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REVIEW ARTICLE

Actin as target for modification by bacterial protein toxins

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Various bacterial protein toxins and effectors target the actin cytoskeleton. At least three groups of toxins/effectors can be identified, which directly modify actin molecules. One group of toxins/effectors causes ADP-ribosylation of actin at arginine-177, thereby inhibiting actin polymerization. Members of this group are numerous binary actin–ADP-ribosylating exotoxins (e.g. *Clostridium botulinum* C2 toxin) as well as several bacterial ADP-ribosyltransferases (e.g. *Salmonella enterica* SpvB) which are not binary in structure. The second group includes toxins that modify actin to promote actin polymerization and the formation of actin aggregates. To this group belongs a toxin from the *Photorhabdus luminescens* Tc toxin complex that ADP-ribosylates actin at threonine-148. A third group of bacterial toxins/effectors (e.g. *Vibrio cholerae* multifunctional, autoprocessing RTX toxin) catalyses a chemical crosslinking reaction of actin thereby forming oligomers, while blocking the polymerization of actin to functional filaments. Novel findings about members of these toxin groups are discussed in detail.

Introduction

The actin cytoskeleton is involved in many cellular motile events like intracellular vesicle transport, phagocytosis and cytokinesis after mitosis and is essential for active cell migration. It plays pivotal roles in the control of epithelial barrier functions and the adherence of cells to the extracellular matrix. It is essential for the recognition and adherence of immune cells and their subsequent phagocytic activity. Furthermore, the actin cytoskeleton is a general regulator in immune cell signaling and is involved in the control of cytokine and reactive O_2^- production. Similarly, cytoplasmic microtubules are essential for the establishment of cell polarity and directed intracellular vesicle transport over long distances as in neuronal axons. Both the F-actin filaments and microtubules are highly dynamic structures, whose supramolecular organization is constantly modified according to cellular needs. Their dynamic behavior is regulated by a large number of binding proteins, which are often the effectors of intracellular and extracellular signaling pathways. It is therefore not surprising that the actin cytoskeleton is one of the main targets of bacterial protein toxins, and thus of major importance for the host–pathogen interaction.

Bacteria have developed numerous toxins and effectors to target the actin cytoskeleton. (Note that toxins are often defined as bacterial products that can act in the absence of the bacteria. The bacterial effectors depend on the presence of the bacteria, e.g. for transport into the target cells.) Probably most of these bacterial products affect the actin cytoskeleton by

Abbreviations
ABP, actin binding protein; ACD, actin crosslinking domain; CDT, *Clostridium difficile* transferase; CST, *Clostridium spiroforme* toxin; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; PA, protective antigen; VIP, vegetative insecticidal protein.

interfering with the endogenous regulation of the cytoskeleton [1,2]. Thus, several bacterial protein toxins have been described that modify the activity of Rho proteins. These master regulators of the cytoskeleton can be manipulated by toxins by ADP-ribosylation [3,4], glucosylation [5], proteolysis [6], adenylylation [7], deamidation [8] and transglutamination [9]. Moreover, several types of bacteria target the actin cytoskeleton by modulating the Rho GTPase cycle of host cells with effectors, acting as GTPase-activating proteins (GAPs) [10–13] or guanine nucleotide exchange factors (GEFs) [14,15]. A direct interaction with actin molecules is the basis for the rearrangement of the actin cytoskeleton by bacterial effectors like *Salmonella* invasion protein A (SipA) and C (SipC). Whereas SipA decreases the critical concentration for F-actin formation leading to polymerization and stabilization of F-actin filaments by acting as a molecular staple [16–18], the SipC functions as an actin nucleator and filament bundling protein [17,19]. Certain bacterial toxins also directly modify the actin molecule. These toxins belong to at least three groups. The first group causes ADP-ribosylation of specific residues of actin, resulting in depolymerization of actin. The second group induces polymerization by ADP-ribosylation of actin. The third group modifies actin by enzymatic crosslinking leading to the formation of stable dimers and higher order oligomers of this microfilament protein. Bacterial toxins that directly modify actin molecules are discussed in this review in more detail.

Three-dimensional structure of monomeric and filamentous actin

Actin is one of the most abundant proteins in eukaryotic cells and is composed of 375 amino acid residues forming a single chain of 42 kDa. Its atomic structure was first solved for its complex with deoxyribonuclease I [20]. G-actin is a flat molecule with dimensions of about $50 \times 50 \times 35$ Å. Figure 1 gives the standard view on the flat face of actin. A deep cleft separates actin into two main domains of almost equal size, each being composed of two subdomains numbered SD1–SD4 (Fig. 1). All subdomains contain a central β-sheet surrounded by a varying number of α-helices. The bound adenine nucleotide (ATP; deep blue in Fig. 1) is located at the bottom of the deep cleft. Both N- and C-terminus are located in SD1 and the peptide chain crosses twice between the two main domains at the bottom of SD1 and SD3, i.e. underneath the nucleotide binding site involving the sequence stretches from residues 140 to 144 and 340 to 345. This region is supposed to form a flexible hinge region, allowing

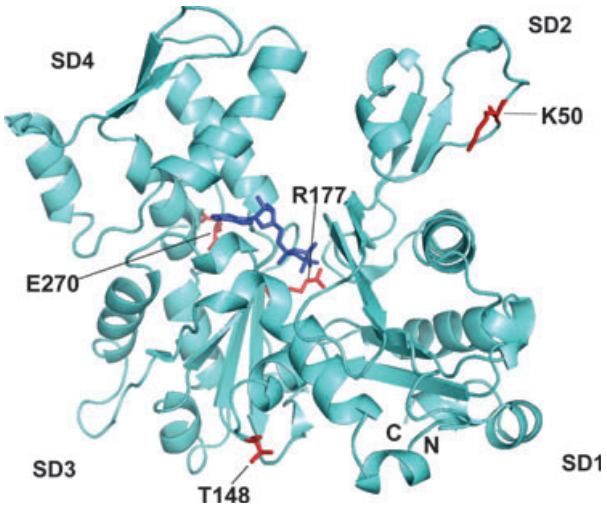

Fig. 1. Structure of the actin molecule. The four subdomains of actin are indicated (SD1–SD4). In red, amino acids are indicated, which are modified by bacterial protein toxins. Arg177 (R177) is ADP-ribosylated by toxins (e.g. binary actin–ADP-ribosylating toxins which prevent polymerization and induce depolymerization of actin). Thr148 (T148) is ADP-ribosylated by *Photorhabdus luminescens* toxin (TccC3), which causes polymerization of actin. Various toxins catalyze actin crosslinked between Lys50 (K50) and Glu270 (E270). For details see text.

movements of the two main domains relative to each other.

Under physiological salt conditions purified monomeric or G-actin polymerizes to its filamentous form, F-actin. F-actin is composed of two strands of linearly arranged actin subunits that are wound around each other forming a helix that can be described either as a two-start left-handed double helix with a half-pitch of about 360 Å or as a one-start genetic right-handed helix with a rotational translocation of 166° and an axial rise of 27.5 Å resulting in a pitch of 360 Å after 13 actin molecules and six turns [21].

G-actin contains firmly bound one molecule of ATP that is hydrolyzed to ADP and Pi after incorporation into a growing F-actin filament. The ADP remains attached to the actin subunit, whereas the Pi dissociates slowly from the filament generating two filament ends with actin subunits differing in their bound nucleotide: either ATP or ADP. Actin polymerization proceeds until equilibrium is established between monomeric and filamentous actin. The concentration of the remaining monomeric actin is the critical concentration of actin polymerization (C_c).

