ADP-Ribosylation and Cross-Linking of Actin by Bacterial Protein Toxins

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Contents

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B.M. Jockusch (ed.), *The Actin Cytoskeleton*,
Handbook of Experimental Pharmacology 235, DOI 10.1007/164_2016_26

Abstract

Actin and the actin cytoskeleton play fundamental roles in host–pathogen interactions. Proper function of the actin cytoskeleton is crucial for innate and acquired immune defense. Bacterial toxins attack the actin cytoskeleton by targeting regulators of actin. Moreover, actin is directly modified by various bacterial protein toxins and effectors, which cause ADP-ribosylation or cross-linking of actin. Modification of actin can result in inhibition or stimulation of actin polymerization. Toxins, acting directly on actin, are reviewed.

Keywords

Actin • Actin cross-linking • ADP-ribosylation • Formins • Host–pathogen interaction • Thymosin-β4 • Toxins

1 Introduction

Actin is an essential constituent of the cytoskeleton and one of the most abundant cellular proteins. The actin cytoskeleton has many crucial functions in host–pathogen interactions. This is especially true not only for immune cells but also for all types of cells targeted by pathogens (Higley and Way 1997; Dramsi and Cossart 1998; Haglund and Welch 2011). The actin cytoskeleton secures epithelium barrier functions and is involved in bacterial adhesion, invasion, endocytosis, and phagocytosis. Actin is crucial for all types of motility and migration and essential for signaling at the immunological synapse. Accordingly, numerous bacterial pathogens target and manipulate the actin cytoskeleton of host cells (Gruenheid and Finlay 2003; Barbieri et al. 2002; Aktories et al. 2011; Popoff 2000). This happens by protein toxins (exotoxins), which are released by bacteria in the environment and are able to enter target cells in the absence of the producing pathogen and by the so-called bacterial effectors, which act on target cells after delivery by injection machines, which depend on the direct contact of the pathogen with host cells. Evolution of host–pathogen interaction allowed the development of different strategies to attack the actin cytoskeleton (Aktories et al. 2011).

1.1 Regulators of the Actin Cytoskeleton Are Targeted by Bacterial Toxins

Many bacterial toxins or effectors[1] act on proteins that are regulators of the actin cytoskeleton or they mimic these regulators and hijack their functions (Barbieri et al. 2002; Aktories and Barbieri 2005; Popoff 2014). The GTP-binding Rho proteins, which act as molecular switches, are master regulators of the actin cytoskeleton and are preferred targets of toxins (Aktories 2011; Popoff 2014;

[1]®In the following both types of bacterial virulence factors will be assigned as “toxins.”

Lemichez and Aktories 2013). Rho proteins, which favor actin polymerization via different mechanisms, including activation of actin nucleators like formins or WH2-domain containing proteins, are inactivated or activated by bacterial toxins. Inactivation occurs by ADP-ribosylation (Aktories et al. 1989, 2004; Paterson et al. 1990), AMPylation (Yarbrough et al. 2009), glycosylation (Just et al. 1995; Jank et al. 2013, 2015), or proteolytic cleavage (Shao et al. 2002). Rho proteins are activated by ADP-ribosylation (Lang et al. 2010) and deamidation (Schmidt et al. 1997; Flatau et al. 1997). Moreover, bacterial toxins manipulate the Rho-dependent control of the actin cytoskeleton by mimicking regulators of Rho proteins. Numerous bacterial toxins have GEF (guanine nucleotide exchange factors)-like function and activate Rho proteins, eventually leading to enhanced polymerization of actin. This group of bacterial toxins includes, for example, *Salmonella enterica* SOP proteins (Hardt et al. 1998), *Burkholderia pseudomallei* BopE (Upadhyay et al. 2008), *Shigella flexneri* IpgB proteins (Klink et al. 2010; Ohya et al. 2005), *E. coli* Map protein (Huang et al. 2009), and others. On the other hand, bacterial toxins switch off the active state of Rho proteins by mimicking GTPase-activating proteins (GAPs), resulting in inhibition of polymerization of G-actin and/or depolymerization of F-actin. These GAP mimics include, for example, *Yersinia* ssp. YopE (von Pawel-Rammingen et al. 2000), *Pseudomonas aeruginosa* ExoS (Goehring et al. 1999), Exo T (Krall et al. 2000), and *S. Typhimurium* SptP (Fu and Galan 1999).

Other bacterial toxins (effectors) interfere with actin polymerization not via Rho proteins but by using actin nucleators like *Listeria monocytogene*s protein ActA (Pistor et al. 1994), which activate Arp2/3, or *Shigella flexneri* surface protein IcsA (Suzuki et al. 1998), which also activate Arp2/3, however, via N-WASP. Still another group of bacterial toxins directly hijacks actin nucleator functions like *Vibrio cholerae* and *Vibrio parahaemolyticus* proteins VopF (Pernier et al. 2013) and VopL (Tam et al. 2007), respectively, or *Rickettsia* ssp. protein Sca2 (Haglund et al. 2010) and others.

However, actin is also directly targeted by bacterial toxins. These toxins modify the actin molecule by covalent modification, including ADP-ribosylation and protein cross-linking (Satchell 2009). In the following, these toxins will be described in more detail.

2 Inhibition of Polymerization by Toxin-Catalyzed ADP-Ribosylation of Actin

ADP-ribosylation is a very common mechanism by which bacterial toxins modify eukaryotic targets. Well-known substrates are heterotrimeric G proteins (e.g., cholera toxin and pertussis toxin), elongation factor 2 (e.g., diphtheria toxin), small GTPases like Rho proteins (*Clostridium botulinum* C3 toxin), and actin. In all these cases the toxins split NAD^+ and transfer the ADP-ribose moiety onto the protein target and release nicotinamide. Two major groups of actin-ADP-ribosylating toxins, which differ in their target amino acid of actin have been

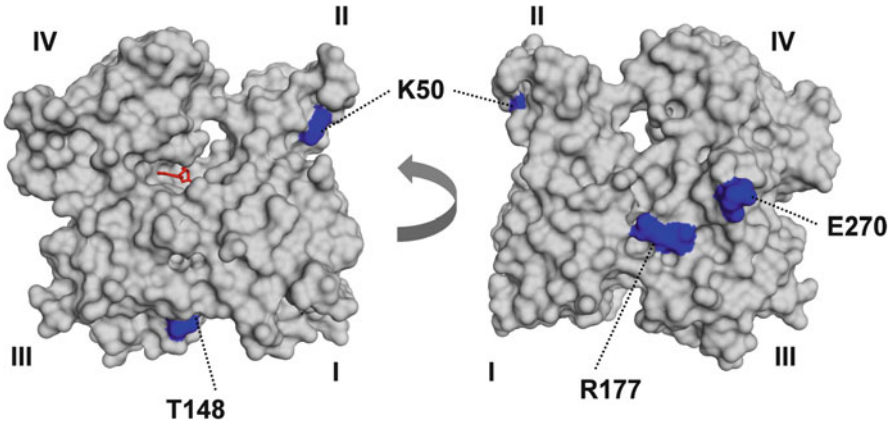

Fig. 1 Space-filling model of actin (PDB 1ATN). The four subdomains of actin are indicated (I-IV). Amino acids, which are modified by bacterial protein toxins, are marked in blue. Whereas arginine177 (R177) is ADP-ribosylated by several toxins like C2 toxin from *Clostridium botulinum* to prevent polymerization and to induce depolymerization of actin, threonine148 (T148) is exclusively ADP-ribosylated by *Photorhabdus luminescens* toxin (TccC3), which causes polymerization of actin. Various other toxins like VgrG1 of *Vibrio cholera* catalyze actin cross-linking between lysine50 (K50) and glutamate270 (E270). For details, see text

described. One group of the ADP-ribosylating toxins modifies actin at arginine177 and the second group ADP-ribosylates actin at threonine148 (Fig. 1). The functional consequence is exactly the opposite. While toxin-induced ADP-ribosylation of arginine177 of actin inhibits actin polymerization, modification of threonine148 increases polymerization. Prototypes of the toxins, which modify actin in arginine177, are the binary actin-ADP-ribosylating toxins.

