The Actin Cross-linking Domain of the Vibrio cholerae RTX Toxin Directly Catalyzes the Covalent Cross-linking of Actin*

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Vibrio cholerae is a Gram-negative bacterial pathogen that exports enterotoxins to alter host cells and to elicit diarrheal disease. Among the secreted toxins is the multifunctional RTX toxin, which causes cell rounding and actin depolymerization by covalently cross-linking actin monomers into dimers, trimers, and higher multimers. The region of the toxin responsible for cross-linking activity is the actin cross-linking domain (ACD). In this study, we further investigated the role of the ACD in the actin cross-linking reaction. We show that the RTX toxin cross-links actin independently of tissue transglutaminase, thus eliminating an indirect model of ACD activity. We demonstrate that a fusion protein of the ACD and the N-terminal portion of lethal factor from Bacillus anthracis (LFNACD) has cross-linking activity in vivo and in crude cell extracts. Furthermore, we determined that LFNACD directly catalyzes the formation of covalent linkages between actin molecules in vitro and that Mg²⁺ and ATP are essential cofactors for the cross-linking reaction. In addition, G-actin is proposed as a cytoskeletal substrate of the RTX toxin in vivo. Future studies of the in vitro cross-linking reaction will facilitate characterization of the enzymatic properties of the ACD and contribute to our knowledge of the novel mechanism of covalent actin cross-linking.

The causative agent of the diarrheal disease cholera is the Gram-negative bacterial pathogen Vibrio cholerae, which is transmitted to the human host following consumption of contaminated food or water. Upon colonization of the upper intestine, V. cholerae produces the major virulence factor cholera toxin, which ADP-ribosylates the α-subunit of the G, GTP-binding protein and constitutively activates the adenylate cyclase complex. The subsequent increase in the cAMP levels in intestinal epithelial cells leads to the opening of Cl⁻ ion channels, resulting in the profuse diarrhea that is the hallmark of cholera. If untreated, the disease can progress to severe dehydration, and case-fatality rates can reach as high as 50% (1).

The V. cholerae RTX⁴ toxin, which is encoded by rtxA, was discovered through a combination of genomic sequence analysis, genetic mapping, and representational difference analysis (2). The rtxA gene, which is tightly linked to the cholera toxin-encoding ctx genes, is deleted in the classical V. cholerae O1 isolates, but is expressed by both the El Tor O1 and O139 strains, which are responsible for the current cholera pandemic (2, 3). The RTX toxin is also produced by non-O1/non-O139 isolates, but it has been suggested that the toxin may contribute to the emergence of pathogenic non-O1/non-O139 strains (4).

The full-length 4545-amino acid RTX toxin is predicted to be 484,000 Da in size and is secreted from the bacterium by an atypical type I secretion system that requires two transport ATPases (5). Although related to the RTX family of pore-forming toxins, the V. cholerae RTX toxin seems to be the founding member of a new family of RTX exoproteins. These proteins, encoded by V. cholerae, Vibrio vulnificus, Photobrabdus luminescens, and Xenorhabdus sp., are all larger than 3500 amino acids and share consensus 20-residue N-terminal glycine-rich repeats in addition to 18-residue C-terminal repeats that include the nonapeptide motif GGGXGDXXXX common to all RTX exoproteins. However, the central regions of these proteins vary dramatically, and it has been predicted that each toxin will have a distinct repertoire of cellular activities (5, 7).

