, monomers are oriented such that the interface is formed between subdomains 2 (1B) and 4 (2B) of the bottom monomer and subdomains 1 (1A) and 3 (2A) on the top monomer, respectively (Fig. 2a, residues at the interface are highlighted in the sequence alignment in Fig. 1b). The domain-swap in FtsA also allows for an actin-like protofilament formation, with the subdomain 1C occupying the position corresponding to subdomain 2 (1B) within a protofilament (Fig. 2a).

Though there is negligible sequence conservation among intra-protofilament interface residues of the different actin family proteins, the positions of the residues at the protofilament interface remain conserved (Fig. 1b). For example, Fig. 1b highlights that residues in the corresponding secondary structure elements of the subdomains form the interface for ParM and MreB. The same is true also for actin and other protofilaments in the family such as psk41 ParM (Popp et al. 2010), pB171 ParM (Rivera et al. 2011), Alp12 (Popp et al. 2012), AlfA (Polka et al. 2014), BtParM (Jiang et al. 2016), and MamK (Ozyamak et al. 2013a). The extent of the interface depends on insertions or deletions present within these sequences



(Fig. 1b). The variation in the sequences at the interface contributes towards changes in the helical parameters of the actin-like filaments (see Ozyamak et al. 2013b for figure and helical parameter comparison).

◀ **Fig. 1** The actin fold. **a** Crystal structures of the representative members of the actin family (Actin, MreB, FtsA and ParM) with the subdomains labeled and *color coded*. PDB IDs: Actin—1YAG, MreB—1JCE, FtsA—1 E4G, ParM—4A62. **b** A structure-based sequence alignment of the actin, MreB, ParM and FtsA obtained using ProMALS3D (Pei et al. 2008). The various subdomains are *color-coded* in the sequence alignment according to (**a**), and the secondary structure elements marked using *arrows* for β-strands and rectangles for α-helices. The residues at the intra-protofilament interface for MreB and ParM are highlighted in *gray* and *pink-shaded boxes* respectively for the *top* and *bottom* monomers in a protofilament. The interface residues were identified using the PDB-ePISA webserver (http://www.ebi.ac.uk/pdbe/pisa/) for the PDB IDs 1JCE (MreB) and 5AEY (ParM)

1.1.3 Filament Architecture

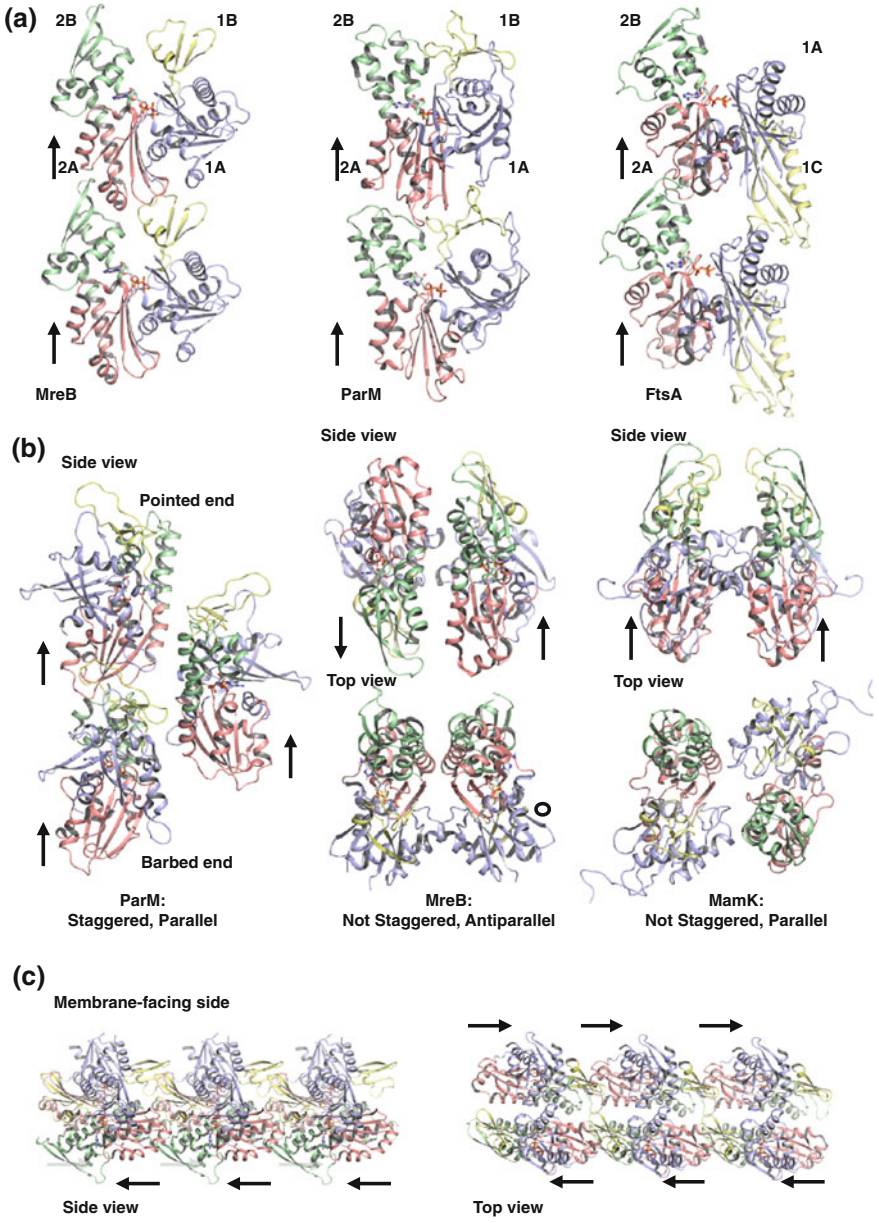
Eukaryotic actin can be described as a two-stranded filament with the two protofilaments coming together in a right-handed twist. Since the protofilaments are arranged in a parallel fashion, there is a polarity for the filament, the two ends termed as pointed end and barbed end, respectively (Fig. 2b). This is conserved across actins of all eukaryotic organisms. In contrast, in bacterial actins, the protofilaments come together in different ways to form a functional filament (Ozyamak et al. 2013b).