2.1 Binary Actin-ADP-Ribosylating Toxins

Members of the family of binary actin-ADP-ribosylating toxins are *Clostridium botulinum* C2 toxin, *C. perfringens* iota toxin, *C. spiroforme* toxin (ST), *C. difficile* transferase CDT toxin, and *Bacillus cereus* vegetative insecticidal proteins (Aktories et al. 1986b; Barth et al. 2004; Stiles et al. 2014). All these toxins are comprised of the components A and B. The B component is involved in receptor-binding and toxin uptake, while the A component possesses the ADP-ribosyltransferase activity (Fig. 2). Both components are separately released from the bacteria. Excellent groundbreaking studies were first performed with *C. botulinum* C2 toxin (Ohishi et al. 1980, 1981; Ohishi 1983; Ohishi and DasGupta 1987).

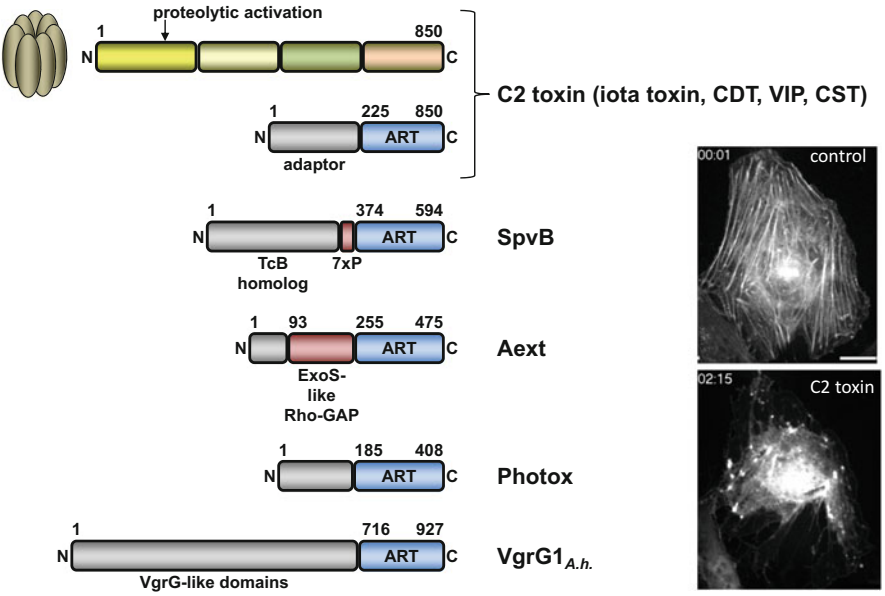

Fig. 2 Scheme of the structures of actin-ADP-ribosylating toxins/effectors, which all modify actin at arginine177. The family of binary toxins consists of *C. botulinum* C2 toxin, *C. perfringens* iota toxin, *C. difficile* transferase (CDT), *Bacillus cereus* vegetative insecticidal toxin (VIP), and *C. spiroforme* toxin (CST). These toxins are binary in structure, because they consist of a four-domain binding/translocation component and the separated enzyme component. After proteolytic activation, the binding/translocation component forms heptamers. The enzymatic component consists of a C-terminal ADP-ribosyltransferase (ART) domain and an N-terminal adaptor domain, which interacts with the binding/translocation component. Numbers given are from *C. botulinum* C2 toxin. The other toxins/effectors are not binary in structure but all possess a C-terminal actin-ADP-ribosylating domain (ART). These toxins are translocated into host cells by type-III secretion systems (SpvB, AexT) or by unknown mechanisms. The effector SpvB from *Salmonella enterica* possesses a C-terminal actin-ADP-ribosylating domain. AexT is produced by *Aeromonas salmonicida* and possesses in addition to the actin-ADP-ribosyltransferase domain (ART), a domain with Rho GTPase-activating activity (GAP), which is related to *Pseudomonas* ExoS protein. Photox is an effector, which is produced by *P. luminescens*. VgrG1 from *Aeromonas hydrophila* possesses an actin-ADP-ribosyltransferase domain at its C-terminus. The right panel shows the effect of the binary *Clostridium botulinum* C2 toxin on HeLa cells after 2.15 h. Actin-staining is shown (bar 10 μm)

2.2 Binding Components of Binary Toxins

The binding and translocation components of all these toxins have molecular masses of 80 to 100 kDa, and share sequence identity between 36 and 85%. Notably, they all share sequence similarity with the binding component of anthrax toxin, the protective antigen PA (Barth et al. 2004; Young and Collier 2007; Stiles et al. 2011). All components (including PA) are produced as precursor proteins and are activated by proteolytic cleavage at the N-terminus (Ohishi 1987). Thereby, an ~20 kDa peptide is released, a process that initiates oligomerization of the rest of

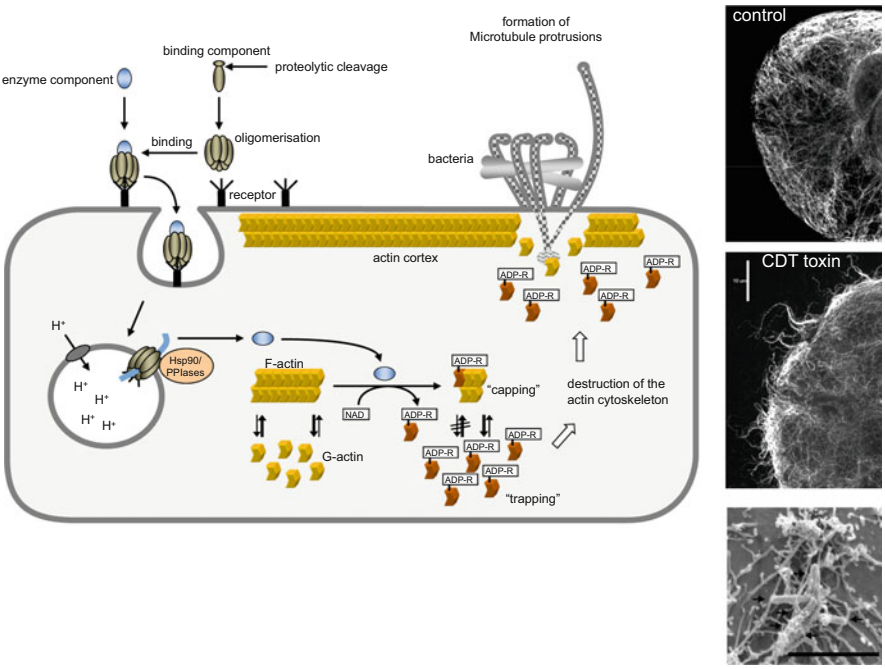

Fig. 3 Effects of binary actin-ADP-ribosylating toxins. Binary actin-ADP-ribosylating toxins consist of a binding component and an enzyme component, which are separate proteins. The binding component is proteolytically activated and forms heptamers, which bind to the cell surface receptor of the toxins. After binding of the enzyme component, the toxin complex is endocytosed. At low pH of endosomes the heptamer inserts into the endosomal membrane and forms a pore. Through the pore the enzyme component is translocated into the cytosol, where G-actin is ADP-ribosylated at arginine177. Thereby actin polymerization is blocked. ADP-ribosylated actin acts like a barbed-end capping protein and prevents further F-actin elongation. Partial destruction of cortical actin induces formation of microtubule based cell protrusions. The cell protrusions are involved in increased adherence of bacteria. Right panel (upper and middle pictures). Effects of the binary *Clostridium difficile* toxin CDT (CDTa 20 ng/ml, CDTb 40 ng/ml) on human colon adenocarcinoma Caco-2 cells. CDT causes formation of microtubule-based cell protrusions. Tubulin is stained (bar 10 μm). Right panel (lower picture). Scanning electron microscopy of Caco-2 cells. Cells were treated as above. After 1 h, *Clostridium difficile* bacteria were added. After 90 min, cells were washed and fixed. Bacteria were caught in toxin-induced protrusions

