

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

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

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

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1 Introduction

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

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

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

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

2 Discovery of ACD

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

3 ACD-Containing Toxins and ACD-Producing Organisms

3.1 ACD Protein Family

3.1.1 Originally Identified ACD Toxins

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

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

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

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

3.1.2 Extended List of ACD Toxins

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

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

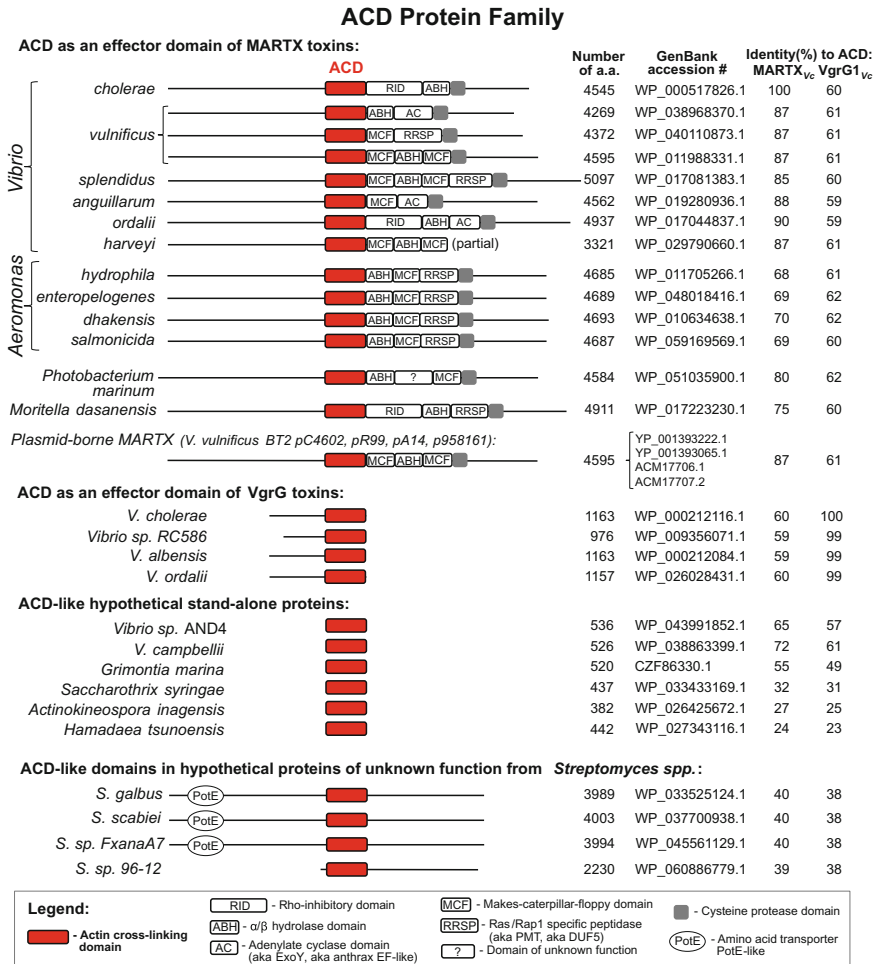


Fig. 1 ACD protein family. The NCBI protein BLAST search identified ACD orthologs present in different microorganisms either as part of larger proteins (both, already known toxins and previously uncharacterized proteins with ACD-like domains) or as stand-alone entities. Proteins are shown in scale with the total number of amino acids indicated for each protein. When available, reference sequence (RefSeq) non-redundant protein accession numbers are provided (O’Leary et al. 2016). The percent of identity of each protein with ACD_{MARTX_{Vc}} and ACD_{VgrG1_{Vc}} is shown

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

3.2 ACD-Producing Organisms

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

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

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

3.3 ACD Pathogenesis

3.3.1 Pathological Role of ACD-Containing Toxins in Animal Models

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

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

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

3.3.2 Cellular Toxicity of ACD

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

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

4 Mechanism of the Cross-Linking Reaction

4.1 Substrate and Cofactors of ACD

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

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

In addition to actin, two other factors, Mg²⁺ cations and ATP, appeared to be *required to support the reaction* (Cordero et al. 2006), but their exact role was obscured by the fact that both molecules have strong and specific influence on the structure of the substrate, i.e., actin. ATP is an irreducible integral part of actin structure, while binding of Mg²⁺ cations at the high-affinity site (in complex with ATP) and several low affinity sites promote conformational changes favoring actin polymerization. Furthermore, actin is an ATPase that hydrolyzes ATP upon polymerization. Hence, replacing ATP with a non-hydrolyzable analog, AMP-PNP, strongly reduced, but did not abolish the ACD-cross-linking reaction, possibly due to a residual ATP leakage from the nucleotide-binding cleft of actin (Cordero et al. 2006).