Antiparallel arrangement of MreB

For MreB, there are in vitro observations and in vivo crosslinking studies suggesting that the two protofilaments come together in an antiparallel fashion (Fig. 2b, van den Ent et al. 2014). This arrangement results in the orientation of the membrane-binding residues towards the membrane in all the monomeric subunits of the filament (Fig. 2c; Salje et al. 2011). This facilitates interaction with RodZ and other proteins of the cell wall synthesis machinery that are located within the cell membrane (van den Ent et al. 2010; see Sect. 2.1).

Bacterial actins in plasmid segregation

The two-stranded filament, similar to eukaryotic actin but with varying helical parameters, is the most common assembly of protofilaments that has been characterized till now. Some examples of bacterial actins performing plasmid segregation that form two-stranded filaments are *E. coli* R1 plasmid ParM (van den Ent et al. 2002; Bharat et al. 2015), pB171 ParM (Rivera et al. 2011), AlfA (Polka et al. 2009), psk41 ParM (Popp et al. 2010), etc. In these filaments, the two protofilaments occur in a staggered and parallel arrangement (Fig. 2b), and hence these have a structural polarity (two structurally dissimilar ends) similar to eukaryotic actin filaments, though the helical parameters including handedness and the twist differ. The existence of structural polarity implies that the adaptor protein that links the bacterial actins to the plasmid DNA can potentially interact only at one end of the filament. Hence, to achieve plasmid segregation, there should be an antiparallel arrangement of structurally polar filaments. In *E. coli* R1 plasmid ParM, it has been demonstrated that ParR*C* complex binds only to the barbed end of the double-stranded actin-like filament, and two such filaments can come together in an



antiparallel manner to achieve plasmid segregation (Gayathri et al. 2012). Variations of this exist in the different bacterial actins involved in plasmid segregation. AlfA also forms filaments composed of two protofilaments in a parallel, staggered arrangement (Polka et al. 2014). During plasmid segregation, bundles of filaments in mixed orientation may be present (Polka et al. 2014).

◀ **Fig. 2** Filament architecture of bacterial actins. **a** Two adjacent monomers within a protofilament for MreB, ParM and FtsA highlight the similarity in subdomain arrangement (PDB IDs: MreB—1JCE, ParM—5AEY, FtsA—4A2B). The *arrows point* in the direction from subdomains 2A–2B. **b** Monomers in staggered versus unstaggered and parallel versus antiparallel arrangements in ParM, MreB and MamK filaments. Two perpendicular views (*side* and *top views*) are shown for MreB and MamK to illustrate the parallel and antiparallel orientations. The *arrows point* in the direction from subdomains 2A–2B, *closed* and *open circle* denotes an *arrow* pointing outside and inside of the plane respectively. PDB IDs: MreB—1JCE, MamK—fitted model in the EM reconstruction (Ozyamak et al. 2013a; http://faculty.washington.edu/jkoll/structures.html) **c** Antiparallel protofilaments of MreB (PDB ID: 4CZJ). The N-terminal residues and the loop between residues 97–104 (not modeled in the crystal structure) of subdomain 1A face the membrane-binding side. Two views of the filament arrangement are shown for illustrating the antiparallel nature. *Light gray arrows* represent the direction of the monomers behind the plane

In addition to two-stranded filament architecture, novel assemblies of four-strand and tubular structures have also been recently characterized for the Alp family of bacterial actins involved in plasmid segregation. In Alp12, the protofilament arrangement itself consists of parallel and antiparallel orientations, forming a four-stranded filament of novel arrangement with mixed orientation within it (Popp et al. 2012). Newly identified filament architecture of *Bt*ParM from *Bacillus thuringensis* demonstrates the formation of a double-stranded filament consisting of two protofilaments in antiparallel orientation in the presence of nucleotide, with an elaborate twist (Jiang et al. 2016). In the presence of *Bt*ParR, two such double-stranded filaments come together to form a tubular structure made of four protofilaments. The four-stranded tubular structure thus formed consists of alternate protofilaments in parallel and antiparallel orientations.