the protein to form heptamers (Barth et al. 2000). According to PA, they consist of four functional domains (Young and Collier 2007; Schleberger et al. 2006). The N-terminal domain I, which is proteolytically cleaved, is involved in activation and in initial binding of component A. While domain II of the binding domain is responsible for membrane insertion, domain III is essential for oligomerization of the binding component. Finally, domain IV is the receptor-binding domain. Domains I-III exhibit highest sequence similarity among the toxins. Whereas the receptor-binding domains of C2 toxin (C2II) and PA are completely unrelated

Table 1 Typical R, STS, and ExE motifs of bacterial actin-ADP-ribosyltransferases

Toxin	Bacterium	R	STS	ExE
SpvB	*Salmonella enterica*	RVVY**R**GLK^{474}	FM**STS**PD^{505}	FKG**E**A**E**ML^{540}
iota a	*Clostridium perfringens*	LIVY**R**RSG^{339}	FI**STS**IG^{383}	YAG**E**Y**E**VL^{423}
VgrG1	*Aeromonas hydrophila*	DFVY**R**GLA^{812}	FM**STS**PD^{843}	FKG**E**A**E**ML^{878}
Photox	*Photorhabdus luminescens*	KKVY**R**GLK^{291}	FL**STS**PD^{322}	FKG**E**A**E**ML^{357}
VIP2	*Bacillus cereus*	ITVY**R**WCG^{338}	YI**STS**LS^{376}	FAN**E**K**E**IL^{416}
AexT	*Aeromonas salmonicida*	QHLN**R**SLR^{306}	YL**STS**RD^{368}	EGD**E**Q**E**IL^{405}
C2I	*Clostridium botulinum*	LIAY**R**RVD^{302}	FS**STS**LK^{352}	FQD**E**Q**E**IL^{391}

Sequence alignment of highly conserved residues of bacterial ADP-ribosyltransferases. The NAD-interacting arginine residue (R), the first serine residues from the STS motif (S), and the catalytic glutamate (second E) from the ExE motif, which form the RSE-motif of ADP-ribosyltransferases are highlighted (Fieldhouse and Merrill 2008). Alignments were made with the following sequences: SpvB (Acc. No. D0ZHS9), iota a (Acc. No. F7J0A4), VgrG1 (Acc. No. A0KHA9), Photox (Acc. No. Q7N8B1), VIP2 (Acc. No. G8FSA8), AexT (Acc. No. Q93Q17), and C2I toxin (Acc. No. D4N871)

between all toxins, the binding domains of iota toxin (Ib), *C. spiroforme* toxin (STb) and CDTb, are highly similar and share the same membrane receptor (see below). These latter three toxins are combined in the iota toxin-family, because the binding components of the toxins are interchangeable and they are able to transport the A components of each other toxin of this family (Stiles and Wilkens 1986; Stiles and Wilkins 1986; Simpson et al. 1989).

The A components of the toxins have molecular masses of ~44–52 kDa and possess ADP-ribosyltransferase activity (Figs. 2 and 3). All toxins ADP-ribosylate actin at arginine177 (Vandekerckhove et al. 1987, 1988). The crystal structures of the enzyme components of *B. cereus* VIP toxin (VIP2), C2 toxin (C2I), and iota toxin (Ia) have been determined. Because iota toxin was crystallized in complex with actin, we now know a lot about the actin–toxin interaction and the ADP–ribosylation reaction. The toxins share the typical folding of ADP-ribosyltransferases. Interestingly, each toxin component consists of two domains with typical ADP-ribosyltransferase folding, which most likely developed by gene duplication (Han et al. 1999). The N-terminal ADP-ribosyltransferase domain is inactive and involved in interaction with the binding domain, while the C-terminal ADP-ribosyltransferase domain harbors the enzyme activity. This domain possesses the highly conserved RSE-motif of ADP-ribosyltransferase, which is involved in NAD^+ binding and catalysis (Table 1).

2.3 Toxin Binding and Uptake

Figure 3 shows a scheme of the binding and uptake of actin-ADP-ribosylating binary toxins. After tryptic activation (e.g., in the gut) the toxin forms heptamers and interacts with cell surface receptors. It appears that also the monomer can bind to receptors. While the functionally important part of the receptor for C2 toxin

seems to be a carbohydrate structure (Eckhardt et al. 2000), the membrane receptor for CDT, iota toxin, and ST has been identified as the lipolysis stimulated lipoprotein receptor (LSR) (Papatheodorou et al. 2011, 2012). This receptor was initially suggested to be involved in lipid metabolism (Yen et al. 1994), later it turned out that it is essential for triangular tight junctions (Masuda et al. 2011).

After receptor-binding, the toxins are endocytosed (Nagahama et al. 2002, 2004), a process that probably involves interaction with lipid rafts (Nagahama et al. 2004; Papatheodorou et al. 2013). The low pH of endosomes induces conformational changes of the binding component and facilitates the membrane insertion of the beta-barrel-like structure of the binding component (Barth et al. 2000) (Stiles et al. 2002; Nagahama et al. 2002; Blöcker et al. 2001). Thereby, pores are formed, which allow transport of the enzyme components into the cytosol (Schmid et al. 1994; Knapp et al. 2002). Because the diameter of the pores is small, partial unfolding of the enzyme component is necessary. Translocation of the enzyme component is facilitated by cytosolic proteins including chaperons like heat shock protein90 (Haug et al. 2003, 2004), and protein-folding helpers like peptidyl-prolyl cis/trans isomerases cyclophilin A, cyclophilin 40 and FK506 binding protein (FKBP) (Kaiser et al. 2011, 2012; Dmochewitz et al. 2011).

2.4 Toxin-Induced ADP-Ribosylation of Actin in Arginine177

After translocation of the enzyme component into the cytosol, the toxin ADP-ribosylates monomeric G-actin at arginine177 (Vandekerckhove et al. 1987, 1988) (Fig. 1). Polymerized F-actin is a very poor substrate (Aktories et al. 1986a, 1987; Schering et al. 1988), and phalloidin, a fungal toxin that binds to F-actin, blocks ADP-ribosylation of actin (Aktories et al. 1986a). This is reasoned in the localization of arginine177 in F-actin. Arginine177 is located near the axes between the long two-start helices. The voluminous ADP-ribose moiety cannot be accommodated between the strands (Holmes et al. 1990; Margarit et al. 2006) (Fig. 4). Therefore, F-actin has to be depolymerized first to be subsequently modified by the toxin. ADP-ribosylation of G-actin at arginine177 causes only minor structural changes (Margarit et al. 2006). Nevertheless, the attachment of ADP-ribose at this position has functional consequences for G-actin. For example, the ATPase activity of actin is blocked. This is observed not only for basal ATPase activity (Geipel et al. 1989; Margarit et al. 2006) but also for cytochalasin-stimulated ATPase activity (Geipel et al. 1990). Moreover, the exchange of ATP bound to actin is enhanced (Geipel et al. 1989). Because the nucleotide is important for actin stabilization, ADP-ribosylation results in a decrease in thermal stability (Perieteanu et al. 2010). Although arginine177 is conserved in all actin isoforms, the toxins differ in their substrate specificity. While C2 toxin exhibits high preference for β/γ-non-muscle actin, the other toxins (so far studied) accept non-muscle as well as muscle actin as protein substrates (Mauss et al. 1990; Schering et al. 1988).