The V. cholerae RTX toxin is multifunctional and has two distinct mechanisms for cell rounding (7). The first mechanism identified involves the covalent cross-linking of cellular actin, resulting in the depolymerization of actin stress fibers and an increase in paracellular permeability (8, 9). A region of the toxin located between residues 1963 and 2375, denoted as the actin cross-linking domain (ACD), has been identified recently as the portion of the toxin responsible for cross-linking activity. Transient expression of this domain in both transformed African green monkey kidney fibroblast (COS-7) and human laryngeal epithelial (HEp-2) cells leads to the formation of cross-linked actin species, demonstrating that expression of 412

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The abbreviations used are: RTX, repeats in toxin; ACD, actin cross-linking domain; LFv, lethal factor N-terminal portion; PA, protective antigen; TGase, transglutaminase; PBS, phosphate-buffered saline; AMP-PNP, adenylyl-5’-yl imidodiphosphate; LatB, latrunculin B; Dol11, dolastatin 11.
amino acids of the toxin in the cytosol is sufficient to initiate actin cross-linking in the target cell (7). It remains unclear whether the ACD can bind actin and catalyze the covalent linkage directly or whether the toxin activity stimulates a host cell cross-linking enzyme.

A truncated form of the *Bacillus anthracis* lethal factor (LF₅₆) has been shown previously to mediate cytosolic delivery of heterologous fusion proteins through the entry mechanism used by the anthrax toxin (10). Briefly, protective antigen (PA) binds to the anthrax toxin receptor; PA is processed to its active 63-kDa form (PA63); and the PA63 fragment oligomerizes into a stable heptamer (11–13). The LF₅₆ fusion protein binds to (PA63)₇; the complex enters the host cell through receptor-mediated endocytosis; and acidification of the vacuole results in PA pore formation and translocation of the LF₅₆ fusion protein to the cytosol (14–16). This system has been used to study the mechanism of action of other bacterial toxins, including *Corynebacterium diphtheriae* diphtheria toxin and *Clostridium difficile* toxin B (10, 17).

In this study, we have further defined the novel mechanism of covalent actin cross-linking by the *V. cholerae* RTX toxin. We found that cross-linking by the RTX toxin does not involve tissue transglutaminase (TГase), as suggested previously (9). We established that a fusion protein of LF₅₆ and ACD (LF₅₆/ACD) has cross-linking activity in *vivo* and in crude cell extracts. LF₅₆/ACD directly catalyzes the actin cross-linking reaction in the absence of host proteins, and Mg⁶⁺ and ATP are required cofactors in *vitro*. Finally, we identified G-actin or actin oligomers as potential cytoskeletal substrates of the cross-linking reaction in *vivo*. These results contribute to our overall understanding of the mechanism of action of the *V. cholerae* RTX toxin and demonstrate that the ACD cross-links actin by directly catalyzing the formation of a covalent linkage between actin monomers in the absence of eukaryotic protein intermediates.

**EXPERIMENTAL PROCEDURES**

**Strains, Cell Lines, and Reagents**—Bah1P (expressing the RTX toxin) and Bah2P (harboring a deletion in *rtxA*) are strains derived from *V. cholerae* El Tor Ogawa strain E7946 (9). KFV119 is a ΔhapAΔhlyA derivative of *V. cholerae* El Tor O1 Inaba strain N1691 containing an intact *rtxA* gene (7). Bacterial cultures were grown for 18 h at 30 °C in Luria broth containing streptomycin (100 μg/ml). TSA201 and HEp-2 cells were cultured at 37 °C with 5% CO₂ in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 units/ml streptomycin. All chemicals were purchased from Sigma.

**Tissue TГase Assay**—Cells treated with bacteria for 70 min were washed with phosphate-buffered saline (PBS), released in PBS with 0.1 mM EDTA, scraped and collected in RPMI 1640 medium, and pelleted at 6000 × g. The cell pellets were resuspended in SDS-PAGE sample buffer, boiled for 10 min, and analyzed by SDS-PAGE. Tissue TGase expression was detected with a 1:400 dilution of mouse anti-TГase monoclonal antibody (Lab Vision Corp., Fremont, CA) followed by a 1:5000 dilution of horseradish peroxidase-conjugated anti-mouse IgG (Sigma). Actin cross-linking was monitored by immunoblotting as described previously (9).