The reasons for the variability of protofilament organization, especially as observed among bacterial actins in the same function of plasmid segregation, are intriguing. ParM, AlfA, pB171, Alp12, and *Bt*ParM are all involved in plasmid segregation. However, the protofilament arrangement in these bacterial actins range from variation of two-stranded filaments as in ParM and AlfA, four-stranded filaments as in Alp12, and tubular architecture of four-stranded filaments as in *Bt*ParM. The variability might be the resultant of regulation of filament dynamics by binding partners. The thickness of the filament might have evolved related to the load (size of the plasmid that acts as the cargo), the mechanism of chromosome segregation and cell division in the particular bacterium, etc. These are factors that remain to be investigated.

Parallel and unstaggered arrangement in MamK

In MamK, the bacterial actin involved in magnetosome formation and positioning in magnetotactic bacteria, the two protofilaments are oriented in parallel, but do not possess a stagger as present in actin or other ParM filaments (Fig. 2b). This arrangement has been attributed to an insertion in the inter-protofilament interface in MamK structures (Ozyamak et al. 2013a). The functional relevance of such an architecture compared to a staggered configuration is unknown. The stagger of monomers between protofilaments can have implications with respect to rate of

growth of the filaments, as the presence of a stagger can contribute towards an added interface for an incoming monomer.

FtsA

Among the bacterial actins, very less is known about the filament architecture of FtsA. Protofilament of FtsA has been observed in its crystal structure (Fig. 2a), however the further assembly of its protofilaments that form the functional state is not known. FtsA interacts closely with the tubulin cytoskeleton, namely the FtsZ protein, and contributes to ring constriction in bacteria leading to cell division.

The next section discusses the effect of filament architecture on dynamics of assembly of bacterial actins.

1.2 Dynamics of Filament Assembly

An important feature of a cytoskeletal filament such as actin or tubulin, which undergoes nucleotide dependent polymerization, is filament dynamics. Filament dynamics refers to the characteristics of filament assembly and disassembly. Dynamics exhibited by actin-like and tubulin-like filaments is the result of the relative stabilities between the NDP versus NTP conformations within the filament. Typical filament dynamics behavior of actin and tubulin family proteins includes treadmilling and dynamic instability, respectively. Similar to the variety of protofilament architectures observed, the filament dynamics of the bacterial actins also tend to vary (Garner et al. 2004, 2007; Popp et al. 2010; Gayathri et al. 2012; Polka et al. 2014).

1.2.1 MreB

The protofilament architecture of MreB and in vivo studies suggest that MreB forms an antiparallel arrangement of protofilaments (van den Ent et al. 2014). This implies that the assembly of MreB filaments is structurally nonpolar, in contrast to the eukaryotic actin filaments that exhibit structural and kinetic polarity. Short MreB filaments have been observed to rotate circumferentially perpendicular to the longitudinal axes of rod-shaped cells (Garner et al. 2011; Domínguez-Escobar et al. 2011; van Teeffelen et al. 2011). The role of filament dynamics in this process, if any, has not been confirmed so far. The rotation seems to be dictated by the cell wall synthesis machinery, as suggested by studies characterizing the role of RodZ and DapI in MreB circumferential movement (Morgenstein et al. 2015; Reuff et al. 2014). It has also been proposed that DapI can bind to unpolymerised MreB, and thus initiates the assembly of the peptidoglycan machinery on DapI, when MreB attaches to the membrane upon polymerization (Reuff et al. 2014). Another study showed that MreB filaments dissociate from the cell membrane on depletion of cell

wall precursors, and hence the filament dynamics depend on the availability of the precursors (Schirner et al. 2014). There have been some earlier studies in *Caulobacter crescentus*, which suggest that MreB filaments treadmill (Kim et al. 2006). With the current idea of antiparallel arrangement of MreB filaments and the concept of circumferential motion of MreB driven by peptidoglycan synthesis, what contributes to the kinetic polarity of MreB filaments is unknown.

1.2.2 Bacterial Actins in Plasmid Segregation

Actin-like proteins in plasmid segregation are most well studied with respect to filament dynamics, since many of the plasmid segregation systems, consisting of three components, have been reconstituted in vitro. Hence, filament dynamics have been characterized both in vitro and in vivo for these. Similar to the filament architecture, the filament dynamics also vary between the different bacterial actins. ParM exhibits dynamic instability (Garner et al. 2004). ParM assembles into filaments in the presence of ATP without the requirement of a nucleating factor, and disassembles once ATP is hydrolysed. ParM filaments are stabilized in the presence of ParR*C* complex (formed by the adaptor protein ParR that links ParM to the centromeric sequence *parC*) (Garner et al. 2007). ParR*C* complex also functions towards increasing the rate of growth of filaments from one end, thus modulating filament dynamics (Gayathri et al. 2012). AlfA filaments are stable and tend to bundle in the absence of AlfB (Polka et al. 2009). In the presence of AlfB and *parN*, AlfA filaments exhibit treadmilling (Polka et al. 2014). The dynamics of filaments such as Alp7A has been looked at within the bacterial cell (Derman et al. 2009). BtParM forms tubular four-stranded filaments in the presence of BtParR (Jiang et al. 2016).