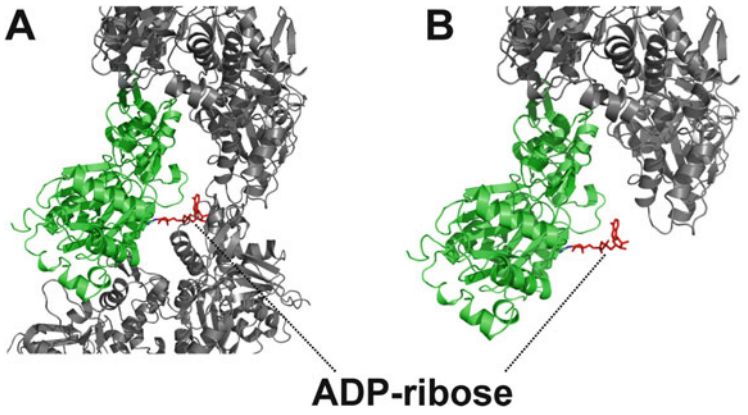

Fig. 4 Effect of ADP-ribosylation of arginine177 on actin–actin interaction. (**a**) Ribbon presentation (PDB 1ATN) of ADP-ribosylated actin (*green*) within the F-actin filament (*grey*); ADP-ribose is marked in *red*. The steric hindrance induced by ADP-ribosylation of arginine177 is shown. (**b**) Binding of ADP-ribosylated actin to the plus end of F-actin. Figure is from Aktories et al. (2011)

2.5 The Physiological Role of Arginine177

Arginine177 is highly conserved in various actin isoforms from different species including yeast actin. It is located in the nucleotide-binding cleft of actin and of major importance for actin functions. Karlsson and coworkers changed arginine177 to aspartate in chicken β-actin (Schüler et al. 2000). They found a 10-fold increase in critical concentration, lowered thermostability, and an increased nucleotide exchange rate. Arginine177 is the pivotal residue in the *cardiofunk* actin mutation in zebrafish. Here, the mutation of arginine177 to histidine causes lack of endocardial cushion formation, defects in cardiac morphogenesis and embryonic lethality (Bartman et al. 2004). The *cardiofunk* mutation (R177H) was biochemically studied in the yeast actin model showing an increase in the critical concentration of actin polymerization, a prolonged nucleation phase and a rapid elongation process of actin polymerization, indicating that this mutation causes increased fragmentation of actin filaments (Wen and Rubenstein 2003).

2.6 Functional Consequences of ADP-Ribosylation of Arginine177

Most important for the functions of actin is its property to reversibly polymerize from G-actin to F-actin and its interaction with actin-binding proteins. What are the functional consequences of the ADP-ribosylation of actin at arginine177 concerning these properties? Early studies showed that C2 toxin and iota toxin inhibit actin polymerization (Aktories et al. 1986b; Schering et al. 1988). As stated above this is explained by steric hindrance of ADP-ribosylated actin to form F-actin

(Holmes et al. 1990) (Fig. 4). However, ADP-ribosylated actin can bind to barbed ends of F-actin filaments (Fig. 4). Here, it acts like a capping protein and blocks F-actin elongation by inhibiting attachment of non-ADP-ribosylated actin (Wegner and Aktories 1988; Weigt et al. 1989; Aktories and Wegner 1989; Perieteanu et al. 2010). By contrast, ADP-ribosylated actin does not interact with pointed ends of actin filaments (Wegner and Aktories 1988; Perieteanu et al. 2010). Thus, depolymerization of F-actin, which preferentially occurs at the pointed ends is still possible. On the other hand, pointed end polymerization can occur, when the concentration of non-modified actin is higher than the critical actin concentration for pointed end polymerization (Wegner and Aktories 1988; Perieteanu et al. 2010). Under in vitro conditions, ADP-ribosylation of actin is reversible in the presence of excessive amounts of nicotinamide (Just et al. 1990; Perieteanu et al. 2010).

The interaction of actin with various actin-binding proteins is crucial for its functions. Toxin-induced ADP-ribosylation affects the interaction of actin with actin-binding proteins. From >100 actin-binding proteins known, only the interaction of gelsolin with ADP-ribosylated actin was studied in some detail. Gelsolin consists of 6 homologous domains (G1-G6), only three of which (G1,G2 and G4) interact with actin resulting in multiple effects. Depending on the Ca^{2+} concentration, gelsolin causes actin sequestering, capping, severing, and nucleation. Actin in complex with gelsolin is modified by toxin-induced ADP-ribosylation in vitro (Wille et al. 1992) and in intact cells (Just et al. 1993). At least four different types of actin-gelsolin complexes (G-Ar, G-Ar-A, G-A-Ar, and G-Ar-Ar) were identified (Wegner et al. 1994; Wille et al. 1992). In vitro studies showed that the G-Ar and G-Ar-A complexes are able to nucleate actin polymerization, while this was not the case for the G-A-Ar and G-Ar-Ar complexes. These data are in line with the finding that ADP-ribosylated actin binds to the barbed ends of actin filaments (see Fig. 4). Moreover, actin dimers and trimers are also ADP-ribosylated by the toxins. As expected, modified oligomers are still able to interact with DNaseI and gelsolin (Perieteanu et al. 2010).

2.7 ADP-Ribosylation of Actin as Tool to Investigate Physiological Actin Functions

Inhibition of actin polymerization by toxin-induced ADP-ribosylation of arginine177 has typical cytotoxic effects in cell culture (Wiegers et al. 1991). The actin cytoskeleton depolymerizes with rounding up of cells (Fig. 2) and, eventually, loss of cell adherence occurs followed by apoptosis (Heine et al. 2008). In numerous previous studies, toxin-induced depolymerization of actin was used to analyze the role of the actin cytoskeleton on various cellular functions. It was shown that toxin-induced depolymerization of the actin cytoskeleton inhibits migration of chemoattractant-evoked human neutrophils but enhances superoxide anion production and secretion of lipid mediators and inflammatory factors (Norgauer et al. 1988, 1989; Al-Mohanna et al. 1987; Grimminger et al. 1991a, b). Effects of actin-ADP-depolymerizing toxins on degranulation of mast cells (Prepens

et al. 1997, 1998; Wex et al. 1997), release of neurotransmitters from PC12 cells (Matter et al. 1989), insulin release from endocrine cells (Li et al. 1994) and on endothelial cell functions (Ermert et al. 1995, 1997; Schnittler et al. 2001; Suttorp et al. 1991) have been reported. Most of these studies were performed with rather high toxin concentrations, which clearly affected the F-actin and the actin cytoskeleton. However, not only the actin cytoskeleton but also the microtubule system is affected by binary actin-depolymerizing toxins.