**Construction, Expression, and Purification of Proteins**—The pABII plasmid is a derivative of the pET15b plasmid (Novagen, Madison, WI) containing the DNA sequence for LF₅₆ with an N-terminal His₆ tag. To create a fusion to the ACD, the ACD sequence was amplified from the *V. cholerae* chromosome using primers LFACD-F (5′-GAAAGATCTCTAAGTGCCTAG-3′) and LFNACD-R (5′-AGAGATCTTAAGAGCGGTAATTTCGTCG-3′). The ~1.3-kb PCR product was cloned into the pCR-BluntII-TOPO vector (Invitrogen) and then subcloned as a BglII fragment into the BamHI site of the pABII expression vector (17). The resulting plasmid, pTCO24, expresses the ACD protein fused to the C terminus of LF₅₆. DNA sequencing of the insert in pTCO24 was performed at the Northwestern University Biotechnology Laboratory to confirm the accuracy of both the coding region and the junction between the LF₅₆ and ACD sequences.

LF₅₆ and the LF₅₆/ACD fusion protein were expressed and purified as described by Milne et al. (10, 18) following standard molecular biology protocols. Briefly, pABII or pTCO24 was transformed into *Escherichia coli* BL21(DE3) cells (Novagen), and bacterial cultures were grown in Luria broth supplemented with ampicillin (100 μg/ml) to _A₅₆₀_ = 0.6–1.0. Protein expression was induced for 2 h after the addition of 1.0 mM isopropyl β-D-thiogalactopyranoside at 37 °C, and bacterial cells were pelleted at 5000 × g for 15 min at 4 °C. The pellet was resuspended in Buffer A (20 mM Tris (pH 8.0) and 0.5 mM NaCl) with 5.0 mM imidazole. Following the addition of lysozyme (1 mg/ml), the cell suspension was incubated for 30 min on ice and sonicated in the presence of 1% Triton X-100 with a Branson Sonifier 450 digital cell disruptor at 35% amplitude (15-s pulses for a total of 4 min). The cell lysate was incubated with Complete EDTA-free protease inhibitor mixture (Roche Applied Science), 5.0 μg/ml DNase I, and 5.0 μg/ml RNase I and cleared by centrifugation at 10,000 × g for 30 min at 4 °C.

The soluble fraction was passed through a 0.45-μm pore syringe filter, loaded onto a 1-ml HisTrap HP column (GE Healthcare) equilibrated in Buffer A, and purified on an AKTA purifier FPLC system (GE Healthcare). The fusion protein was eluted at 107 mM imidazole using a linear gradient of 5–250 mM imidazole in Buffer A. All fractions containing the His₆-tagged protein were pooled, analyzed by SDS-PAGE for purity, and concentrated on a Centricon YM-30 microconcentrator (Millipore, Billerica, MA). The proteins were loaded onto a 5-ml HiTrap desalting column (GE Healthcare) and exchanged into Buffer A. Fractions containing the desalted protein were pooled, concentrated, and stored in 10% glycerol at −80 °C. PA was purified using plasmid PA-pET15b as described by Voth et al. (19).

**SDS-PAGE and Immunoblot Analysis of Purified Proteins**—Protein samples were analyzed by SDS-PAGE and visualized with Coomassie Brilliant Blue R-250 or transferred to Hybond-ECL nitrocellulose membranes (GE Healthcare). The proteins were immunoblotted either with a 1:20,000 dilution of mouse anti-His₆ monoclonal antibody (Sigma) followed by a 1:5000 dilution of horseradish peroxidase-conjugated anti-mouse IgG or with a 1:5000 dilution of rabbit anti-ACD polyclonal anti-
body followed by a 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Sigma). The anti-ACD antibody was generated by immunization of a New Zealand White rabbit with the synthetic peptide VESRKEAMLWLAKEFTDH-COOH, which corresponds to amino acids 2066–2083 of the RTX protein sequence (2), using standard procedures (Protein-Tech Group, Inc., Chicago, IL).