To summarize the observed trends, the adaptor-DNA complex modulates the filament dynamics in plasmid partitioning systems, such that proper segregation through directional movement of plasmids towards opposite ends is achieved after the plasmid DNA is captured at the structurally polar filament ends.

1.2.3 MamK

In vitro characterization of polymerization dynamics using an untagged version of MamK protein showed that MamK polymerizes in the presence of ATP and not ADP (Ozyamak et al. 2013a). Filament disassembly is triggered once ATP is hydrolysed, as observed from measurements using light scattering and ATP hydrolysis. MamK filaments are more stable compared to other bacterial actins such as ParM due to bundling dependent on salt concentration. Single molecule fluorescent studies to observe growth differences between the two ends have not yet been performed for MamK. An interesting study in the filament dynamics of MamK involves the characterization of polymerization properties along with the

MamK-like protein present in a magnetosome island (Abreu et al. 2014). More details on MamK dynamics in the presence of interactors are reviewed in a later section (Sect. 2.3).

1.2.4 FtsA

FtsA filaments have been observed along with FtsZ as a sandwich between the cell membrane and FtsZ filament in cryotomography of *Caulobacter* cells (Szwedziak et al. 2014). The same study also observed FtsA and FtsZ filaments in liposomes. Swirling movement of fluorescently labeled FtsZ was observed in an in vitro reconstitution of FtsZ and FtsA filaments on a lipid layer (Loose and Mitchison 2014). The filament formation requires the presence of either ADP or ATP, and nucleotide hydrolysis is not a requirement. ATP hydrolysis activity has not been detected yet for FtsA (Lara et al. 2005). It is probable that FtsA does not hydrolyse ATP especially since the canonical catalytic residues of the actin fold are absent (Fig. 1b). FtsA filaments act cooperatively along with FtsZ to assemble the FtsZ ring to the cell membrane and to cause ring constriction. One of the mechanisms of ring constriction proposes that the mismatch in the filament dimensions between FtsA ($\sim$5 nm repeat distance) and FtsZ filaments ($\sim$4 nm repeat distance) contributes towards the generation of curvature required for constriction (Szwedziak et al. 2014).

With the above brief introduction to the structure, function, and dynamics of the prominent members of the bacterial actin families, I now move on to the various interacting proteins that have been characterized for bacterial actins. Eukaryotic actins are very well conserved, and one of the reasons proposed for its high conservation is the wide variety of interacting proteins that regulate its polymerization (Gunning et al. 2015). One of the distinguishing features between prokaryotes and eukaryotes was proposed to be the absence of interacting proteins to the bacterial cytoskeletal family that are similar to nucleators and molecular motors that walk along the tracks (Theriot 2013). The next section gives an overview of the various interacting proteins of bacterial actins that affect their dynamics and the functional significance of these interactions. However, the molecular basis of the correlation between dynamics and function of many interactors of the bacterial cytoskeleton is not known yet.

2 Interactors of Bacterial Actins

A large number of proteins that interact with the eukaryotic actin cytoskeleton are known. Eukaryotic actins require accessory proteins that assist in nucleation, elongation, etc. Formins, profilin, cofilin, Spire, fascin, etc., are some of them (Chesarone and Goode 2009; Campellone and Welch 2010; Dominguez and Holmes 2011; Breiher 2013). Bacterial pathogens very often hijack the host actin cytoskeleton with the help of nucleating factors that enable formation of actin

filaments that aid their entry or movement within the host cell (Bugalhão et al. 2015). They can also manipulate the cytoskeleton through controlling the GTPases that regulate the cytoskeleton. In bacteria, the information about interactors and how they modulate the bacterial cytoskeleton dynamics is limited. The current section reviews some of the available information on the bacterial cytoskeleton in this regard.

2.1 Interactors of MreB

MreB is the chromosomally encoded bacterial actin responsible for the rod shape in bacteria (Jones et al. 2001). The rotation of MreB filaments within the cell was proposed to be the result of the action of the cell wall biosynthesis machinery (Garner et al. 2011; Domínguez-Escobar et al. 2011; van Teeffelen et al. 2011). MreB is located near to the cell membrane, and it directly interacts with the cell membrane through an amphipathic helix at the N-terminus (in Gram-negative bacteria), or in combination with a hydrophobic loop (in Gram-positive bacteria) (Salje et al. 2011). The direct interaction of MreB with the membrane also brings it into proximity for interactions with other components of the cell wall elongation machinery such as MreC, MreD, RodZ, and the penicillin binding proteins PBP-1a and PBP2.