2.8 Actin-Depolymerizing Toxins Induce Microtubule-Based Protrusions

At rather low concentrations, the binary actin-ADP-ribosylating toxins induce formation of cell membrane protrusions that form a network of up to 150 μm long tentacle-like structures on the surface of epithelial cells (Schwan et al. 2009) (Fig. 3). These protrusions contain microtubules. Mainly 1–2 microtubule filaments but no actin filaments are detected in the protrusions. The plus ends of the microtubules are at the distal ends of protrusions and typically decorated with EB1-3 proteins. The microtubule-based structures are highly dynamic, grow and retract quite rapidly at the beginning of the intoxication process (Schwan et al. 2009). Later (>4 h) they are more stable. Precise mechanisms of the development of the protrusions are still enigmatic. However, it has been suggested that capture proteins like ACF7 and Clasp2, which are usually involved in stabilization of growing microtubules at the actin cell cortex, are mislocalized into the cytosol after toxin treatment, thereby losing the ability to capture growing microtubules (Schwan et al. 2009). Changes in structure of microtubules induced by binary actin-depolymerizing toxins have been also reported for leukemia cells (Uematsu et al. 2007) but in this case, protrusion formation was not prominent. Role and functions of the protrusions, which form after partial depolymerization of F-actin, are not clear. Since toxins play a crucial role in host–pathogen interaction, it was a surprising but plausible finding that the toxin-induced protrusions are involved in bacterial adhesion (Fig. 3). The protrusions form a network on the cell surface where *C. difficile* bacteria are caught.

Furthermore, it is suggested that the protrusions are involved in signaling between host cells and pathogens. Electron microscopic studies of human adenocarcinoma Caco-2 cells revealed that the toxin-induced microtubule-based protrusions contain vesicles showing antero- and -retrograde trafficking (Schwan et al. 2014). Moreover, tubes of endoplasmic reticulum (ER) are in the protrusions. The ER membranes are connected to the distal tips of microtubules via the ER-membrane protein Stim1, which is a well-known regulatory protein of the calcium channel protein Orai (Fahrner et al. 2013). Notably, in the protrusions, Stim1 couples to Orai proteins, which function as channels in store-operated calcium entry (SOCE) (Taylor 2006). Thus, vesicle movements, Stim1–Orai interaction, and toxin-induced calcium signaling suggest that the protrusions are capable of specific signaling in a cilia-like fashion.

Another aspect of toxin-induced actin depolymerization is related to actin-integrin interaction, which plays a pivotal role in cell migration and interaction with the extracellular matrix (ECM). ECM proteins (e.g., fibronectin) interact with integrin receptors at the basolateral side of epithelial cells. After partial cleavage by proteases, ECM proteins are taken up via their integrin receptors and reach Rab5- and Rab11-associated vesicles (Pellinen and Ivaska 2006). Usually the vesicles recycle with the integrins in an actin-dependent manner to the basolateral membrane (Sheff et al. 2002; Durrbach et al. 2000; Powelka et al. 2004). These cycling processes are suggested to be essential for cell movement and motility. However, toxin-induced partial depolymerization of actin disturbs re-cycling of vesicles (Schwan et al. 2014). Moreover, using microtubules as tracks, the vesicles are re-routed to the apical membrane where the microtubules form protrusions. Here, fibronectin and other ECM proteins are released and function as receptor for bacterial surface proteins, thereby bacterial adhesion is increased (Schwan et al. 2014).

2.9 ADP-Ribosylation of Actin by Single Chain Toxins

Various bacterial single chain protein toxins and effectors, which do not belong to the family of binary toxins, modify actin also at arginine177 (Fig. 2). These toxins include *Salmonella Typhimurium* effector SpvB, *Aeromonas salmonicida* and hydrophila toxin AexT, *Photorhabdus luminescens* toxin Photox and *Aeromonas hydrophila* effector VgrG1. These 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. The uptake mechanism of Photox is not clear so far but it may also use type III secretion. By sequence comparison SpvB, Photox, and VgrG1 share maximal sequence similarity. AexT appears to be more distantly related. As shown in Table 1, the typical features of ADP-ribosyltransferases like the RSE motif are highly conserved.

2.10 *Salmonella typhimurium* Effector SpvB

SpvB is an ~65 kDa virulence factor of *Salmonella typhimurium* (Otto et al. 2000; Tezcan-Merdol et al. 2001; Lesnick et al. 2001). It is a two-domain protein (Fig. 2). The N-terminus part exhibits sequence similarity with parts of the linker (TcB) of the tripartite *Photorhabdus luminescens* Tc toxin (see below). The C-terminus harbors the ADP-ribosyltransferase activity that modifies actin. After invasion of host cells and formation of the so-called Salmonella containing vacuole, where the pathogens proliferate, SpvB is microinjected by Salmonella into the cytosol of host cells. SpvB ADP-ribosylates actin at arginine177 (Hochmann et al. 2006). This is responsible for cytotoxicity and apoptosis of macrophages and crucial for *Salmonella* proliferation and infection.

2.11 Photorhabdus Luminescens Photox Toxin

Photox is an ~46 kDa two-domain protein produced by *P. luminescens*, which shares 39% sequence identity overall with SpvB (Visschedyk et al. 2010) (Fig. 2). However, the C-terminal 200 amino acids exhibit 61% sequence identity with the ADP-ribosyltransferase domain of SpvB. While binary actin-targeting ADP-ribosyltransferases possess significant NAD-glycohydrolase activity and are able to split NAD^+ in the absence of their substrate actin, Photox like SpvB does not possess NAD-glycohydrolase activity, again showing the high similarity of the ADP-ribosyltransferase domain of Photox with the Salmonella effector SpvB. However, the N-terminus of Photox is largely different to SpvB. It consists of a domain that is also found by other type-III secretion effectors and might be involved in chaperone and membrane interaction. As SpvB, Photox ADP-ribosylates skeletal and non-muscle actin at arginine177. This was confirmed by mass spectrometry, also showing that arginine177 is the only modification site (Visschedyk et al. 2010). A detailed analysis of cross-linked actin-ADP-ribosylated by Photox has been performed showing no significant differences to actin-ADP-ribosylated by binary toxins with respect to actin polymerization and interaction with actin binding proteins like gelsolin (Perieteanu et al. 2010).

2.12 AexT, an ADP-Ribosylating Toxin with GAP Activity

AexT (~50 kDa) is a type III secretion effector, which is produced by *Aeromonas salmonicida* (Fehr et al. 2007) and *A. hydrophila* (Vilches et al. 2008). Also *A. salmonicida* AexT is a two-domain protein (Fig. 2). The C-terminus has ADP-ribosyltransferase activity and modifies actin at arginine177 (Fehr et al. 2007). In addition, AexT contains an N-terminal Rho-GAP domain, which possesses high enzyme activity and efficiently stimulates the GTP-hydrolysis of Rho proteins thereby it inactivates these master regulators of actin. Accordingly, the cytopathic effects of AexT, which is characterized by destruction of the cytoskeleton and rounding up of cells, depend on both direct modification of actin and inactivation of Rho proteins by its GAP activity (Fehr et al. 2007).

2.13 *Aeromonas hydrophila* VgrG1

Aeromonas hydrophila effector VgrG1 proteins possess an ADP-ribosyltransferase domain at the C-terminus of the 102 kDa protein, which exhibits high sequence similarity with SpvB and Photox (Suarez et al. 2010) (Fig. 2). VgrG (Valine-glycine repeat G) proteins are components of the bacterial type VI secretion system, which forms a syringe-like structure and is involved in cell puncturing and effector delivery into target cells (Pukatzki et al. 2009) (see below). Some of these proteins have not only puncture functions but possess also domains with effector functions. In case of *A. hydrophila* VgrG1, this domain ADP-ribosylates actin (Suarez

et al. 2010). So far, the site of actin modification has not been determined. However, VgrG1 of *A. hydrophila* causes redistribution of the actin cytoskeleton with rounding-up of cells and increase of G-actin and depolymerization of F-actin, suggesting a similar action on actin as SpvB, Photox, and binary toxins.