In Vivo Actin Cross-linking Assay—Approximately $2.5 \times 10^5$ HEP-2 cells seeded in a 12-well dish were incubated with PA and either LF$_N$ or LF$_N$ACD or treated with PBS-washed KFV119 bacteria at a multiplicity of infection of $\sim 200$. Cell rounding was monitored with a Nikon Eclipse TS100 inverted microscope. Phase-contrast images were acquired at $\times 200$ magnification with an inverted Leica DMIRE2 microscope equipped with a C4742-95–12ERG digital charge-coupled device camera (Hamamatsu Photonics, Tokyo, Japan) and the Openlab software program (Improvision, Coventry, UK). For some experiments, cells were washed and scraped in PBS, centrifuged at $4500 \times g$ for 5 min, resuspended in SDS-PAGE sample buffer, and boiled for 10 min in preparation for the detection of actin cross-linking by immunoblotting as described previously (9).

In Vitro Actin Cross-linking Assay—For the preparation of cell extracts, $\sim 4 \times 10^6$ HEP-2 cells were plated onto two 100-mm dishes, washed and scraped in PBS, and sonicated with a Conoco Sonicl disruptor (5-s pulses for 1 min). Cell lysates were pelleted at $1000 \times g$ for 10 min at $4{\degree}C$. Protein concentrations were determined with a BCA protein assay kit (Pierce), and 120 $\mu$g of cleared lysates were added to 5.0 $\mu$g of either purified LF$_N$ or LF$_N$ACD protein. Each reaction was incubated at $37{\degree}C$ for 90 min and then boiled in SDS-PAGE sample buffer for 5 min. Actin cross-linking was detected by immunoblotting as described previously (9).

Rabbit skeletal actin was purified from rabbit back muscle as described by Spudich and Watt (20). Actin was added at a concentration of 10 $\mu$M to either purified LF$_N$ or LF$_N$ACD at 0.018 $\mu$M, and the reactions were incubated at $37{\degree}C$ for 20 min in Buffer B (5 mM HEPES (pH 7.5), 0.2 mM ATP, and 0.2 mM CaCl$_2$). Samples were boiled for 5 min in SDS-PAGE sample buffer, and cross-linked actin bands were detected on gels by staining with Coomassie Brilliant Blue R-250. The in vitro experiments were also performed at $22{\degree}C$, and the results were identical.

Actin Polymerization and Light Scattering—Actin polymerization was initiated following the addition of 2.0 mM CaCl$_2$, 2.0 mM MgCl$_2$, or 50 mM KCl. Light scattering measurements were performed in a Photon Technology International spectrofluorometer with the emission and excitation wavelengths set at 325 nm.

RESULTS

The RTX Toxin Cross-links Actin Independently of Tissue TGase Activity—The formation of covalent linkages between actin monomers following the treatment of eukaryotic cells with V. cholerae strains expressing the RTX toxin may be attributed either to the activity of the ACD directly or to an ACD-mediated modification of a eukaryotic cellular enzyme. Tissue TGase, a member of the family of Ca$^{2+}$-dependent enzymes that introduce e-$\gamma$-glutamyl)lysine side chain linkages between proteins, is the only known host enzyme that catalyzes the covalent cross-linking of cellular actin (21, 22). To address a model in which the ACD functions by activating tissue TGase, actin cross-linking was monitored in TSA201 cells, a human embryonic kidney cell line with undetectable tissue TGase production (23), by immunoblotting (Fig. 1A). When cells were incubated with a V. cholerae strain producing the RTX toxin, cross-linked actin species were detected by immunoblotting (Fig. 1B). These data indicate that actin is cross-linked in the absence of tissue TGase, showing that tissue TGase is not required for the actin cross-linking reaction.