During polymerization ATP-bound G-actin preferentially associates to the end containing ATP-actin subunits, the fast growing end, which has also been termed the plus or barbed end. After reaching

equilibrium actin monomers associate to the barbed end and an identical number dissociates preferentially from the opposite end, which has also been termed the minus or pointed end. Thus, under these conditions and in the presence of ATP actin subunits constantly associate to the barbed end and travel through the whole filament until they dissociate from the pointed end [22]. This behavior has been termed treadmilling or actin cycling and represents for a number of motile processes the sole basis for force generation [23,24]. The critical concentrations for the barbed end C_cb and pointed end C_cp are 0.1 and 0.8 μM, respectively. Under polymerizing conditions the critical concentration of polymerization C_c is 0.2 μM, i.e. closer to that of the barbed end [24].

Actin is one of the most highly conserved proteins in nature. In mammals there exist six tissue-specific actin isoforms: α-skeletal, α-cardiac, α- and γ-smooth muscle, and β- and γ-cytoplasmic actins [25]. α-Skeletal and γ-cytoplasmic actins differ only by 25 amino acid exchanges most of them being conservative and located on the surface of the molecule. The mammalian actins exhibit about 90% sequence identity with those from distant organisms like yeast.

The physiologically active form of actin is F-actin; therefore much effort has been undertaken to elucidate the orientation and the F-specific structural alterations of the actin monomer [21]. A recent study using high magnetic fields to obtain optimal alignment of F-actin filaments has led to the resolution of the F-actin structure being increased to about 4 Å [26].

Actin binding proteins

Actin is a highly 'promiscuous' protein that interacts with many different kinds of proteins. About 150 different specific actin binding proteins (ABPs) are known both at extracellular (only a few) and intracellular localizations that modify particular properties or its supramolecular organization [27,28]. The ABPs can be grouped into at least eight classes: (a) proteins that stabilize or sequester the monomeric actin; (b) proteins that bind along F-actin filaments (like tropomyosin); (c) motor proteins that generate the force for the sliding of F-actin filaments; (d) proteins that nucleate actin polymerization [29,30]; (e) proteins that bundle F-actin filaments; (f) proteins that stabilize filament networks; (g) proteins that sever F-actin filaments; and (h) proteins that attach filaments to specialized membrane areas. Even if they have different functions many of these proteins attach to a few target zones on the actin surface such as the hydrophobic region mentioned above. It is probably because of these multiple interactions that the sequence and three-dimensional structure of actin has been so highly conserved during the billions of years of evolution.

Many ABPs are at the end of signaling cascades and regulated by phospholipid interaction, Ca^{2+}-ion concentrations, phosphorylation or small GTPases [31]. These signals either deactivate or activate the supramolecular organization of actin during cell migration, exocytosis or endocytosis, or cytokinesis.

Binary actin–ADP-ribosylating toxins

Actin is ADP-ribosylated by various bacterial protein toxins (Fig. 2). The prototype of these toxins is

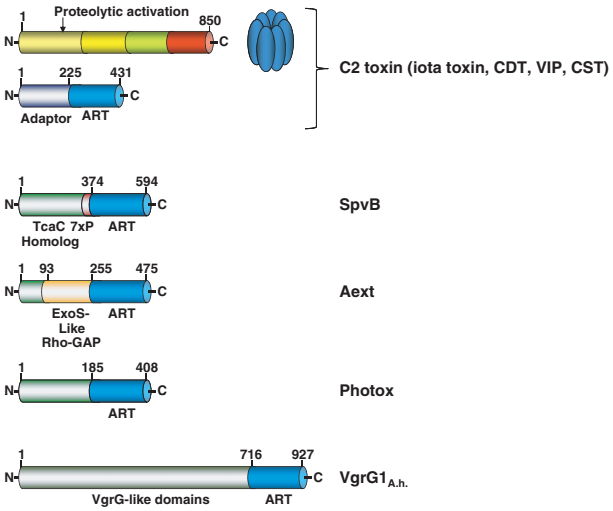

Fig. 2. Different structures of actin–ADP-ribosylating toxins/effectors, which all modify actin at Arg177. The family of binary toxins consists of *Clostridium botulinum* C2 toxin, *Clostridium perfringens* iota toxin, *Clostridium difficile* transferase (CDT), *Bacillus cereus* vegetative insecticidal toxin (VIP) and *Clostridium spiroforme* toxin (CST). The toxins are binary in structure. They consist of a binding/translocation component and the separated enzymatic component. The activated binding/translocation domain 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 domain. Numbers given are from *C. botulinum* C2 toxin. The other toxin/effectors are not binary in structure but all possess a C-terminal actin–ADP-ribosylating domain. These toxins are introduced into host cells by a type III secretion system (SpvB, AexT) or by unknown mechanisms. *Salmonella enterica* produces the effector SpvB, which possesses a C-terminal actin–ADP-ribosylating domain. AexT is produced by *Aeromonas salmonicida* and possesses, in addition to the actin ART domain, a domain with Rho GTPase-activating activity (GAP), which is related to *Pseudomonas* ExoS protein. Photox is an effector, which is produced by *Photorhabdus luminescens*. VgrG1 from *Aeromonas hydrophila* possesses an actin–ADP-ribosyltransferase domain at its C-terminus. This protein is probably part of the type VI secretion system and also effector (see also Fig. 8).

Clostridium botulinum C2 toxin [32–34], which is the founding member of the family of binary actin–ADP-ribosylating toxins. Other members are *Clostridium perfringens* iota toxin [35,36], *Clostridium difficile* transferase (CDT) [37], *Clostridium spiroforme* toxin (CST) [38,39] and the *Bacillus cereus* vegetative insecticidal protein (VIP) [40]. All these toxins ADP-ribosylate Arg177 of actin (marked in Fig. 1); they are binary in structure and consist of an enzyme component, which harbors ADP-ribosyltransferase activity, and a separated binding component, which is responsible for the uptake of the toxin [2,41–43].

The binding component (C2II) of C2 toxin has to be activated by proteolytic cleavage (Fig. 2), which releases an ~ 20 kDa fragment from C2II [44]. The activated C2II fragment forms heptamers, which have a prepore structure [45]. These heptamers bind to carbohydrate structures (complex and hybrid carbohydrates) on the surface of target cells [46]. Recent crystal structure analysis provided a preliminary model of the structure of the binding component [47], which is very similar to the prepore structure of *Bacillus anthracis* protective antigen (PA), the binding component of anthrax toxin [48,49]. In fact, sequence comparison and structural data revealed a high similarity of the binding components of all binary actin ADP-ribosyltransferases throughout the whole molecule with the exception of the C-terminal receptor-binding domain.