3 Actin Polymerization by *P. luminescens* Toxins

Bacterial protein toxins, which covalently modify actin, induce not only depolymerization of actin but also polymerization. This is true for TccC3, the active component of the tripartite Tc toxins of *P. luminescens*. *P. luminescens* are motile Gram-negative enterobacteria, which possess insecticidal activity. They live symbiotically in the intestine of entomopathogenic nematodes of the family *Heterorhabditidae* (Forst et al. 1997; Joyce et al. 2006; Waterfield et al. 2009). The nematodes invade insect larvae, where the bacteria are released by the worms by regurgitation to produce toxins, which kill the insects (Waterfield et al. 2001a, b). Thereby, an enormous source of nutrients is generated for proliferation of nematodes and bacteria. Eventually, the nematodes take up bacteria again to find and invade new insect preys.

Among a large array of insecticidal toxins produced by *P. luminescens* (see also Photox), the tripartite Tc (toxin complex) toxins have been shown to target the actin cytoskeleton (Fig. 5). Tc toxins consist of 3 components TcA, TcB, and TcC (ffrench-Constant and Waterfield 2006) and form a large complex of >1.5 MDa. TcA is the binding component, which interacts with the cell membrane of target cells (e.g., enterocytes of insect larvae) and initiates toxin uptake (Fig. 5). TcC possesses catalytic activity and TcB acts as a structural linker between TcA and TcC. Several homologs of Tc toxins exist not only in *Photorhabdus* bacteria but also in other species like *Yersinia* (Waterfield et al. 2007). Recently, the crystal structure of the TcA, TcB, and TcC complex has been obtained, exhibiting a novel type of a bacterial injection machine for translocating the toxin into target cells (Gatsogiannis et al. 2013; Meusch et al. 2014). According to these data, TcA forms pentamers of >1.5 MDa, which combine with TcB to form a cage or cocoon-like structure of TcB and TcC. The Tc component, which consists of a highly conserved N-terminal region and a C-terminal variable part, participates in cage formation with its N-terminal region, while the variable region is suggested to be inside the cage in an unfolded conformation. By gross conformational changes of the syringe-like translocation machine, the variable part of TcC, which is probably cleaved by an inbuilt protease activity, is injected into host cells. Two of the various TcC isoforms known, namely TccC3 and TccC5 possess ADP-ribosyltransferase activities. TccC5 affects the actin cytoskeleton via Rho proteins. It ADP-ribosylates Rho proteins at glutamine61/63. Thereby, Rho proteins are persistently activated and induce strong formation of stress fibers and lamellipodia.

The TcC isoform TccC3 is an ADP-ribosyltransferase that directly induces polymerization of actin. The toxin ADP-ribosylates actin in threonine148 (Figs. 1 and 6). This residue is part of the binding site of thymosin-β4. Thymosins are

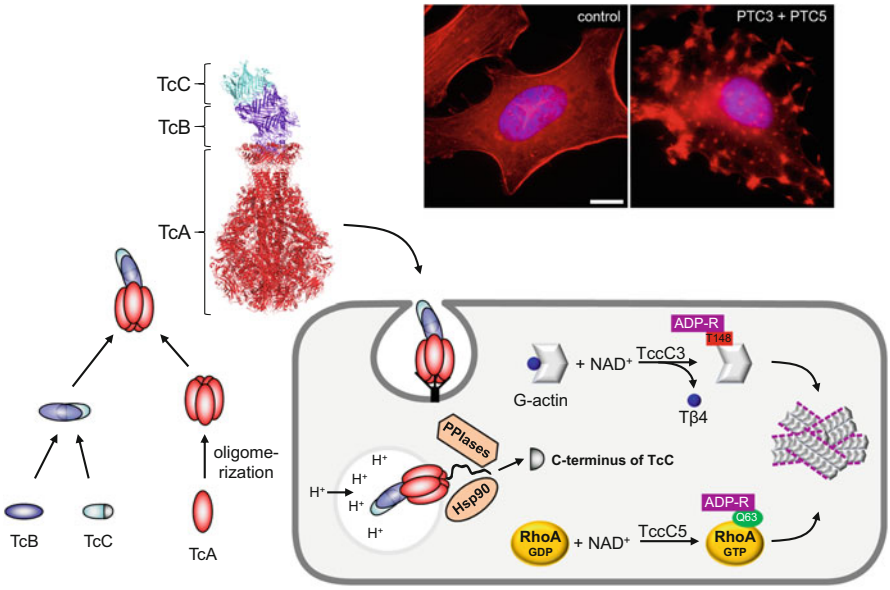

Fig. 5 Model of the structure and uptake of *Photorhabdus* toxin complexes. *P. luminescens* toxin complexes consist of three different types of proteins called TcA, TcB, and TcC. TcA forms pentamers and is the receptor-binding component, which interacts with the cell membrane of target cells and initiates uptake of the biologically active component TcC. TcB and the N-terminal region of TcC form together a large cocoon-like structure while the C-terminal part of TcC is suggested to be inside the cage in an unfolded conformation. This C-terminal domain of TcC is cleaved in an autoproteolytic process and enters the translocation channel of TcA after holotoxin formation. After receptor-mediated endocytosis and endosomal acidification, the translocation channel of TcA penetrates endosomal membranes and the C-terminal part of TcC is injected into the cytosol of host cells. The TcC isoforms TccC3 and TccC5 possess ADP-ribosyltransferase activities. TccC5 affects the actin cytoskeleton via Rho proteins. It ADP-ribosylates Rho proteins at glutamine61/63. Thereby, Rho proteins are persistently activated and induce strong formation of stress fibers and lamellipodia. TccC3 ADP-ribosylates actin at threonine148, thereby preventing the binding of the actin sequestering protein thymosin-β4 to actin and favoring actin polymerization. Together, TccC3 and TccC5 cause clustering of F-actin. The upper panel shows intoxication of HeLa cells with *Photorhabdus luminescens* ADP-ribosylating toxins PTC3 (TcA + TcB-TccC3) and PTC5 (TcA + TcB-TccC5). Cells were fixed and stained with TRITC-conjugated phalloidin and DAPI (scale bar, 10 μm). Crystal structure of TcA is from PDB 4O9Y and of TcB-TccC3 is from PDB 4O9X

~5 kDa peptides of 42–45 amino acids (Mannherz and Hannappel 2009). They bind to G-actin in an extended conformation, which sequesters actin in its monomeric form. Therefore, thymosin-β4 can inhibit salt-induced actin polymerization. ADP-ribosylation by TccC3 inhibits thymosin-β4-actin interaction, and this might increase G-actin availability for polymerization. Notably, in contrast to actin-ADP-ribosylation at arginine177, toxin-induced attachment of ADP-ribose to actin at threonine148 also occurs with polymerized actin (Lang et al. 2010). This is in line with the localization of threonine148 in F-actin (Fig. 6).