In addition to cell-shape determination, MreB also plays a role in various functions such as chromosome segregation (Kruse et al. 2003; Gitai et al. 2005), cell motility, positioning of molecules involved in pili formation (e.g., *Pseudomonas*, Cowles and Gitai 2010) and motility complexes (e.g., *Myxococcus xanthus*, Mauriello et al. 2010). MreB has also been shown to directly interact with FtsZ (Fenton and Gerdes 2013). Most of these functions of MreB have been implicated based on experiments carried out with a small molecule inhibitor of MreB named A22 (Iwai et al. 2002). However, a direct interaction and a molecular mechanism have been shown for very few proteins. The following subsections give details about the proteins that are known to interact with MreB.

2.1.1 RodZ

MreB is linked to the motion of the cell wall synthesis machinery through contacts with RodZ, which has also a periplasmic domain (Alyahya et al. 2009; Bendezú et al. 2009). Interaction between the cytoplasmic domain of RodZ and MreB has been characterized biochemically and structurally (van den Ent et al. 2010). Since the interface is away from the membrane-binding face of the monomer or the filament, RodZ binds MreB in both the monomeric and filament form, and facilitates membrane binding of the polymerized MreB. This is the only crystal structure published till date of an interactor in complex with MreB (Fig. 3a; PDB ID: 2WUS).

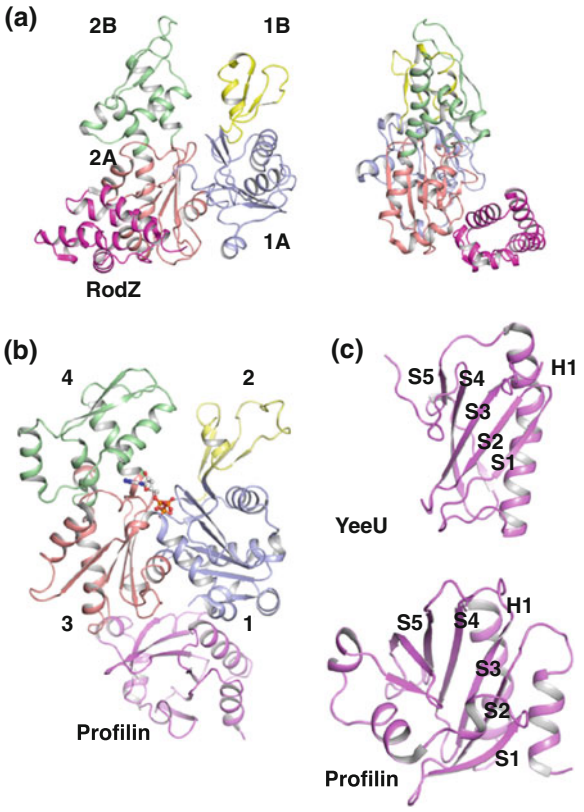


Fig. 3 Interacting proteins of MreB **a** Crystal structure of MreB complexed with RodZ. Two perpendicular side views are shown to highlight the interaction with subdomain 2A. (PDB ID: 2WUS) **b** Crystal structure of actin complexed with profilin (PDB ID: 2BTF) **c** Structural comparison of YeeU (PDB ID: 2H28) with profilin. The corresponding secondary structure elements between YeeU and profilin in DALI (Holm and Rosenström 2010) superposition are labeled

2.1.2 MbiA

A filament disruptor of MreB is MbiA in *Caulobacter crescentus* (Yakhnina and Gitai 2012). The function of MbiA is not known. It was identified as a protein that colocalises with MreB. Overexpression studies of MbiA showed that it disrupts MreB filaments, producing an effect very similar to that of A22, the small molecule inhibitor specific to MreB.

2.1.3 YeeU and YeeV

YeeU and YeeV form a toxin–antitoxin (TA) system in *E. coli* (Brown and Shaw 2003). The TA pair interacts with both MreB and FtsZ cytoskeletons in bacteria (Tan et al. 2011). YeeV acts to disrupt or destabilize both MreB and FtsZ filaments, resulting in the formation of lemon-shaped cells. Hence, YeeV has been renamed as CbtA (Cytoskeleton Binding Toxin A). In contrast, the corresponding antitoxin of

YeeV, YeeU functions as an antitoxin by exerting an antagonist effect on the cytoskeleton (Masuda et al. 2012). YeeU also interacts with both FtsZ and MreB. It causes bundling of the cytoskeletal filaments, thereby preventing their disassembly. The effects of YeeU makes the filaments insensitive to other inhibitors such as A22, M265 of MreB, and SulA, MinC, etc., of FtsZ. YeeU has been consequently named as CbeA (Cytoskeleton Bundling Enhancer A). The action of these could be similar to the actin severing and bundling proteins, such as cofilin and fascin, though sequence or fold similarities have not been detected. Structural comparison using DALI server (Holmes and Rosenström 2010) shows that the crystal structure of YeeU (Arbing et al. 2010; PDB ID: 2H28) has structural similarities to proteins of the profilin fold (Fig. 3b). The interaction site on the actin monomer or filament has to be identified for such comparisons to obtain mechanistic insights.