Most probably the heptameric structure of C2II generates a polyvalent binding platform of high affinity for the proposed carbohydrates on the surface of target cells, which function as cell receptors or are at least an essential part of the receptors [46] (Fig. 3). Then, the enzyme component C2I binds to the heptameric C2II and subsequently the toxin–receptor complex is endocytosed. At the low pH prevailing in endosomes a

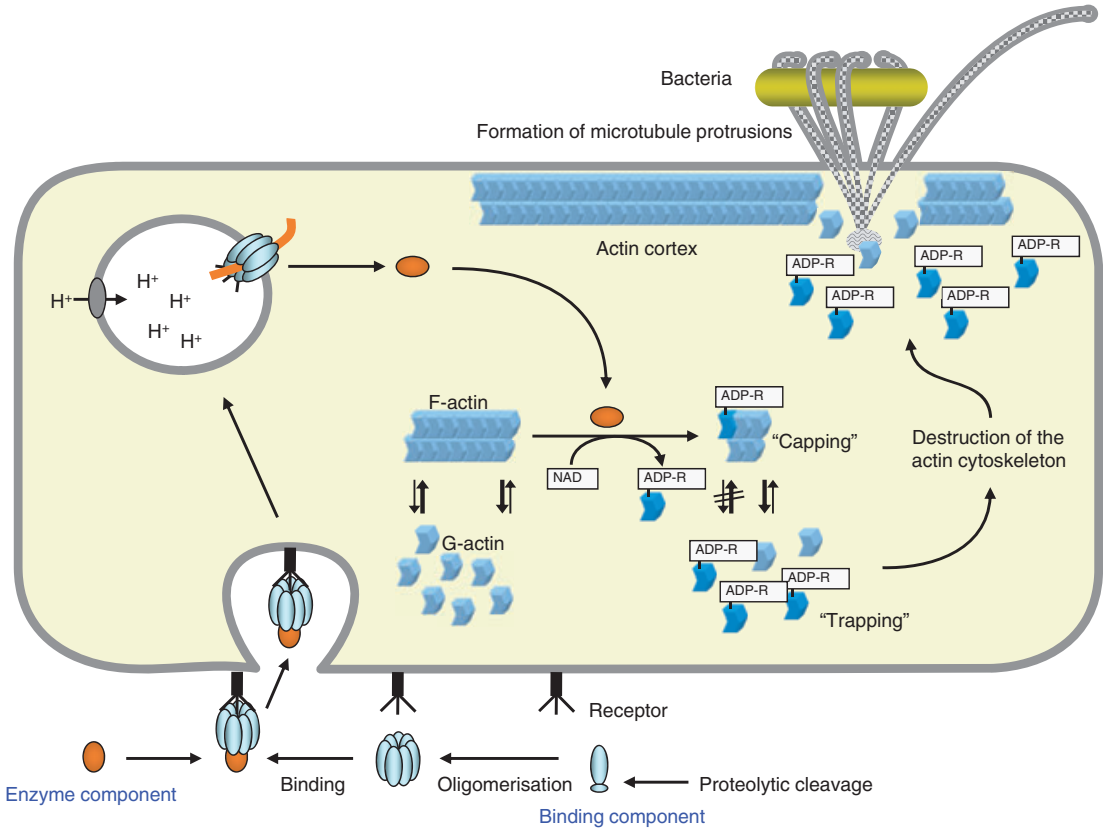

Fig. 3. Model of the action of binary actin–ADP-ribosylating toxins. The binary toxins consist of the binding component and the enzymatic ADP-ribosyltransferase component. The binding component is proteolytically activated and forms heptamers. After binding to cell surface receptors, the enzyme component interacts with the binding component and the toxin complex is endocytosed. At low pH of endosomes, the binding and translocation component inserts into membranes and finally allows the delivery of the enzyme component into the cytosol. Here actin is ADP-ribosylated at Arg177. ADP-ribosylation of actin at Arg177 causes inhibition of actin polymerization and destruction of the actin cytoskeleton. This has consequences for the microtubule system. Growing microtubules are no longer captured at the cell membrane and form long protrusions extending from the cell surface. These protrusions facilitate adherence and colonization of bacteria.

conformational change of the prepore occurs. This is characterized by the conversion of a loop (most probably loop 2β2–2β3 as in PA [48]) in domain 2 of each monomer to form a β-barrel structure, forcing the insertion into the endosomal membrane resulting in formation of a pore. Through this pore (with help of the ψ-clamp-like residue Phe428 [50]) the enzyme component is transported into the cytosol, a process which depends on the cytosolic heat shock protein Hsp90 [51]. Recent studies suggest that, in addition to the heat shock protein Hsp90, cyclophilin A is involved in the translocation of the enzyme component into the cytosol [52].

The binary actin–ADP-ribosylating toxins can be divided into two subfamilies. One subfamily is formed by *C. botulinum* C2 toxin, and the other subfamily is the so-called iota-like toxin family composed of the toxins iota, CST and CDT [43,53]. Within the family of iota-like toxins the binding components can be exchanged. Thus, the binding component Ib of iota toxin is able to translocate the enzyme components of CST or CDT into target cells [54]. The iota toxin appears to gain access to the cytosol by entering the cells through a different pool of endosomes [55]. Another difference between the toxin subfamilies is their substrate specificity. The iota-like toxins ADP-ribosylate all actin isoforms studied so far. The C2 toxin, however, appears to modify β,γ-actins but not – or to a much lesser extent – the α-actin isoforms [56,57].

The ADP-ribosyltransferase component of binary toxins

During the last few years we have learned much about the structure–function relationship of the ADP-ribosyltransferase components of the toxins [47,58–60]. Early analysis of the sequences of the enzyme components revealed that the ADP-ribosylating enzyme components consist of two related domains of almost identical fold, which were probably generated by gene duplication [40]. However, only the C-terminal domain is a functional ADP-ribosyltransferase possessing the typical active site residues. The N-terminal part, which during evolution has lost a number of crucial amino acid residues for the ADP-ribosyltransferase activity, functions as an adaptor for the interaction with the binding/transport components. Nevertheless, a recent crystal structure analysis of the complex of the enzyme component of iota toxin with its substrate actin showed that not only the active C-terminal domain but also the N-terminal domain of Ia interacts with actin (see Fig. 4 later). The finding that the N-terminal part of the enzyme component is important for the interaction with the translocation domain was used to construct a delivery system for fusion proteins.

All known binary ADP-ribosylating toxins possess a very similar catalytic fold with a highly conserved NAD^+ binding core, consisting of a central six-stranded β-sheet [61,62]. Within this core, three highly conserved motifs, which are often abbreviated RSE, can be identified in β-strands 1, 2 and 5. The 'R' located in β-strand 1 and the 'STS' motifs positioned in β-strand 2 are both crucial for NAD binding. The β-strand 5 contains the EXE motif including two glutamate residues, which are essential for ADP-ribosylation of actin at Arg177. The first glutamate is part of the ARTT (ADP-ribosylating turn-turn) loop in front of β-strand 5, which is involved in substrate recognition (see also below). The second glutamate of this motif is the so-called catalytic glutamate.

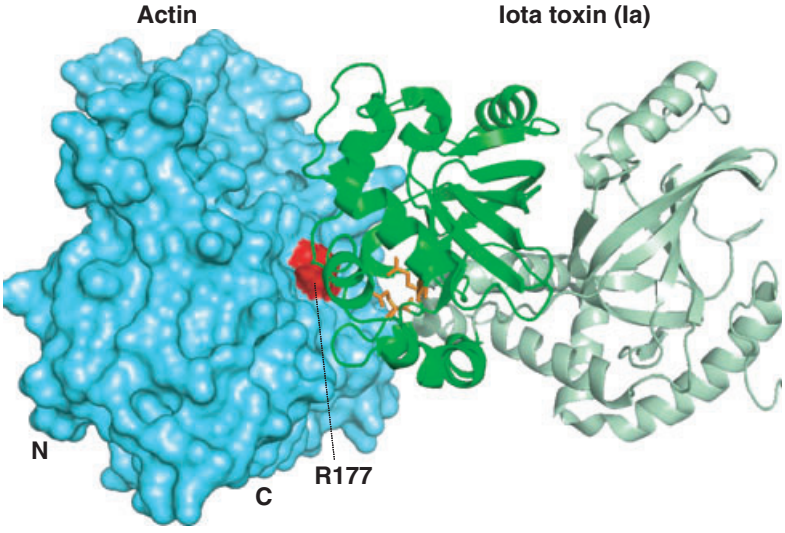

Fig. 4. Complex of *Clostridium perfringens* iota toxin with actin. Actin is shown in blue. Arg177 (R177) of actin is modified by toxin-catalyzed ADP-ribosylation. The enzymatic component of *C. perfringens* iota toxin (Ia) is on the right. The enzyme domain, possessing ADP-ribosyltransferase activity, is in green and the adaptor domain, which interacts with the binding component (not shown), is in grey. The data are from Protein Data Bank 3BUZ.