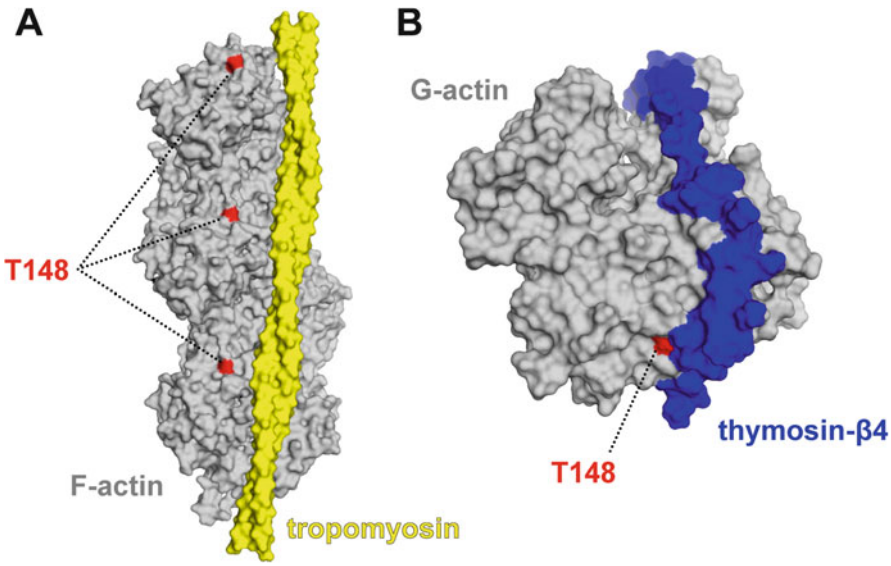

Fig. 6 Localization of threonine148 (T148) in actin. (**a**) Space-filling model of F-actin (*grey*) in complex with tropomyosin (*yellow*). Data are from PDB 3J8A. In F-actin, T148 (*red*) is accessible for modification by *Photorhabdus luminescens* toxins TccC3. (**b**) Interaction of thymosin-β4 with actin in a space-filling model. The ~5 kDa thymosin-β4 (*blue*) interacts with actin (*grey*) in an extended conformation partially covering residue T148 (*red*) of actin. Data are from PDB 1UY5

4 Cross-Linking of Actin by Bacterial Toxins

Cross-linking is another type of toxin-induced covalent modification of actin. Various bacterial pathogens produce toxins that possess a conserved actin cross-linking domain (ACD). At first, the actin cross-linking activity was identified in a *Vibrio cholerae* toxin called MARTX (multifunction-autoprocessing repeats-in-toxin) (Fullner and Mekalanos 2000; Satchell 2015). *Vibrio cholerae* MARTX ($MARTX_{V.c.}$) is a multimodular single chain toxin that contains glycine rich repeats at the N and C-terminus, which are suggested to be involved in pore formation and translocation into the cytosol (Satchell 2015) (Fig. 7). $MARTX_{V.c.}$ possesses three effector domains, which are located in the middle part of the toxin. In addition, it carries an autoprocessing protease domain. The effector domains are the Rho-inhibitory domain (RID) that inactivates Rho proteins at the cell membrane, the alpha/beta hydrolase (ABH), which was shown to activate Cdc42 (Dolores et al. 2015) and the actin cross-linking domain ACD. All three domains are translocated into the cytosol where they are released by activity of the protease domain. This protease is related to the autoprocessing protease of large clostridial glycosylating toxins and is similarly activated by cytosolic inositol hexakisphosphate.

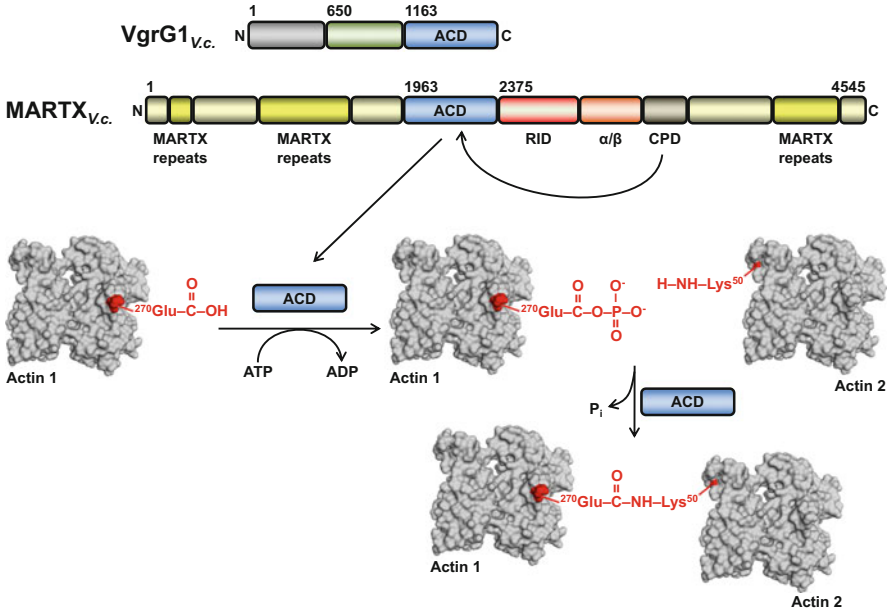

Fig. 7 Structure and function of actin-cross-linking toxins. VgrG1 from *Vibrio cholerae* harbors an actin cross-linking domain (ACD) at its C-terminus. VgrG1 proteins are part of the type VI secretion system, which is present in many Gram-negative pathogens. The N-terminal and middle part of VgrG1 harbors domains with similarity to bacteriophage tail spike complex like proteins, which might function as a translocon. MARTX (Multifunctional, autoprocessing RTX toxin) of *Vibrio cholerae* is a large multimodular protein, which consists of several conserved glycine-rich RTX repeats (MARTX repeats), a Rho GTPase inactivating domain (RID), a α/β hydrolase (α/β), a cysteine protease domain (CPD), and an actin cross-linking region (ACD). The CPD is involved in mobilization and release of the ACD (arrow), which then catalyzes cross-linking of G-actin. Cross-linking is caused by bond formation between glutamate270 and lysine50 of two actin molecules. ATP is required for formation of the glutamyl phosphate intermediate. Structural data are from PDB 1ATN

ACD catalyzes the cross-linking of G-actin to form dimers, trimers, and higher order oligomers. Thereby, the formation of actin filaments is inhibited and the physiological dynamics of actin polymerization/depolymerization are blocked. In cell culture, this results in rounding up of cells.

ACD domains are also present in MARTX toxins produced by *A. hydrophilia* (Suarez et al. 2012) and *Vibrio vulnificus*, which are both aquatic pathogens and involved in intestinal and extra-intestinal infections in humans (Satchell 2015). Similarly, as for $MARTX_{V.c.}$, the toxin from *A. hydrophilia* causes actin cross-linking in vitro and disrupts the actin cytoskeleton in vivo which results in apoptosis of target cells (Suarez et al. 2012).

4.1 Functional Consequences of Actin Cross-Linking by ACDs

ACDs cause cross-linking of actin between lysine50 and glutamate270 (Kudryashov et al. 2008) (Fig. 7). The reaction is highly specific. Exchange of lysine50 or glutamate270 blocks cross-linking. Lysine50 is located in the DNase-I binding loop or D-loop of subdomain 2 of actin (Kabsch et al. 1990). This region is often disordered in G-actin crystal structures, can adopt different kinds of conformation, and is flexible. The D-loop is involved in intersubunit contacts also explaining why cross-linked actin cannot polymerize. Glutamate270 is located in subdomain 3 and is part of a loop, which is the connection to subdomain 4. In the F-actin structure, lysine50 and glutamate270 are not in close contact. Therefore, cross-linking is not compatible with the F-actin structure. The functional consequence is the inhibition of actin polymerization and blockade of actin functions. For host–pathogen interactions, this means inhibition of migration of immune cells or blockade of phagocytosis.