The site of TGase cross-linking on the actin molecule is located at the glutamine residue at position 41 of the protein sequence (24). The involvement of this residue in the RTX-dependent cross-linking reaction was investigated by site-directed mutagenesis of the $\beta$-actin sequence from pEGFP-actin (Clontech, Mountain View, CA). An asparagine substitution (Q41N) had no effect on the cross-linking of green fluorescent protein-actin in the presence of an RTX-expressing V. cholerae strain (data not shown). In addition, the TGase-specific cross-link was not detected by immunoblotting with an anti-$N$-$\gamma$-glutamyl)lysine antibody (Abcam Inc., Cambridge, MA) in lysates from V. cholerae-treated cells (data not shown). These data provide further evidence that the actin cross-linking reaction is tissue TGase-independent. As there are no other enzymes known to covalently cross-link actin, an alternative model that favors a direct interaction between the ACD of the RTX toxin and cellular actin monomers was investigated.

Construction and Purification of LF$_N$ACD—An in vitro assay was developed to assess whether the ACD is sufficient to cross-link actin in the absence of other cellular proteins. Previous attempts to catalyze the cross-linking reaction with partially
purified holotoxin or recombinant ACD proteins were unsuccessful, and inactivation of the ACD may have been due to decreased stability or improper protein folding (9). To circumvent this problem, it was essential to first establish that the purified ACD protein has actin cross-linking activity in vivo before monitoring actin cross-linking in vitro. A fusion protein was constructed to take advantage of the entry mechanism of B. anthracis anthrax toxin, which has been used to translocate the catalytic domains of various bacterial toxins into eukaryotic cells (17).

The ACD sequence was amplified from the V. cholerae chromosome by PCR and cloned into a bacterial LFN expression vector (17). The resulting fusion protein, LFNACD, was purified on a nickel-chelating column and further desalted by gel filtration chromatography. The protein was analyzed by SDS-PAGE (Fig. 2A), and the observed mobility of the LFNACD fusion protein corresponded to an expected molecular mass of 81 kDa. This protein reacted with both the anti-His tag antibody and a peptide antibody raised against the ACD (Fig. 2, B and C). The molecular mass of the purified LFNACD protein was verified by matrix-assisted laser desorption ionization mass spectrometry to be 81,407 Da, which is accurate to 0.09% of the predicted mass. The protein containing the LFN portion alone (purified under similar conditions) reacted only with the anti-His tag antibody and not with the ACD-specific antibody.

**FIGURE 2. Characterization of LFNACD.** LFN (32 kDa) and LFNACD (81 kDa) were purified by affinity chromatography on a nickel-chelating column and desalted by gel filtration. Purified proteins were loaded onto 10% SDS-polyacrylamide gels and stained with Coomassie Blue (A), immunoblotted with mouse anti-His tag monoclonal antibody (B), or immunoblotted with rabbit anti-ACD polyclonal antibody (C). The positions of the Invitrogen BenchMark unstained (A) and prestained (B and C) protein standards are indicated on the left.

**FIGURE 3. LFNACD has actin cross-linking activity in vivo.** HEp-2 cells were incubated with PBS (untreated (Unt)), exposed to PBS-washed V. cholerae strain KFV119 (V.c.), or intoxicated with 31.7 nM purified PA and either 13.6 nM LFN or LFNACD. A, cell rounding was observed after 90 min, and phase-contrast images were acquired at ×200 magnification. B, treated cells were harvested; lysates were separated on 8% SDS-polyacrylamide gels; and the formation of cross-linked actin species was monitored by immunoblotting. The protein standards are labeled on the left.