Recently, iota toxin has been crystallized in complex with actin and the non-hydrolyzable NAD analog betaTAD [58] (Fig. 4). Structure analysis has shown that the iota toxin binds to actin through subdomains 1, 3 and 4. The structure of actin was hardly changed, whereas the substrate–enzyme interaction induced specific changes in the enzyme component of the toxin. It was demonstrated that the recognition of actin depended on five loops of the enzyme component. Surprisingly, the structural data demonstrated that the N-terminal domain of the enzyme domain also, which was previously suspected to be only involved in the interaction with the binding component, is essential for the interaction with actin [58]. Comparison of the actin-binding interface of iota toxin with other actin-binding proteins like gelsolin, profilin or DNaseI revealed that the toxin binds in a completely different manner to actin.

Bacterial actin ADP-ribosyltransferases, which are not binary toxins

ADP-ribosylation of actin is also caused by bacterial toxins or effectors which differ in their structure and delivery system from the binary toxins [63–65] (Fig. 2). *Salmonella* SpvB is a bacterial effector which is transported into eukaryotic target cells by the type III secretion system [66]. The protein consists of 594 amino acid residues. The C-terminus, covering residues 374–594, shares similarities with actin–ADP-ribosylating toxins like Vip2 (identity 19%). The N-terminus is similar to the N-terminal part of *Photorhabdus luminescens* toxin complex component TcC (see below). However, the function of this part is not known. SpvB modifies actin (most probably all isoforms) also at Arg177 and therefore the functional consequences for actin are probably the same as with binary toxins [64,67].

Photox is a ~ 46 kDa protein which is produced by *P. luminescens* (see also below) and possesses a two-domain structure [68]. The complete protein shares 39% identity with SpvB. Even higher is the sequence identity (~60%) of the C-terminal 200 amino acid residues of photox with the catalytic core of SpvB. The role of the N-terminal part of the protein is unclear. However, it might play a role in toxin entry into target cells; indeed for this process a type VI secretion has been proposed [68].

Photox, like SpvB, does not possess any detectable NAD hydrolase activity. Photox targets all actin isoforms and like other toxins it modifies Arg177 and does not accept polymerized actin as substrate [68].

Aeromonas salmonicida is a fish pathogen which produces the bifunctional *Aeromonas* exotoxin T (AexT) [69,70]. The toxin consists of at least two functional modules. The complete protein is ~60% identical with ExoT and ExoS from *Pseudomonas aeruginosa*. The bacterial type III secretion effectors ExoT and ExoS possess N-terminal Rho-GAP and C-terminal ADP-ribosyltransferase activities, modifying the Crk (C10 regulator of kinase) protein and Ras, respectively [71]. The N-terminal 210 amino acids of AexT are also ~33% identical with the Rho-GAP-like effector from *Yersinia pseudotuberculosis* YopE [69]. Thus, AexT possesses GAP activity towards Rho, Rac and Cdc42, while the C-terminal ADP-ribosyltransferase activity causes modification of actin at Arg177 [70]. AexT modifies non-muscle actin much more efficiently than skeletal muscle actin. Of special interest is the diversity in the active site of the ADP-ribosyltransferase of AexT. Whereas all arginine-modifying transferases possess an EXE motif, AexT appears to use an EXXE motif for its catalytic activity [70].

Recently, the type-VI secretion effector protein VgrG1 (~ 100 kDa) from *Aeromonas hydrophila* was shown to ADP-ribosylate actin and to cause depolymerization of the actin cytoskeleton and finally apoptosis. The site of actin modification by VgrG1 is not known so far. However, because the C-terminal part of VgrG1 covering ~ 200 residues is very similar to the ADP-ribosyltransferase domain of VIP2 from *B. cereus* it is feasible that this effector also modifies Arg177 [72].

Functional consequences of the ADP-ribosylation of actin at Arg177

All binary actin–ADP-ribosylating toxins studied so far modify G-actin at Arg177 [64,68,70,73,74] (Fig. 3). This residue is located near the interaction site between the two helical strands of F-actin filaments [21] and has been shown to be directly involved in the interstrand interaction. Using SpvB transferase, actin was ADP-ribosylated and subsequently crystallized. The data obtained from the crystal structure analysis confirmed previous suggestions [21] that the polymerization of actin ADP-ribosylated at Arg177 is blocked by steric hindrance [67]. Figure 5A illustrates this fact by showing the steric effect of ADP-ribosylation of Arg177 of one actin within the F-actin filament. It can be clearly seen that the ADP-ribosyl group can extend towards the neighboring strand like the so-called hydrophobic loop that links the two strands (Fig. 5A).

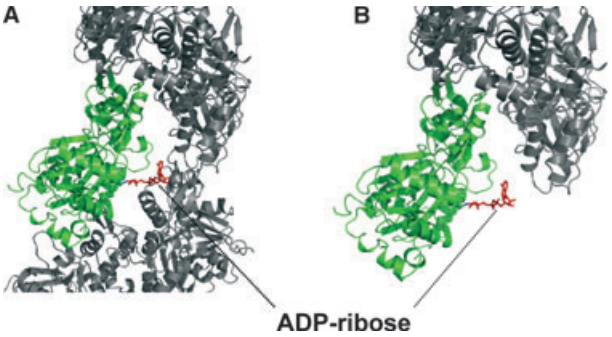

Fig. 5. Effect of ADP-ribosylation of Arg177 on actin–actin interaction. (A) Ribbon presentation of ADP-ribosylated actin (green) within the F-actin filament (grey); ADP-ribose is colored in red. The steric hindrance induced by ADP-ribosylation of Arg177 is shown. (B) Binding of ADP-ribosylated actin to the plus end of F-actin. The data are from Protein Data Bank 1ATN.

Thus, actin ADP-ribosylated at Arg177 cannot be polymerized and conversely F-actin is not a substrate or is only a very poor substrate for ADP-ribosylation by these toxins [56]. Indeed, it is completely blocked when F-actin is stabilized by phalloidin as shown biochemically [68,75]. It is conceivable, however, that monomeric actin in equilibrium with F-actin or dissociating from the pointed ends during treadmilling may become accessible for ADP-ribosyltransferases, and by this effect the cellular actin will be completely converted into polymerization-incompetent ADP-ribosylated actin (see also Fig. 4). Although Arg177 ADP-ribosylated actin is unable to polymerize, it is still able to bind to and cap the barbed ends of native (unmodified) actin filaments [76–78], inhibiting further growth of actin filaments from the barbed end. Figure 5B gives a model of binding of one ADP-ribosylated actin to the plus end, thus inhibiting the addition of further subunits. By contrast, the pointed ends of filaments are not affected and depolymerization or exchange of actin subunits can occur at this site [77,78].