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

4.2 Nature of the Cross-Link

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

4.3 Structure of ACD and Its Homology to Glutamine Synthetases

4.3.1 ACD Homology to Glutamine Synthetases

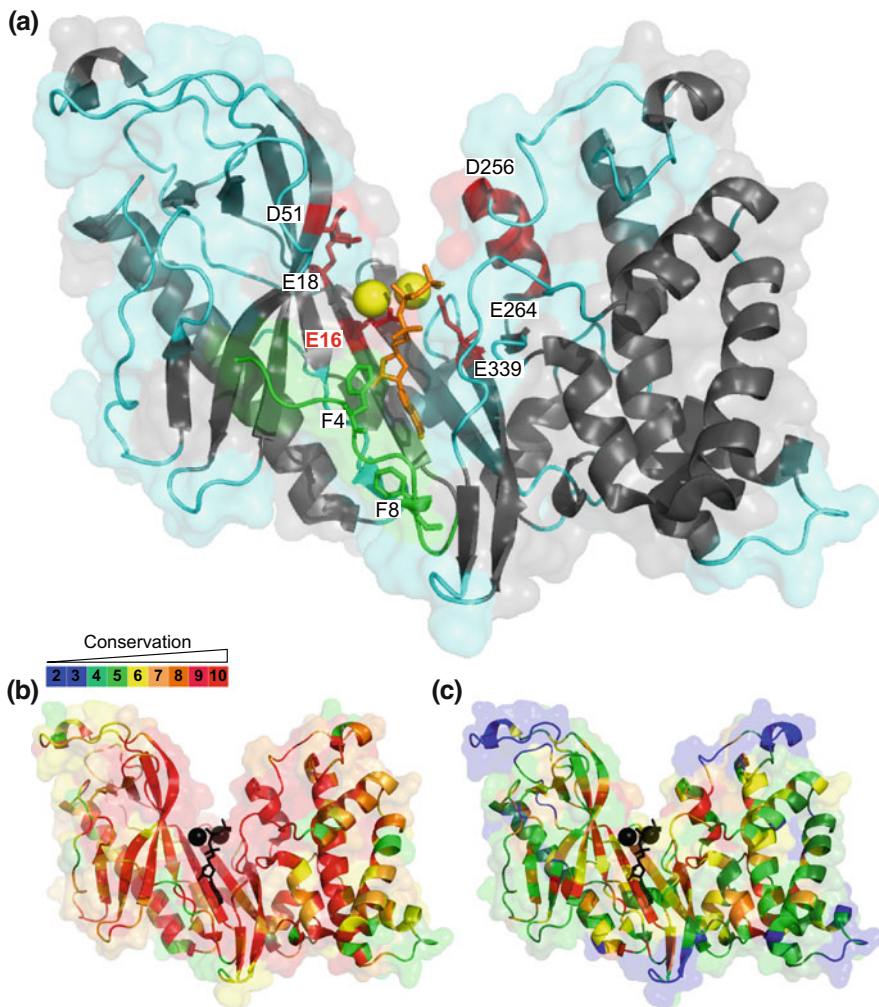
A lack of substantial sequence similarity to any known protein precluded homology-based identification of the ACD active site and called for different approaches. In an elegant and extensive study, Geissler and colleagues employed a combination of linker scanning mutagenesis and error-prone PCR mutagenesis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

The first step in the reactions catalyzed by GS/GCS enzymes is the activation of glutamate by transferring the terminal phosphoryl group from ATP to the glutamate's side chain carboxyl with the formation of an acyl-phosphate intermediate (Midelfort and Rose 1976; Orłowski and Meister 1971). Based on the overall similarity of the active sites of these enzymes with the active site of ACD (Durand et al. 2012; Geissler et al. 2009), it had been first proposed and then confirmed experimentally (Kudryashova et al. 2012) that in a similar manner *ACD activates the side chain of E270 of one actin monomer for its subsequent cross-linking to the amine group of K50 on another actin molecule*. In vitro, treatment of K50C yeast actin mutant with ACD in the presence of γ ATP³² resulted in the incorporation of the radioactive phosphoryl group into the mutant. The resulted *acyl-phosphate derivative* can be detected as a radioactive band on an SDS-gel. The addition of the phosphoryl group to E270 was confirmed by the lack of radioactivity in the E270Q yeast actin mutant, while the role of the acyl-phosphate in the catalysis was corroborated by an observation that the radioactive phosphate was removed from E270 of K50C-actin upon its cross-linking to K50 of the E270Q-mutant with a formation of actin dimer (Kudryashova et al. 2012).