Purified LFNACD Cross-links Actin in Vivo—Fusion of the ACD sequence to LFN allows for the cytosolic delivery of purified LFNACD proteins into target cells. To monitor the activity of LFNACD in vivo, HEp-2 cells were incubated with purified PA and either LFN or LFNACD at a molar ratio of 7:3 (25). Cell rounding was observed in LFNACD-intoxicated cells, and the rounding was markedly similar to that of cells incubated with KFV119, a V. cholerae strain expressing the full-length RTX toxin (Fig. 3A). Actin dimers, trimers, and higher order multimers were detected in cells treated with LFNACD or KFV119 by immunoblotting (Fig. 3B). These data demonstrate that the purified LFNACD protein has covalent actin cross-linking activity in vivo and is suitable for the development of an in vitro actin cross-linking assay.

The results from this purified protein assay confirm previous findings based on transient transfection that actin cross-linking activity is carried by the ACD region of the RTX toxin and that the ACD functions within the cell (7). In addition, these data show that the LFNACD protein must be translocated to the cytosol through the activity of PA and that LFNACD alone is incapable of functioning extracellularly. These data indicate that ACD activity does not lead to actin cross-linking by stimulating an endogenous signal transduction pathway that ultimately results in the formation of cross-linked actin proteins and that the ACD does not gain access to the target cell through a receptor interaction independent of PA. It can be further extrapolated that the ACD in the holotoxin is likely transported...
to the cell cytoplasm via the entry mechanism of the full-length RTX toxin and not by an ACD-specific pathway.

**LFN ACD Has Actin Cross-linking Activity in Crude Cell Extracts**—To further investigate the role of the ACD in the actin cross-linking reaction, purified LFN or LFNACD proteins were added to crude HEp-2 cell extracts. Cross-linked actin species were detected by immunoblotting in the LFNACD-treated extracts, but not in extracts incubated with LFN (Fig. 4).

To determine whether the reaction requires divalent cations, cell extracts were preincubated with either 5.0 mM EDTA or EGTA for 5 min before the addition of LFNACD. The formation of cross-linked actin was inhibited in the presence of EDTA, suggesting that Mg$^{2+}$ is needed for the reaction, although it is possible that the addition of EDTA caused actin denaturation. The detection of actin cross-linking was inconsistent in EGTA-treated samples, a result that may be attributed either to a requirement for Ca$^{2+}$ in the reaction or to a low or variable concentration of Mg$^{2+}$ in different extract preparations. Taken together, these data show that the ACD can cross-link actin in vitro and that divalent cations are needed for the reaction either as a cofactor or to stabilize actin.

**LFN ACD-catalyzed Cross-linking of Purified Actin Is Dependent upon Mg$^{2+}$**—To determine whether the ACD directly catalyzes the formation of covalent linkages between actin proteins, purified actin was tested as a substrate in the actin cross-linking reaction. Purified rabbit skeletal actin was incubated with LFN or LFNACD in the presence of 2.0 mM CaCl$_2$, MgCl$_2$, MnCl$_2$, or ZnCl$_2$ as the divalent cation cofactor. Upon the addition of Mg$^{2+}$, covalently cross-linked actin dimers, trimers, and higher multimers were detected on Coomassie Blue-stained SDS-polyacrylamide gels (Fig. 5A). Reduced ACD activity was observed in the presence of Mn$^{2+}$, and there was no actin cross-linking following the addition of Ca$^{2+}$ or Zn$^{2+}$ ions.

![Figure 4](image.png)

**Figure 4.** Divalent cations are important for actin cross-linking in crude cell extracts. HEp-2 cells were sonicated in PBS and cleared by centrifugation at 1000 $\times$ g. Cell extract (120 $\mu$g) was incubated with 5 $\mu$g of purified LFN or LFNACD for 90 min. EDTA or EGTA was added to a final concentration of 5.0 mM as indicated. Actin cross-linking activity was monitored by immunoblotting. The positions of the protein standards are indicated on the left.