2.1.4 Interaction Partners in *Myxococcus xanthus* Motility

Motility in *Myxococcus xanthus* and the frequent reversals between its leading pole and the lagging pole involve movement of motor complexes such as AglQRS (equivalent to the MotA and MotB complexes of flagellar motor; Nan et al. 2011, 2013) and AglZ (Mignot et al. 2007) and regulatory components namely the small GTPase MglA and its GTPase activating protein MglB (Nan et al. 2015). These complexes move in a helical track along the body of the organism. Since the components of the motor complexes extend till the substratum and facilitate the movement of the organism, these have been termed as focal adhesion-like complexes, drawing parallels from the eukaryotic cell crawling (Mignot et al. 2005, 2007; Nan and Zusman 2016). The helical track has been hypothesized to be MreB based on the disruption of movement upon treatment with A22 (Mauriello et al. 2010; Nan and Zusman 2016). Interaction of *Myxococcus xanthus* MreB with AglZ, one of the cytoplasmic components of the focal adhesion-like complex, has been demonstrated based on pull-down assays (Mauriello et al. 2010). A recent in vitro experiment involving *Myxococcus* MreB and MglA, a small Ras-like GTPase involved in spatial positioning of motility complexes during reversals show that MglA interacts with MreB (Treurner-Lange et al. 2015; Nan et al. 2015). The interaction between MreB and the small Ras-like GTPase homologue in spatial positioning of motility complexes requires to be understood at a molecular level in order to delineate the role of the cytoskeleton in the process. The effect, if any, of the interaction on the filament dynamics of MreB will also be interesting to understand. Many other components in *Myxococcus xanthus* motility such as FrzCD, a part of the chemosensory pathway, also interacts with MreB either directly or indirectly through unknown mediator proteins (Mauriello et al. 2009).

2.1.5 Filament Interactors of MreB

EF-Tu

In addition to its role in translation, EF-Tu has been shown to form a helical localization near the cell membrane, similar to MreB localization. The filaments formed by EF-Tu were observed to be static as opposed to the dynamic filaments of MreB, and the two proteins were shown to interact in vivo and in vitro (Defeu Soufo et al. 2010). An in vitro study characterizing the filament dynamics of MreB using light scattering and fluorescence microscopy showed that the polymerized content of MreB is accelerated in the presence of EF-Tu (Defeu Soufo et al. 2015). Further studies on the significance of this interaction and its role in cell shape determination, and the molecular mechanism behind the modulation of MreB filament dynamics by EF-Tu have to be carried out. Interestingly, an interaction between the eukaryotic orthologue of EF-Tu, EF-1A and the eukaryotic actin has also been observed (Yang et al. 1990; Gross and Kinzy 2005).

Spiroplasma Fibril

An example of plausible interfilament interaction between bacterial cytoskeletal filaments is the interaction between Fib, a novel bacterial cytoskeletal protein in the helical cell wall-less bacterium, *Spiroplasma* and MreB (Kürner et al. 2005). There are five different MreBs in *Spiroplasma* and the role of these MreBs in shape determination and motility has not been established yet (Ku et al. 2014). Based on electron cryotomography images of *Spiroplasma*, and the spacing between the layer lines, the interacting filaments have been hypothesized to be Fib and MreB (Kürner et al. 2005). The role of filament dynamics of MreB, if any, in the kinking motion of *Spiroplasma* (Shaevitz et al. 2005) is yet to be established.

2.2 *Filament Stabilizers and Nucleators of Bacterial Actins in Plasmid Segregation*

Examples of interactors that modulate filament dynamics are known for bacterial actins involved in plasmid segregation. Among the three components of the plasmid segregation system, the adaptor protein and its complex with the plasmid DNA formed at the centromeric sequence influences the dynamics of the actin-like cytoskeleton (Garner et al. 2007; Gayathri et al. 2012; Polka et al. 2014).

Structural information on the interaction between the bacterial actin and the adaptor protein has been characterized for the ParMR*C* system (Gayathri et al. 2012). The crystal structure of ParM complexed with interacting C-terminal peptide of ParR provided a description of the interaction between a bacterial actin and a modulator of filament dynamics. Structural information of the interacting complex (PDB ID: 4A62), along with the ParR*C* ring structure and a high-resolution filament

reconstruction provided mechanistic insights into the stabilization of ParM filaments by ParR*C* (Gayathri et al. 2012). The dimensions of the ParR*C* ring matches with the short pitch helix dimensions of the ParM filament, and hence provides an explanation of how ParR*C* could accelerate the growth of ParM filaments at one end only. The mechanism of elongation stimulated by ParR*C* has been proposed to be analogous to the formin-assisted elongation of actin filaments (Gayathri et al. 2012). The interaction between ParM and ParR also reveals the striking similarity with the interaction of actin polymerization modulators such as Spire, formin, cofilin, twinfilin, etc., which all interact through an amphipathic helix recognizing the cleft between subdomains 1 and 3 of actin (Fig. 4). Thus the interactors of bacterial actin filaments also appear to utilize a similar strategic location on the actin fold for regulating filament dynamics and comes within the purview of the unifying hypothesis put forward for eukaryotic actin-binding proteins (Dominguez 2004; Dominguez and Holmes 2011).