4.3.2 Crystal Structure of ACD

Initial attempts of several groups to crystallize ACD_{MARTXVc} failed, while the homologous ACD_{VgrG1Vc} was successfully crystallized and the structure solved at a 2.5 Å resolution (Durand et al. 2012). Structures were solved with ADP, ATP, AMP-PNP, or in the nucleotide-free state in the presence of either Mg²⁺ or Mn²⁺ and confirmed a high structural homology of the ACD active site with those of GS/GCS enzymes. ACD has a characteristic butterfly-like shape with two well-defined relatively flat domains (“wings”) and the nucleotide positioned in the cleft between the domains (Fig. 2a). The N-terminal domain is largely composed of β -strands, while the C-terminal domain is largely α -helical.

At the core of the enzyme is an eight-stranded antiparallel β -sheet, six strands of which belong to the N-terminal domain and two others are contributed by the C-terminal domain, which also contains seven α -helices. One of these helices (residues 256–264 in ACD_{VgrGVc} crystallized sequence) is highly conserved in all ACD orthologs from MARTX and VgrG toxins and throughout different species as it comprises part of the catalytic site and is involved in coordination of the substrates (Fig. 2a; red helix). The ATP molecule (Fig. 2a; orange sticks) is bound in the “saddle” formed by the core β -sheet and coordinated by two divalent cations, which can be either Mg²⁺ or Mn²⁺ (Fig. 2a; yellow spheres). In the apo-state, the structure of ACD is stabilized upon binding of a conserved N-terminal peptide (Fig. 2a; shown in green) to the nucleotide cleft region between the domains. Given the overall low stability of ACD ($\Delta G = 11.5$ kJ/mol; Kudryashova et al. 2014a), such stabilization can be critical for protein integrity in the absence of the nucleotide (e.g., upon secretion into extracellular milieu before entering into the host cell cytoplasm). Recombinant removal of the peptide destabilizes ACD to the extent that it cannot be produced in *E. coli* (Durand et al. 2012). In the presence of ATP, the peptide is displaced from the cleft, but in this state, it contributes to the formation of the front-bottom part of the nucleotide-binding pocket, particularly with its two highly conserved phenylalanine residues (Fig. 2a; green sticks) contacting the nucleoside moiety of ATP. It is tempting to speculate that the mechanism of protein destabilization by dislodging the N-terminal peptide from the cleft is essential for spontaneous unfolding of ACD toxins, critical for crossing the host cell membranes by MARTX effector domains via narrow pores. Notably, in all MARTX toxins containing ACD, it always precedes all other effector domains (Fig. 1), suggesting that it may play an important role in priming the unfolding of the entire effector domain region needed for the translocation process. This hypothesis is indirectly supported by the observation that the ACD domain of MARTX from psychrotrophic *A. hydrophila* is less stable (by ~ 10 °C) than its counterpart from mesophilic *V. cholerae*, whereas all other effector domains from MARTX toxins of both bacteria share similar thermal stability (Kudryashova et al. 2014a).



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

◀ **Fig. 2** Structure and conservation of ACD toxins. **a** PyMOL-generated image of the X-ray structure of ACD_{VgrG1Vc} (PDB 4DTH; Durand et al. 2012). Four ACD active site residues (E16, E18, D51, and E339, numbered as in crystallized ACD_{VgrG1Vc}) critical for catalysis (Geissler et al. 2009) are shown as *red sticks*; essential catalytic residue E16 is highlighted in *red*. Highly conserved helix (residues 256–264) comprising part of the catalytic site is shown in red. N-terminal peptide is colored in *green*, with two highly conserved phenylalanine residues (F4 and F8; *green sticks*). ATP molecule is represented as *orange sticks*; Mg²⁺ cations are shown as *yellow spheres*. **b, c** The conservation scoring was performed using the PRALINE multiple sequence alignment program (Simossis and Heringa 2005) and color-coded on PyMOL-generated images of the X-ray structure of ACD_{VgrG1Vc} (PDB 4DTH). Residues that are 100 % conserved across all compared sequences are colored in *red*; those with the lowest degree of conservation are in *blue* (color scheme is provided). ATP molecule and Mg²⁺ cations are shown in *black*. **b** Conservation of highly homologous ACD orthologs from all MARTX and VgrG1 toxins as well as stand-alone ACDs of *Vibrio* spp. (AND4 and *V. campbellii*). **c** Conservation of newly identified, distantly related ACD-like proteins of *Streptomyces* spp. and non-*Vibrio* stand-alone proteins from *S. syringae*, *Grimontia marina*, *A. inagensis*, and *H. tsunoensis*, as compared to each other and to ACD_{VgrG1Vc}.