It has been shown for *C. perfringens* iota toxin, *C. botulinum* C2 toxin [79] and *P. luminescens* toxin photox [78] that the toxin-induced ADP-ribosylation of actin is reversible in the presence of an excess of nicotinamide. De-ADP-ribosylation restores the property of actin to polymerize. In *Acanthamoeba rhysodes*, which can be infected by SpvB-producing specific serovars of *Salmonella enterica*, actin is rapidly degraded after toxin-catalyzed ADP-ribosylation [80]; however, this is not observed in mammalian cells.

ADP-ribosylation has effects on the binding and hydrolysis of ATP. The affinity of ATP for ADP-ribosylated actin is decreased (the dissociation rate of ε-ATP is increased after ADP-ribosylation at Arg177 by a factor of 3). Concomitantly, the thermal stability is slightly reduced [78]. Moreover, ATP hydrolysis is largely inhibited by ADP-ribosylation of actin at Arg177 [81,82]. These data are in agreement with recent findings that ADP-ribosylation of actin at Arg177 by SpvB toxin causes conformational changes in the so-called W-loop (residues 165–172) of actin, a putative nucleotide-state sensor and an important region for interaction with profilin, cofilin and MAL [83].

It has been shown that actin also when bound to gelsolin is a substrate for ADP-ribosyltransferases. Gelsolin is a multifunctional protein that can cap, nucleate or sever F-actin filaments depending on the free Ca^{2+}-ion concentration and the presence of either G- or F-actin. Gelsolin is built from six homologous domains of identical fold (G1–G6), but only three are able to bind actin: G1, G2 and G4. The N-terminal segment G1 binds G-actin independently of the Ca^{2+} concentration with high affinity, whereas binding of G4 to G-actin occurs only in the presence of micromolar Ca^{2+}. G2 binds F-actin preferentially. At low Ca^{2+} intact gelsolin binds only one actin molecule, most probably by its G1 segment. At micromolar Ca^{2+}-ion concentration it forms stable complexes with two actin molecules presumably by its G1 and G4 segments. The isolated N-terminal half of gelsolin (G1–3) is able to nucleate and to sever F-actin and also to form a complex with two actin molecules independent of the Ca^{2+} concentration. Therefore in the presence of ADP-ribosylated actin (Ar) several types of gelsolin–actin complexes can be formed. Quite early studies showed that the gelsolin–actin complexes can be modified, resulting in three types of complexes (G–Ar–A, G–A–Ar and G–Ar–Ar) [84]. However, whereas the G–Ar and G–Ar–A complexes, in which the Ar was most probably attached to G1, nucleated the actin polymerization, this was not the case with the G–A–Ar complex. The nucleation of actin polymerization occurred not before the ADP-ribosylated actin was exchanged for non-modified actin. A recent study confirmed the formation of a ternary complex of gelsolin with two ADP-ribosylated actins. Moreover, at least two different modes of binding of ADP-ribosylated actin to gelsolin were shown. However, the complex obtained was readily able to nucleate actin polymerization [78].

As in the test-tube, intracellular ADP-ribosylation of actin at Arg177 favors the depolymerization of F-actin filaments, and finally results in destruction of the actin cytoskeleton [85]. Toxin-induced depolymerization of actin causes dramatic effects on the physiological responses of target cells, e.g. of mast cells [86,87], leukocytes [88,89], PC12 cells [90], fibroblasts [91] smooth

muscle [92], axons of spinal nerve cells [93] and endothelial cells [94,95], which have been described in detail in previous reviews [34,41,96,97]. Recent studies reported also the induction of apoptosis by actin–ADP-ribosylating toxins [98].

Effect of ADP-ribosyltransferases on the microtubule system

More recently, an unexpected effect of the binary actin–ADP-ribosylating toxins on the microtubule system has been observed. When epithelial cells are treated with CDT the formation of cell protrusions with diameters of 0.05–0.5 μm and a length of > 150 μm is observed (Fig. 6) [99]. These protrusions form a dense network at the surface of epithelial monolayers. Interestingly, the protrusions generated in the presence of the actin–ADP-ribosylating toxins are formed by microtubule structures.

The cellular microtubule system consists of long filaments formed by α- and β-tubulin heterodimers. Microtubules, like F-actin filaments, are polarized and possess a fast growing plus end and a slowly growing minus end [100]. The minus end of most microtubules is anchored and stabilized at the microtubule organizing center. The dynamic plus ends are directed towards the peripheral cell cortex. These plus ends undergo phases of rapid polymerization and depolymerization, a phenomenon called dynamic instability. This dynamic behavior of microtubules is controlled and modified by several regulatory proteins. Of special importance are the plus end binding proteins EB1 (end binding protein 1) and CLIP-170 (cytoplasmic linker protein 170), which are called +TIPs (plus end tracking proteins). +TIPs are essential for growth of microtubules [101]. However, some +TIPs (so-called capture proteins) like CLASP2 (CLIP-associated protein) and ACF7 (actin crosslinking family 7) stop microtubule polymerization when the growing microtubules reach the actin cortex located below the cell membrane [102–104]. Apparently, actin microfilaments and microtubule structures regulate each other in a dynamic fashion. Thus, ADP-ribosylation of actin, which results in depolymerization of F-actin, affects the regulation of the dynamic behavior of microtubules [105] and causes formation of tubulin protrusions [99]. Immunofluorescence microscopy revealed that the actin–ADP-ribosylating toxins increase the length of

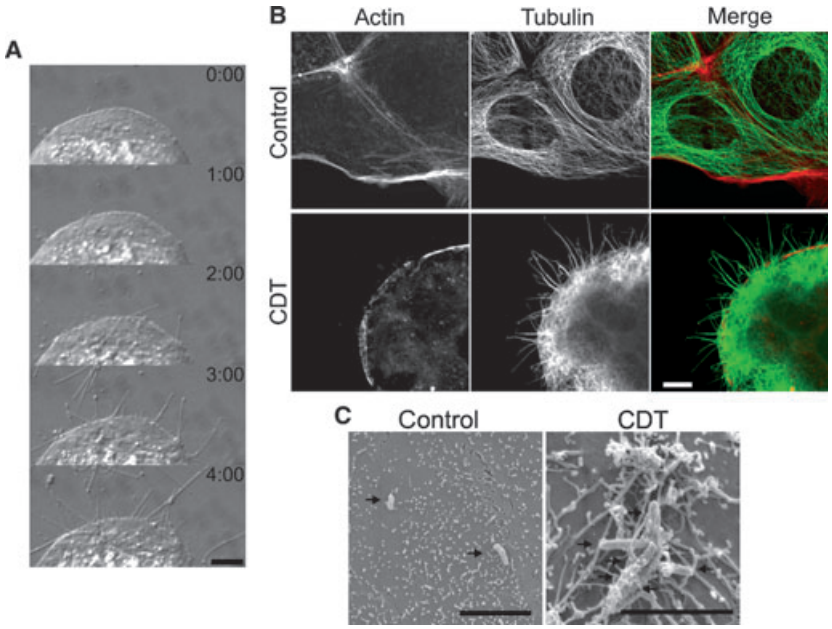

Fig. 6. Effects of ADP-ribosylation of actin at Arg177 on the microtubule system. (A) Subconfluent Caco-2 cells were treated with the actin–ADP-ribosylating toxin *Clostridium difficile* transferase (CDT). The number and length of cell processes increase over time. In each panel the incubation time (h) is indicated. Scale bar represents 10 μm. (B) Indirect immunofluorescence of α-tubulin (green) and actin staining by TRITC-conjugated phalloidin (red) in Caco-2 cells. CDT causes disruption of the actin cytoskeleton and concomitant formation of microtubule-based protrusions. Cells were treated for 2 h. Scale bar represents 10 μm. (C) Scanning electron microscopy of Caco-2 cells. Cells were treated without and with CDT. After 1 h, *C. difficile* bacteria were added. After 90 min cells were washed and fixed. Scale bar represents 5 μm. After CDT treatment *Clostridia* were caught and wrapped in protrusions (arrows). The figure is reproduced from [99].

the plus ends decorated with EB1. Probably more importantly, ADP-ribosylation of actin causes the translocation of the capture proteins ACF7 and CLASP2 from the actin cortex into the cell interior apparently resulting in blockage of their capture functions [99].