![Figure 5](image.png)

**Figure 5.** LFNACD directly cross-links actin in the presence of Mg$^{2+}$. A, 10 $\mu$M purified rabbit skeletal actin and 0.018 $\mu$M LFN or LFNACD were incubated for 20 min in Buffer B. The reactions were supplemented with 2.0 mM CaCl$_2$, MgCl$_2$, MnCl$_2$, or ZnCl$_2$ as indicated. B, 0.018 $\mu$M LFNACD, 10 $\mu$M actin, and 2.0 mM MgCl$_2$ were co-incubated, and the reactions were terminated at the designated time points. For both A and B, samples were boiled in SDS-PAGE sample buffer for 5 min and then loaded onto 8% SDS-polyacrylamide gels. Covalent actin cross-linking was detected by Coomassie Blue staining.

To further characterize the *in vitro* cross-linking reaction, LFNACD, actin, and Mg$^{2+}$ were co-incubated, and actin cross-linking was monitored at various time points. Covalently cross-linked actin dimer proteins were detected after an incubation period of 0.5 min (Fig. 5B). The distribution of actin laddering remained unchanged after 10 min, and we concluded that the cross-linking reaction had reached completion. These data indicate that, in the presence of Mg$^{2+}$ as a cofactor, LFNACD catalyzes the actin cross-linking reaction in a time-dependent manner.

Actin is present in the cell as either globular monomers (G-actin) or polymeric filaments (F-actin), with polymerization occurring under physiological salt conditions (26). However, Mg$^{2+}$ alone causes efficient polymerization of actin *in vitro*, and the requirement for Mg$^{2+}$ in the actin cross-linking reaction suggests that actin polymerization needs to be initiated prior to the formation of covalent linkages by the ACD. To investigate the contribution of Mg$^{2+}$, G-actin was polymerized into F-actin following the addition of 2.0 mM CaCl$_2$, 2.0 mM MgCl$_2$, or 50 mM KCl. As shown in Fig. 6A, all conditions resulted in actin polymerization as detected by light scattering. In parallel experiments, actin polymerization was initiated in the presence of LFNACD, and actin cross-linking was detected...
 ATP Is a Required Cofactor for ACD-catalyzed Actin Cross-linking—Cation binding is known to be important for the association of ATP molecules with proteins, including actin (27). The requirement for Mg\(^{2+}\) in the cross-linking reaction implies that ATP is also a necessary cofactor, and it was noted that ATP was a common component in all buffers used for both actin purification and in vitro actin cross-linking. To better characterize the role of ATP in the actin cross-linking reaction, MgATP-actin and MgADP-actin were prepared as described by Gershman et al. (28) with slight modifications (29) and tested as substrates of the cross-linking reaction. In addition, actin purified in the presence of ATP was passed over a PD-10 gel filtration column to remove ATP and then used as a substrate in cross-linking reactions supplemented with either free ATP or the non-hydrolyzable ATP analog AMP-PNP. Actin polymerization was maintained under each of these conditions as monitored by light scattering (data not shown). Covalent actin cross-linking was detected in the presence of LFN\(_2\)ACD and MgATP-actin as well as LFN\(_2\)ACD, Mg\(^{2+}\), and free ATP (Fig. 7, lanes 2 and 4). However, cross-linked actin proteins were not observed following incubation of LFN\(_2\)ACD and MgADP-actin, despite an incubation period of 60 min to ensure adequate polymerization (Fig. 7, lane 1). The combination of free AMP-PNP with Mg\(^{2+}\) and LFN\(_2\)ACD led to the production of a small amount of the cross-linked dimer, which was most likely due to contamination with traces of ATP released from the nucleotide pocket of the actin molecule (Fig. 7, lane 3). Only in the presence of both Mg\(^{2+}\) and ATP was ACD-catalyzed actin cross-linking detected through the higher multimer protein species. Because the structure of AMP-PNP-actin is not substantially different from that of ATP-actin (30) and because its polymerization properties are not perturbed (31), these results strongly suggest that ATP is an essential cofactor for ACD-catalyzed actin cross-linking rather than for maintaining actin structure.