The molecular basis of how other adaptor proteins and DNA complexes effect changes in their respective filament dynamics and enables plasmid segregation is yet to be elucidated. Though the filaments are of the same fold, the interacting proteins need not necessarily belong to the same fold. The number and arrangement of the repetitive sequences in the centromeric sequences (iterons) that are the binding sites of the adaptor protein also differ resulting in different geometries of the adaptor-DNA complexes. This corroborates with the different helical geometries observed for the corresponding actin-like filaments. The variety of plasmid segregation mechanisms based on actin-like filaments with varied filament architecture ensures that there are a large number of possible ways of manipulation of the dynamics of actin-like filaments. This will also ensure that a particular filament motor associated with the plasmid segregation machinery exclusively segregates the related plasmid.

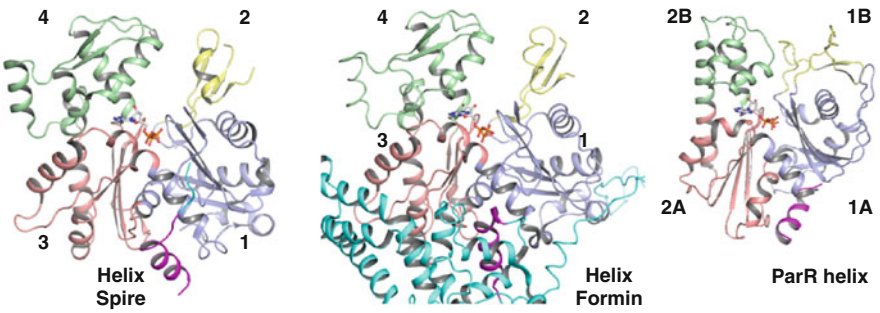


Fig. 4 ParR and the actin-binding proteins Spire and formin interact with the actin fold at a pocket formed between subdomains 1A and 2A (subdomains 1 and 3 for actin). The interacting helix is highlighted in pink and the rest of Spire and formin is shown in cyan. (PDB IDs: Actin-Spire—3MMV, actin-formin—1Y64, ParM-ParR—4A62)

E. coli R1 ParM is a two-stranded staggered filament and the organization of ParR on *parC* iterons forms a helical structure that matches the filament geometry. Similarly, it is probable that the short oligomeric structures of BtParR direct the formation of the tubular structure of BtParM. This can be hypothesized because the four-stranded structure is formed only in the presence of BtParR (Jiang et al. 2016). AlfB, the adaptor protein of AlfA is also capable of nucleating AlfA filaments on one end and destabilizing the other end (Polka et al. 2014). The mechanism of regulation of AlfA dynamics by AlfB is also not known. These are probable examples of how the adaptor protein-DNA complex assemblies can nucleate actin filaments of varied architecture.

2.3 *Interactors of MamK*

MamK, the bacterial actin involved in magnetosome positioning, has a few potential interactors present on the magnetosome genomic island (Draper et al. 2011; Pan et al. 2012). Potential interactors of MamK were identified based on screening for candidate genes encoded by the magnetosome genomic island. Interactors that have been identified till date are MamJ and the MamJ-like protein LimJ (Draper et al. 2011). Both of them affect the dynamics of MamK filaments in vivo. Filament turnover of MamK-GFP filaments in vivo were observed only in the presence of MamJ and LimJ expression. Destabilization of the MamK filaments within the cell by MamJ and LimJ is required for the proper formation of the magnetosome cluster. It is not yet established if their interaction with MamK is direct or mediated through other proteins.

Another interesting example of an interacting protein of MamK is Amb0994, a protein similar to a methyl-accepting chemotaxis protein (MCP), with a cytoplasmic localization in the magnetotactic bacteria *Magnetospirillum* (Philippe and Wu 2010). The protein localizes at the tip of MamK, towards the poles. MamK filaments sense the change in the magnetosome alignment in response to the magnetic field, which in turn transmits the signal to the MCP-like protein. This signals to the rest of the machinery equivalent to the chemotaxis pathway and results in directed movement of the bacterium. Cytoskeletal filaments in eukaryotic organisms also respond to a magnetic field, as observed in sockeye salmon and honey bee (Mann et al. 1988; Hsu et al. 2007). In these, torque linked to magnetic crystal clusters result in the opening of ion channels through signaling mediated by the cytoskeleton.