Toxin-induced formation of the microtubule-based network of protrusions on the surface of epithelial cells has major consequences for the adherence of bacteria. Electron microscope studies as well as colonization assays revealed that the toxin-producing bacteria adhere more strongly to epithelial cells. Moreover, a mouse infection model revealed elevated dissemination of bacteria with increasing activity of the actin–ADP-ribosylating toxin [99]. All these data indicate a novel role of the toxins, which by actin ADP-ribosylation at Arg177 appear to influence the host–pathogen interaction.

ADP-ribosylation of actin by *P. luminescens* toxin

Recently, it was shown that *P. luminescens* produces toxins that target actin. *P. luminescens* are motile Gram-negative entomopathogenic enterobacteria, which live in symbiosis with nematodes of the family Heterorhabditidae [106,107]. The nematodes, which carry 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 (hemocoel) of the insect. Here, the bacteria replicate and release various toxins, which kill the insect host usually within 48 h. Subsequently, the insect body is used as a food source for the bacteria and the nematodes [107,108].

Photorhabdus luminescens produce a large array of toxins, which are only partially characterized. However, the actin-modifying toxins appear to be the most important ones. This toxin type has a high molecular mass ($\sim$ 1 MDa) and belongs to the toxin complex (Tc) family of *P. luminescens*. Tc toxins are trimeric toxins consisting of the three components TcA, TcB and TcC. A number of homologs exist for each toxin component and several of these homologs are present in *Photorhabdus* [109]. The TcA components appear to be involved in toxin uptake, the TcC components possess biological activity and the TcB components are suggested to have a chaperone-like function. The nomenclature of the toxins is rather complicated, because several gene loci are found for the various toxin homologs. Recently, the activity of the TcdA1, TcdB2 and TccC3 toxin complex, which targets actin, has been elucidated [110]. The complex, consisting of these three components, caused formation of actin clusters in insect hemocytes (e.g. *Galleria mellonella* hemocytes) and in mammalian HeLa cells. Further studies revealed that the TcC component TccC3 exhibits the actin-clustering activity.

Studies on the enzyme activity of TccC3 showed that this component possesses ADP-ribosyltransferase activity and modifies actin in cell lysates. Also isolated β, γ- and α-actin isoforms are substrates for ADP-ribosylation by the toxin. Studies performed in parallel with C2 toxin, which ADP-ribosylates actin at Arg177, revealed that modification by TccC3 occurs at a different site. Moreover, analysis of the chemical stability of the ADP-ribose–actin bonds showed major differences. While the Arg–ADP-ribose bond in actin, which was catalyzed by C2 toxin, was cleaved by hydroxylamine, this was not the case for the ADP-ribose bond to actin catalyzed by TccC3.

Mass spectrometric analysis of peptides obtained from TccC3-modified actin revealed that this toxin caused ADP-ribosylation of Thr148 or Thr149. Finally, mutagenesis studies clarified that in fact TccC3 modifies Thr148 (marked in Fig. 1). So far, threonine residues were not known to be acceptor amino acids for modification by ADP-ribosylation. The finding of a different modification site of actin compared with the binary actin–ADP-ribosylating toxins provides an explanation for the different stability of the ADP-ribose–actin bonds observed after C2 toxin and TccC3 induced ADP-ribosylation.

Of special interest is the localization of Thr148 within the actin molecule (see Figs 1 and 7C). In the standard view of actin it is localized at the base of subdomain 3 and points into the hydrophobic pocket, which represents the docking site for a number of ABPs (Fig. 7C). Of particular interest is its overlap with the binding site of the N-terminal part of thymosin-β4, but it appears conceivable that ADP-ribosylation of Thr148 also modifies the binding of gelsolin, of proteins of the ADF/cofilin family and of profilin.

The β-thymosins

The β-thymosins are a group of highly homologous peptides of about 5 kDa usually built from 42–45 amino acid residues (43 residues for the main representative, thymosin-β4). The β-thymosins occur extracellularly and intracellularly [111,112]. Extracellularly, they appear to fulfil a large array of diverse functions like wound healing, angiogenesis and tissue cell protection. Intracellularly, they are expressed in many eukaryotic cells (except in yeast cells), often in high concentration, and fulfil as sole function the sequestration of

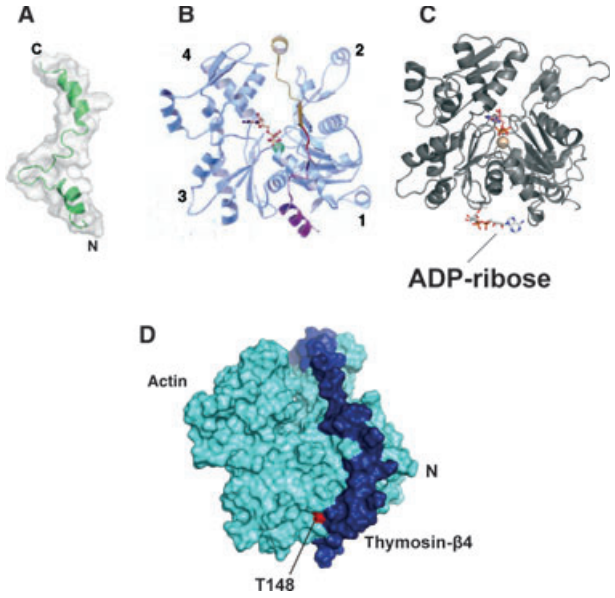

Fig. 7. Interaction of thymosin-β4 with actin. (A) The extended conformation of thymosin-β4 with its N-terminal (bottom) and C-terminal helix (top). (B) Model of binding of thymosin-β4 to actin. It can be seen that the N-terminal helix binds to the small lower groove between subdomains 1 and 3, thereby blocking the barbed end area of actin. The C-terminal helix binds to the top of actin at its pointed end area. (C) An actin molecule with ADP-ribosylated T148 pointing into the groove between SD1 and SD3 indicating the possible steric hindrance of this binding site. (D) Interaction of thymosin-β4 with actin in a space-filling model. The ~ 5 kDa thymosin-β4 interacts with actin in an extended conformation partially covering residue Thr148 (T148) of actin. Data from Protein Data Bank 1UY5.

monomeric actin [113]. The β-thymosin peptides bind to actin in an elongated conformation (Fig. 7A) stretching from the barbed to the pointed end regions of actin and thereby inhibiting association to either end of F-actin (Fig. 7B and 7D as space filling model). This kind of binding to actin is also observed in a large family of proteins that contain the so-called WH2 domain (Wiskot–Aldrich homology domain 2). Their WH2 domains also share high sequence homologies to the N-terminal 35 residues of the β-thymosins (for a review see [112]).