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

4.3.3 Metal Cofactors

Out of the several divalent cations tested experimentally (Ca²⁺, Mg²⁺, Zn²⁺, and Mn²⁺) only Mg²⁺ and Mn²⁺ supported the cross-linking reaction (Cordero et al. 2006; Durand et al. 2012). However, one study reported that the ACD_{VgrG1Vc} toxin was more active in the presence of Mn²⁺ (Durand et al. 2012), whereas another group found that ACD_{MARTXVc} was notably more active in the presence of Mg²⁺ (Cordero et al. 2006). To resolve this uncertainty, we reexamined the specific activity of the ACD domains from MARTX_{Vc}, MARTX_{Ab}, and VgrG1_{Vc} sharing 60–68 % identity and 74–80 % homology. We found that at concentrations of the divalent cations below 250 μM, all three ACD orthologs were more active in the presence of Mn²⁺. The situation was completely reversed, however, at higher concentrations of the cations, as the reaction was strongly potentiated by Mg²⁺ concentrations above 1 mM, but strongly inhibited by equal concentrations of Mn²⁺, either in the absence or presence of Mg²⁺. As a result, the rates were 17- to 30-fold higher at 2 mM of Mg²⁺ as compared to the ones at equal concentration of Mn²⁺ (data not shown). Interestingly, similar inhibition by high Mn²⁺ was reported for brain avian and mammalian glutamine synthetases (Tholey et al. 1987; Yamamoto et al. 1987) suggesting that these properties are not accidental but rather dictated by a common enzymatic mechanism. Such dependence reflects a higher affinity of Mn²⁺ to both glutamine synthetases (Wedler et al. 1982) and ACDs, as confirmed in thermal denaturation experiments by a very prominent 8–10 °C raise in the enzyme stability in the presence of Mn²⁺/ATP as compared to only 1–2 °C stabilization by Mg²⁺/ATP (our unpublished data). Even though our results indicate higher activity of all three

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

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

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

The actin cytoskeleton is a complex hierarchy of interdependent structures brought together through regulated interactions of G- and F-actin with hundreds of ABPs. Taking these interactions into consideration is essential for understanding of pathogenic mechanisms triggered by actin-targeting toxins. Manipulation with the G/F-actin equilibrium in cultured cells and experiments with purified actin unequivocally pointed on G- rather than F-actin as the substrate for ACD. Yet, G-actin is not a likely physiological substrate as in the cell it is rarely found in a pure form. The monomeric pool of cellular actin is maintained by several ABPs, mainly profilin and thymosin- β 4. Importantly, complexes of both proteins with actin can be cross-linked by ACD as efficiently or better than pure G-actin (Kudryashov et al. 2008a), likely owing to the ability of these proteins to inhibit spontaneous polymerization and thereby preserve the cross-linking-competent form of actin. Accordingly, both proteins bind to actin away from the E270 and K50 residues and do not interfere with binding of actin to ACD in the model proposed by Durand et al. (2012) (Fig. 3). Therefore, the actual physiological substrates of ACD are complexes of G-actin with one of the actin-sequestering proteins. In contrast to profilin and thymosin- β 4, another essential actin partner, cofilin, strongly inhibited the cross-linking of monomeric actin, while accelerating the formation of oligomers from prepolymerized actin (Kudryashov et al. 2008a). Cofilin is both a G- and F-actin-binding protein, whose major cellular function is to promote high rates of actin dynamics via accelerated severing and depolymerization of aged filaments (Bernstein and Bamburg 2010). The facilitated cross-linking of polymerized actin can be explained by faster filament recycling (i.e., a higher amount of actin passing through the monomeric state per unit of time). The inhibited cross-linking of initially monomeric actin likely has two components: (1) the ability of cofilin to accelerate polymerization (and thus, promptly deplete the