In the magnetosome islet outside the genomic island, another protein similar to MamK, called MamK-like has been found encoded in the bacterium, *Magnetospirallum magneticum*. Interplay between MamK-like and MamK in magnetosome positioning has been characterized (Abreu et al. 2014). It has been observed in vivo that both MamK and MamK-like can coexist in filaments in vivo. The presence of an alanine corresponding to the conserved catalytic glutamate of

actins raised a suspicion that MamK-like may not perform ATP hydrolysis and might result in more stable MamK filaments if they were capable of copolymerization. However, studies showed that MamK-like also possessed ATP hydrolysis activity, though slightly weaker than MamK. The presence of MamK and MamK-like monomers within the same filaments or interactions between MamK-like and MamK filaments might act as a novel means of regulation of MamK filament dynamics.

A molecular mechanism behind any of the interacting partners with MamK has currently not been characterized.

2.4 *Interactors of FtsA*

As a component of the divisome, FtsA, is a bacterial actin that connects the FtsZ filaments in the divisome ring with the cell membrane and the other divisome components in the membrane. Filaments of FtsA have been observed in vivo. The C-terminal tail of FtsZ binds to FtsA at the domain 2B (Fig. 5; Szwedziak et al. 2012). More than other interactors that affect FtsA dynamics, FtsA seems to be a major player in affecting FtsZ dynamics and curvature, thereby assisting ring constriction. The combined action of FtsA and FtsZ is an example of an interaction between two different cytoskeletal filaments, which contribute towards performing the cytoskeletal function of ring constriction. It is interesting to note here that the dynamics of FtsA may not play a role in this process, since there is no requirement of ATP hydrolysis and FtsA does not appear to hydrolyse ATP. The architecture of FtsA monomer with the swapped 1C domain might also imply that the regulatory region of the actin fold between domains 1 and 3 is not accessible to external factors.

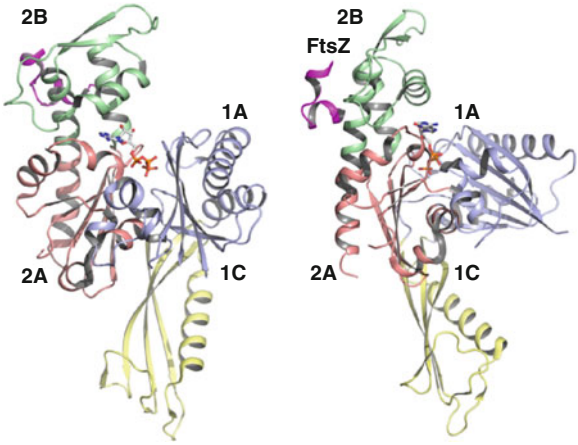


Fig. 5 Interaction between FtsA and FtsZ C-terminal tail. Two side views are shown to highlight the interaction of FtsZ with subdomain 2B. (PDB ID: 4A2A)

3 Comparison Between Interacting Proteins of Eukaryotic and Bacterial Actins

The existence of multiple types of actin filaments for multiple functions (structural diversity) of bacterial actins ensures that these are available for carrying out the various functions and hence the requirement for a large number of regulatory components is avoided in a bacterial system. The variability in a common scaffold (e.g., actin fold) results in the formation of a variety of filament structures ranging from single-stranded filaments to tubules.

An interesting feature of cytoskeletal filaments is the assembly of filaments into higher order structures such as cytoskeletal organization in muscle, spindle formation, formation of cilia, or flagella in eukaryotes. Such an organization is brought about by orientational arrangement of cytoskeletal filaments aided by molecular motors such as myosin, dynein, and kinesin (Theriot 2013). In bacteria, in the absence of molecular motors equivalent to myosin, kinesin, and dynein, a definite arrangement of cytoskeletal filaments has not been characterized yet. The discovery of a directional arrangement of ParM filaments to form a bipolar spindle demonstrates that such arrangements are possible without the presence of molecular motors, if the surface characteristics of the filaments allow for complementarity and the interaction energetics permits interfilament sliding (Gayathri et al. 2012).

Another function carried out by molecular motors in eukaryotes is transport of cargo. Analogous spatial positioning in bacteria appears to be effected by the dynamics of the filament itself. Hence, the interactors that modulate the filament dynamics assist in spatial positioning, as exemplified by the bacterial actin-like filaments in plasmid partitioning.

The study of the bacterial actin family demonstrates that diversification according to the functional necessity seems to be the path chosen for carrying out the various functions in bacteria. In contrast, in eukaryotic systems, the various functional requirements are executed by the regulatory components that interact with the actin cytoskeleton, while the cytoskeleton itself is highly conserved. Analogues of many cytoskeletal interactors and functions carried out by the eukaryotic cytoskeletal components appear to be present also in the bacterial cytoskeletal systems. However, the absence of sequence or structural similarity precludes the search of such equivalents by existing similarity-based computational search methods. Hence one of the approaches to identify eukaryotic analogues in prokaryotic systems is to perform searches based on functional similarity. This can be achieved through understanding the molecular mechanism of fundamental processes such as spatial positioning, chromosome segregation, cell division, etc., which are the functions carried out by the cytoskeleton in a typical eukaryotic scenario. This will help us to obtain profound insights on the evolution of the unity and diversity in cytoskeletal systems across bacteria, archaea, and eukaryotes.

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