In resting cells the β-thymosins bind to monomeric actin and by their ability to inhibit the salt-induced actin polymerization are responsible for maintaining a high fraction of the intracellular actin in monomeric form despite the high ion concentration that would otherwise lead to its complete polymerization [114]. After cell stimulation this monomeric actin pool is readily activatable for the re-polymerization of new F-actin filaments by the action of actin nucleating proteins [112,115]. The activity of the β-thymosins themselves is not regulated directly; they act as mere G-actin sequestering proteins or buffers and the amount of thymosin-β4-sequestered actin is dependent on the activity of other depolymerization or polymerization promoting proteins (for a review see [112]).

Since Thr148 is located within the binding area of thymosin-β4, the effects of ADP-ribosylation of Thr148 of actin (see Fig. 7C) on the interaction with thymosin-β4 were studied in greater detail. Chemical crosslinking and stopped-flow experiments demonstrated that TccC3-mediated ADP-ribosylation leads to a decrease in binding of thymosin-β4 to actin, which might be responsible for the enhanced polymerization of actin, as observed in cells after toxin treatment.

Further effects of *P. luminescens* toxins

Moreover, the actin cytoskeleton is also targeted by *P. luminescens* toxins via the Rho proteins, which are master regulators of the cytoskeleton [31,116,117]. TccC5 of *P. luminescens*, which is also introduced into target cells by means of TcdA1 and TcdB2, ADP-ribosylates and thus activates Rho GTPases (in particular RhoA), which control actin polymerization and stress fiber formation, resulting in clustering of the actin cytoskeleton (Fig. 8).

What are the pathophysiological consequences of the modification of actin at Thr148? To elucidate the functional consequences of the effects of TccC3, the phagocytic activity of insect larvae hemocytes was studied in the presence of *Escherichia coli* particles. The cellular uptake was monitored by fluorescence of internalized particles into low-pH endosomes. These studies showed that the TcdA1, TcdB2 and TccC3 complex potently inhibits the phagocytosis by hemocytes [110]. Therefore, ADP-ribosylation of actin at Thr148 in immune cells of insect larvae might be an important strategy for the bacteria to prevail in an otherwise extremely efficient immune system of insect hemocytes.

As already mentioned, *P. luminescens* also produces the binary actin–ADP-ribosylating toxin photox, which modifies actin at Arg177 to inhibit actin polymerization. Thus, a bidirectional modulation of actin (induction of polymerization of actin by TccC3 and induction of depolymerization of actin by photox) appears to be necessary for the optimal interaction of *P. luminescens* with its host nematodes and its host insect larvae.

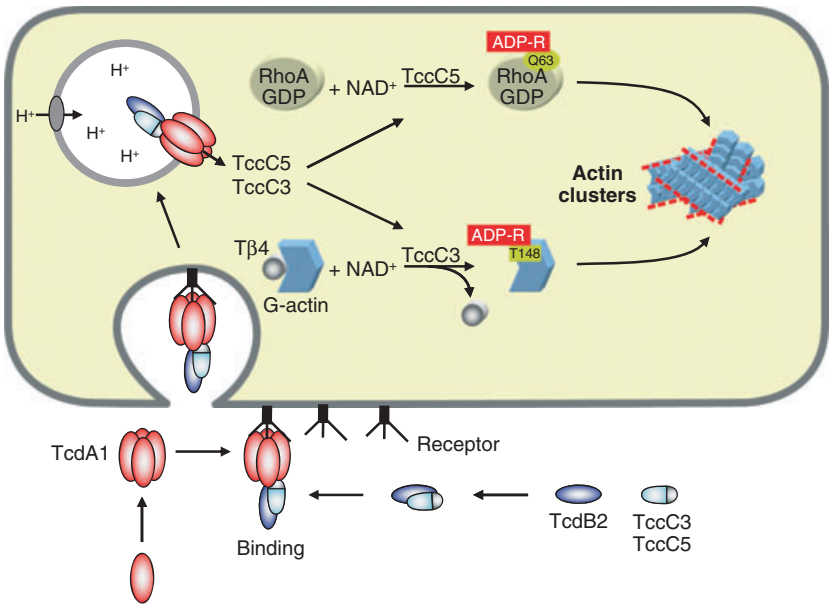

Fig. 8. Action of *Photorhabdus luminescens* toxins on the actin cytoskeleton. The *P. luminescens* toxin complex consists of at least three different types of toxin proteins called TcA, TcB and TcC. Many orthologs and paralogs of the components exist. Component TcA of the toxin complex forms tetramers and is most probably involved in receptor binding and protein translocation of the biologically active component TcC. The role of the TcB component is not clear so far. Component TcC, which has a highly conserved N-terminal region, possesses a C-terminal ADP-ribosyltransferase activity, the substrate specificity of which varies in paralogs. TccC3 ADP-ribosylates actin at Thr148 thereby preventing the binding of the actin-sequestering protein thymosin-β4 to G-actin and favoring actin polymerization. TccC5 ADP-ribosylates Rho proteins at Gln63, thereby persistently activating Rho GTPases, which cause stress fiber formation and facilitate actin polymerization. Together, TccC3 and TccC5 cause clustering of F-actin.

Toxins inducing actin crosslinking

Actin is directly affected also by a family of toxins which catalyze its chemical crosslinking [118]. The prototype of these toxins is $MARTX_{vc}$ (multifunctional, autoprocessing RTX toxin) from *Vibrio cholerae* with a mass of about ~ 500 kDa (Fig. 9). MARTX toxins are multimodular proteins, having different functional domains, which most probably are processed and released during the uptake mechanism in target cells. Release of toxin modules is achieved by auto-catalytic processing by an inherent cysteine protease activity, which is typically activated by inositol hexakisphosphate binding [119]. In the case of $MARTX_{vc}$ an actin crosslinking domain (ACD), a Rho GTPase inactivating domain (RID) and a domain of unknown function are released. MARTX containing ACD domains are also produced by *A. hydrophila* and *Vibrio vulnificus* [118] (Fig. 9).

The major effect of these toxins in target cells is the depolymerization of the actin cytoskeleton by covalent crosslinking of actin monomers to dimers, trimers and high molecular mass oligomers that are polymerization incompetent and therefore lead to cell rounding [120]. By mass spectrometric analyses and crystallographic approaches it was shown that ACD causes covalent crosslinking of actin by forming iso-peptide bonds between Lys50 and Glu270 of actin (see Fig. 1 for the location of these residues). Crosslinking causes dimer, trimer or higher order oligomer formation [120]; however, in all cases Lys50 and Glu270 are involved [121]. Crosslinking preferentially starts with monomeric actin [120] even when the actin is complexed to monomer stabilizing proteins like thymosin-β4 or profilin. The further crosslinking of dimers to higher order oligomers occurs at a lower rate. Also yeast actin is substrate for this modification, but exchange of Lys50 or Glu270 for other amino acids completely blocks crosslinking.