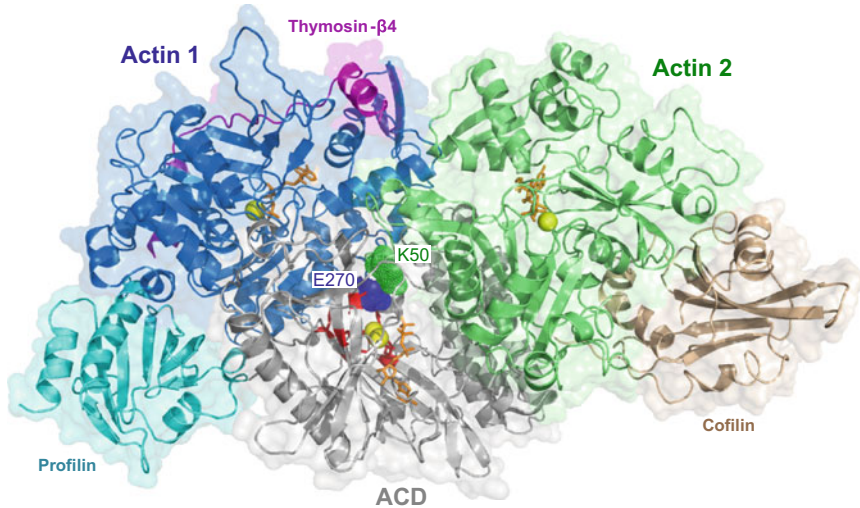


Fig. 3 Model of ACD binding to two molecules of the substrate (actins). As proposed and modeled by Durand et al. (2012), actin molecule donor of E270 (Actin 1 colored in dark blue) binds at one face of ACD (gray), whereas the actin molecule donor of K50 (Actin 2 in green) binds to the opposite face of ACD. The orientation of the actin monomers is such that the H-plug of one and the D-loop of another are positioned in the ACD nucleotide-binding cleft with K50 and E270 residues (green and blue spheres, respectively) oriented toward each other and in proximity to the terminal phosphoryl group of ATP (orange sticks). Notice that major G-actin-sequestering proteins profilin (cyan) and thymosin-β4 (magenta) as well as a G-/F-actin-binding protein cofilin (beige) do not clash with ACD, permitting cross-linking of physiologically relevant G-actin complexes. Four ACD active site residues (E16, E18, D51, and E339; Geissler et al. 2009) are shown as red sticks. ATP molecules bound to actin and ACD are represented as orange sticks; Mg²⁺ cations are shown as yellow spheres. The image was generated in PyMOL by aligning a model of ACD_{V_{gr}G1Vc} bound to two actin monomers (generously provided by Dr. Cambillau) with the following PDB structures: ACD_{V_{gr}G1Vc} (PDB 4DTH; Durand et al. 2012) and G-actin complexes with profilin (PDB 2BTF; Schutt et al. 1993), thymosin-β4 (PDB 4PL7; Xue et al. 2014), and cofilin (PDB 3DAW; Paavilainen et al. 2008). Profilin and thymosin-β4 are positioned at the same actin monomer for presentation purposes and do not represent an actual physiological complex

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

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

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

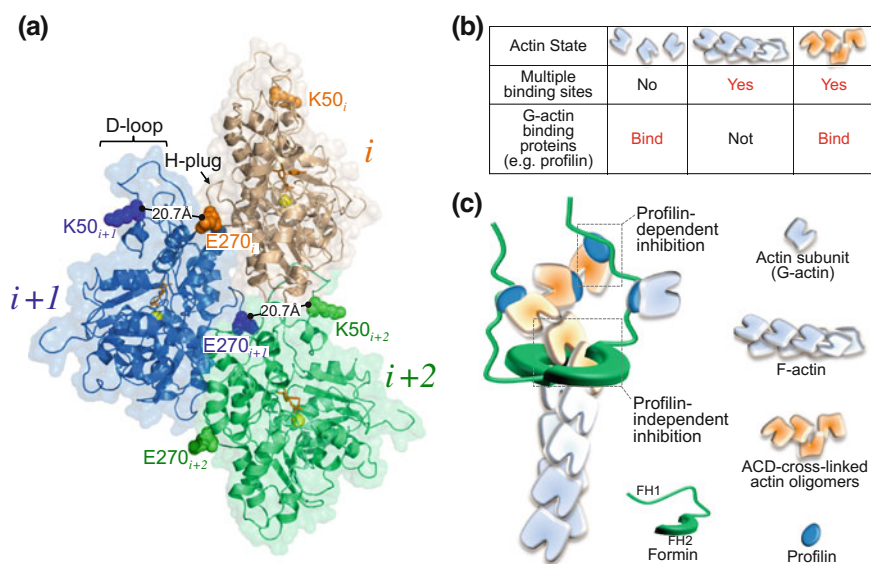


Fig. 4 Two layers of ACD toxicity. **a** Three individual actin subunits [*i* (cream/orange), *i* + 1 (blue), and *i* + 2 (green)] are shown in PyMOL-generated image of the F-actin structure (PDB 3J8I; Galkin et al. 2015). K50 and E270 residues of each actin subunit are labeled and represented by colored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

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

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

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

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

6 Conclusions and Future Perspectives

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

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

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

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