At least in vitro, the rate of actin cross-linking induced by ACD is rather low (Heisler et al. 2015). Considering the large pool of actin in cells, the rapid effects of ACD on cell morphology are difficult to explain. Moreover, only a small fraction of actin modification by ACD appears to be sufficient to induce major cellular effects. The answer to these questions may be the recent finding of the role of cross-linked actin on formin-regulated actin polymerization (Heisler et al. 2015) (Fig. 8). Formins are crucial regulators of actin nucleation and elongation. Humans possess 15 different formins, which can be listed in 7 subfamilies. Typical for formins are three highly conserved formin homology regions (FH1, FH2, and FH3). The proline rich FH1 region, which plays an important role in enhancing the velocity of formin-induced F-actin elongation (Kovar et al. 2006) binds profilin-actin. The FH2 domain forms dimers, which arrange in a donut-like fashion by head to tail assembly and causes nucleation of actin polymerization and elongation of actin filaments. The N-terminal FH3 domain forms an auto-inhibitory connection with the C-terminal Dia-Autoregulatory-Domain (DAD). Inhibition is released by binding of Rho proteins to the GTPase-binding domain (GBD), which allows FH2 dimer formation and nucleation and elongation of actin filaments. Heisler et al. found that ACD-cross-linked actin binds with high affinity (low nanomolar range) to formins (e.g., mDia1/mDia2), thereby inhibiting nucleation as well as elongation of actin. The inhibitory effects were less prominent with ACD-induced actin dimers than with oligomers, suggesting multivalent interaction. Thus, ACD induces the formation of toxic products (cross-linking products) that block actin regulation with high potency, although only a small fraction of actin is modified.

4.2 Structure of ACD and Mechanism of Actin Cross-Linking by VgrG1

ACD is not only a toxin component of multimodular MARTX but is also a bacterial effector of type-VI secretion systems. Type-VI-secretion systems are encoded by

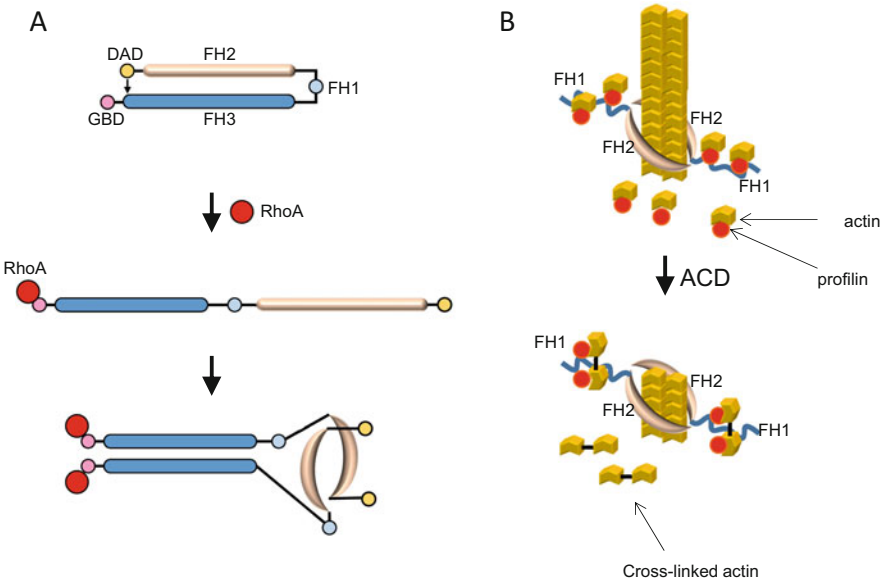

Fig. 8 Cross-linked actin inhibits formin-dependent actin elongation. (**a**) Activation of formins. Formins possess a proline rich FH1 domain, which interacts with profilin-actin. The FH2 region is involved in actin nucleation and elongation. The FH3 domain is involved in autoinhibition by the DAD (DIA autoregulatory domain). Binding of Rho proteins (RhoA) to the GTPase-binding domain (GBD) disinhibited formins and the FH2 region forms a doughnut like structure, which is important for actin nucleation. (**b**) Under normal conditions, profilin-actin binds to FH1 domain of formins to accelerate actin filament nucleation and elongation. Cross-linked actin interacts with profilin and binds with high affinity to FH1 but blocks actin elongation. Modified from (Baarlink et al. 2010)

conserved clusters of 15–25 genes, which are found in many Gram-negative bacteria (Pukatzki et al. 2009; Ho et al. 2014). Many essential proteins of this secretion system are related to the tail spike of T4 bacteriophage components. In many cases, Hcp (hemolysin-coregulated protein) proteins are the structural and functional basis of this system. Hcps form hexameric rings with a central lumen of ~40 Å. Around this tail tube is a sheath of contractile elements. Contraction of the sheath causes extension or injection of the tail tube into target cells, which can be bacterial or eukaryotic cells. On top of the Hcp tail is the VgrG protein, which is involved in piercing the target cell. The VgrG1 protein of *V. cholerae* contains a C-terminal extension with an effector domain that is ~60% identical to ACD of $MARTX_{V.c.}$ and able to cross-link actin (Pukatzki et al. 2007, 2009). The crystal structure of VgrG1 ACD has an overall V-shape, exhibiting the proposed catalytic site in a cleft between the two arms of the three-dimensional structure. The left arm of the V is mainly formed by β-strands, while the right arm is formed by seven α-helices and does not contain any β-strand. Although the fold of ACD is unique, the protein exhibits some similarity with glutamine ligase/γ-glutamyl-cysteine

synthetase (GSC ligase, PDB 1VA6) (Durand et al. 2012). Importantly, most of the catalytic residues of GSC ligase are shared with ACD. Cross-linking of actin by ACD depends on divalent cations and ATP (Fig. 7). Here, ATP is required for ACD activation and not for actin. Without ATP the N-terminal part of ACD is located in the catalytic cleft. Upon ATP binding this N-terminal part is dislodged from the catalytic cleft and catalysis can occur. Moreover, ATP is essential for catalysis, because glutamine270 interacts with the γ-phosphate of ATP to form a glutamyl phosphate intermediate, which could be directly detected. Subsequent hydrolysis of the intermediate allows cross-linking with lysine50 of a second actin molecule (Kudryashova et al. 2012; Durand et al. 2012; Satchell 2009). Similar mechanisms have been proposed for all ACDs including MARTX toxins.

5 Conclusions

Actin is a highly conserved protein, which plays a crucial role in numerous essential cellular functions. So far three different types of bacterial protein toxins have been identified, which affect the physiological functions of actin. One large toxin group causes depolymerization of F-actin by ADP-ribosylation of arginine177. So far, only one toxin is able to enhance actin polymerization by ADP-ribosylation of threonine148. A third group of toxins causes cross-linking of actin between glutamate270 and lysine50, thereby inhibiting actin polymerization. Thus, it appears that the spectrum of toxin mechanisms directly targeting actin is rather limited. By contrast, a large number of toxins and effectors, target regulators of actin functions like Rho proteins or their GEF and GAP proteins. One can speculate that bacteria favor the manipulation of the actin cytoskeleton by attacking actin regulators compared to covalent modifications of the actin itself, especially when a reversible manipulation is more appropriate than a permanent modification of actin.

Although the progress in research on actin-targeting toxins has been highly successful during recent years, many important questions remain. In many cases, the precise pathophysiological role of toxin-induced actin modification is not well understood. What are the consequences of actin modification for the infection process and what are the consequences of the attack of the actin cytoskeleton for the inflammatory responses of the host. Because many studies with toxins have been performed in cell culture, the answers to these intriguing questions remain elusive. Therefore, it is crucial to get further insights into pathophysiological consequences of toxin-induced actin modification in whole tissues and, better, in intact organisms.

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