Lys50 is located at the so-called DNaseI binding loop [20] and Glu270 on the subdomain 3/4 loop (also termed the hydrophobic plug, see Fig. 1) and both are essentially involved in intrastrand and interstrand interactions respectively of F-actin subunits [122]. In F-actin these two hydrophobic loops do not contact each other; therefore their crosslinking distorts the normal F-actin interfaces and forces them into an orientation that is incompatible with polymerization to a

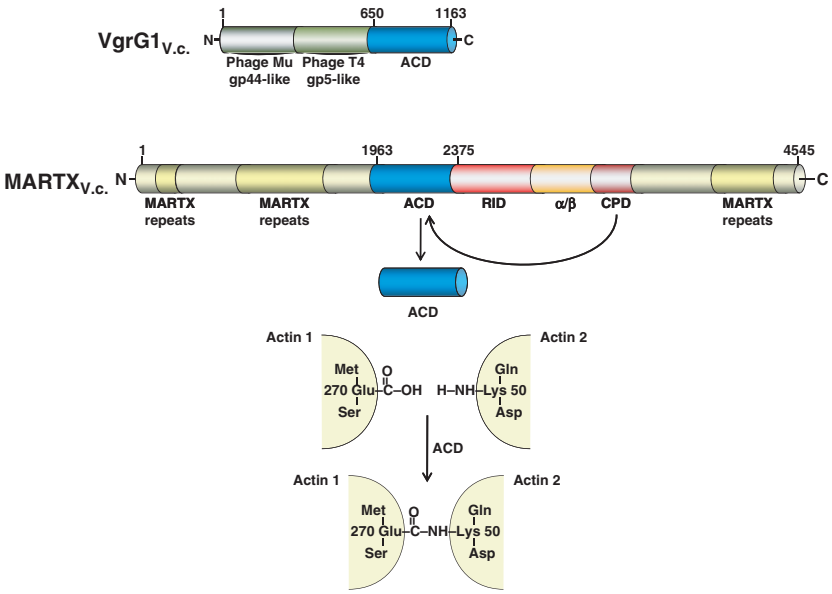

Fig. 9. Structure of actin crosslinking toxins. MARTX (multifunctional, autoprocessing RTX toxin) of *Vibrio cholerae* is a very large multi-module protein, which consists of several conserved glycine-rich RTX motifs (MARTX repeats), a Rho GTPase inactivating domain (RID), an α/β hydrolase (α/β), a cysteine protease domain (CPD) and an actin crosslinking region (ACD). The CPD is suggested to be involved in mobilization and release of (arrow) the ACD, which then catalyzes crosslinking of G-actin. Crosslinking is caused by bond formation between Glu270 and Lys50 of two actin molecules. The ACD domain is also found at the C-terminus of VgrG1 protein from *V. cholerae*. 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.

functional F-actin filament. Surprisingly, it was reported that polymerization was partially rescued in the presence of phalloidin or cofilin [121]. Indeed, electron microscopy after negative staining revealed the formation of short albeit often distorted filaments in the presence of phalloidin or cofilin; however, the functionality of these polymers was not further analyzed. Nevertheless, this particular mode of actin modification aims to severely compromise actin-dependent cytoskeletal functions like phagocytosis allowing the pathogens to escape immune cell surveillance and to increase their dissemination within the host organism.

Molecular mechanism of crosslinking

Crosslinking of actin by the toxin occurs *in vitro* and *in vivo*. Actually, the *in vitro* crosslinking of actin by ACD requires G-actin, ATP and magnesium [123]. Interestingly, ATP is not essential for actin but for the toxin-catalyzed reaction. The toxin domain ACD is an ATPase, which needs ATP for the catalytic reaction of the iso-peptide bond formation [121]. The catalytic mechanism appears to be similar to that caused by glutamate synthetase [124]. It has been proposed that first Glu270 of actin is activated by phosphorylation and subsequently the crosslinking occurs by release of the phosphate. This reaction is very similar to the attachment of ammonia to glutamate to form glutamine [123] (Fig. 9).

Actin crosslinking enzymes as part of VgrG1 proteins

ACD is also found in VgrG1 proteins from *V. cholerae* strains. VgrG proteins are part of the complex type VI secretion system of various Gram-negative bacteria [125–128]. They are essential for the secretory function of this machine but are also secreted by themselves via this system. The proteins exhibit high similarity with the tail parts of various bacteriophages. The C-terminal part contains specific effector domains. As mentioned above, a VgrG protein from *A. hydrophila* harbors a C-terminal actin ADP-ribosyltransferase domain, which modifies Arg177. In the case of *V. cholerae* VgrG1, the ACD domain forms the C-terminus of the protein (Fig. 9).

Conclusions

For efficient invasion and dissemination many bacteria have developed convergent strategies to escape the immune surveillance of the host organism and to

modify particular cellular activities for their intracellular or extracellular survival. Frequently, bacteria do not conquer the genetic material of their hosts in order to reprogram it in favor of their own replication; instead they manipulate the host cell metabolism and/or its intracellular organization to form a niche to prevail. Very often, they do this by producing toxin/effector proteins, which possess specific enzymatic activities allowing them to efficiently modify particular host cell target proteins. Using highly sophisticated delivery systems, including pore formation and cellular uptake from acidic endosomal compartments, cell entry after retrograde transport from the endoplasmic reticulum via the sec61 translocator or direct cell delivery by microsyringe-like nanomachines, the bacterial toxins/effectors enter the cytosol and modify eukaryotic targets by glycosylation, adenylylation, deamidation, proteolysis or ADP-ribosylation.

The cytoskeletal protein actin is a frequently targeted substrate protein, modified in a manner that compromises its proper functions. Actin is constantly cycling between monomeric and polymeric state in order to fulfil its dynamic functions, including its diverse roles in innate and adapted immune responses. Therefore, disturbing the dynamic behavior of actin as achieved by ADP-ribosylation will profoundly disturb the cellular response to pathogen invasion. Notably, the bacterial ADP-ribosyltransferases have been specifically tailored to modify residues like Arg177, which are essential for its proper function, i.e. the ability to polymerize to F-actin filaments. Indeed, it was only the analysis of the toxin specificity that led to the recognition of the importance of this particular residue for this process. Similarly, ADP-ribosylation of Thr148 by the TccC3 toxin of *P. luminescens* clearly emphasized the essential role of the actin–thymosin-β4 interaction for the maintenance of the correct dynamic behavior of actin for cell survival.

However, one has to keep in mind that in most cases the targeting of the cytoskeleton by bacterial protein toxins and effectors is much more complex. Studies from recent years have shown that numerous pathogens produce toxins and bacterial effectors during host–pathogen interactions in a precise time- and space-dependent manner to specifically support defined phases of the infection process. This explains the frequent findings that the same species of bacteria may produce different toxins and effectors, which cause polymerization as well as depolymerization of the actin cytoskeleton. For example, *P. luminescens* produces one toxin which inhibits actin polymerization (photox) and another which induces actin polymerization (TccC3/TccC5). Another example is *S. enterica*, a producer of effectors which indirectly or directly induce actin polymerization (SipA/C, SopE) or cause depolymerization of the actin cytoskeleton (SptP) [129–131]. A large number of bacterial factors have been identified that act via Rho GTPases, which are master regulators of the actin cytoskeleton, on target cells. Many of these bacterial factors hijack the physiological control mechanism by mimicking the regulatory role of Rho GAP or Rho GEF proteins, thereby fine-tuning the activity state of Rho GTPases and modulating the specific function of the cytoskeleton. This may lead, for example, to inhibition of phagocytosis of pathogens by macrophages but to enhanced adhesion of bacteria and stimulation of non-professional phagocytosis of invasive bacteria. Thus, bacteria are capable of modulating the cytoskeleton, thereby using the multitude of functions of the cytoskeleton for their own advantage.

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