G-actin Is a Substrate of the Actin Cross-linking Reaction—As both Mg\(^{2+}\) and ATP are involved in the initial stages of actin polymerization, it was difficult to distinguish from these in vitro experiments whether the cytoskeletal substrate of the cross-linking reaction was G-actin or F-actin. Covalently cross-linked proteins have been detected as early as 0.5 min after the initiation of polymerization (see above), at which point actin is present as a mixed pool of monomers and polymers. Therefore, an in vivo approach was used to further clarify the actin substrate.

Previously, cytochalasin D was used to depolymerize actin in vivo prior to incubation with a strain of V. cholerae that expresses the RTX toxin, and G-actin was proposed as the substrate of the actin cross-linking reaction (9). However, cytoskeletal inhibition with cytochalasin D does not fully eliminate F-actin as a possible target for actin cross-linking, as there may have been partially polymerized F-actin in the treated cells. Two additional cytoskeletal inhibitors were used in vivo to further support the idea that G-actin was the substrate of the cross-linking reaction. Latrunculin B (LatB) is an inhibitor of actin polymerization that binds and sequesters G-actin (32). Dolastatin 11 (Dol11), a depsipeptide of Dolabella auricularia,
LF\textsubscript{N}ACD Directly Cross-links Actin

**FIGURE 8.** Dol11 (but not LatB) inhibits actin cross-linking in vivo. HEp-2 cells were pretreated with either 5.0 [\mu]M LatB or Dol11 for 90 min. Cells were incubated with *V. cholerae* strain KFV119 (V.c.) for an additional 90 min, harvested, and boiled in SDS-PAGE sample buffer. The lysates were separated on 8% SDS-polyacrylamide gels, and covalent actin cross-linking was detected by immunoblotting with an anti-actin antibody. The positions of the protein standards are indicated on the left.

Hyperpolymerizes actin monomers and stabilizes F-actin (33). HEp-2 cells were incubated with either LatB or Dol11 for 90 min to ensure sufficient disruption of the actin cytoskeleton (data not shown). Following treatment, the cells were exposed to *V. cholerae* strain KFV119, and the formation of cross-linked actin proteins was monitored by immunoblotting (Fig. 8). These experiments were performed with RTX-expressing *V. cholerae* bacteria because PA translocation of LF\textsubscript{N}ACD would be inhibited by LatB (34). Actin cross-linking was observed in cells pretreated with LatB, which implies that the inhibition of actin polymerization had no effect on the cross-linking reaction. However, in the presence of Dol11, a molecule that depletes the G-actin pool by increasing F-actin assembly, actin cross-linking by the RTX toxin was inhibited. During actin polymerization, actin monomers self-associate into trimers and short oligomers, and F-actin is formed following the addition of monomers to the ends of the growing filament (26, 35). These data suggest that either G-actin or short actin oligomers may be the cytoskeletal substrates of the actin cross-linking reaction in vivo.

**DISCUSSION**

In this study, we have shown that the ACD of the *V. cholerae* RTX toxin is directly responsible for the covalent cross-linking of cellular actin (9). These models included both a direct model in which the RTX toxin binds actin and catalyzes the cross-linking reaction and indirect models in which the toxin functions by interacting with cytoplasmic host proteins to initiate actin cross-linking or binding to extracellular receptors to stimulate a signaling cascade that generates cross-linked actin proteins.

Previously, we demonstrated that expression of the ACD as a transgene in vivo results in actin cross-linking, suggesting that the ACD is active in the cell cytoplasm and not in initiating a signal transduction pathway at the plasma membrane (7). Here, we have shown that cytosolic delivery of LF\textsubscript{N}ACD, a protein dependent upon uptake by receptor-mediated endocytosis and transfer to the cytoplasm by anthrax toxin PA, is sufficient to cause actin cross-linking. Taken together, these data establish that the mechanism of actin cross-linking by the RTX toxin requires cytosolic delivery of the ACD into the target cell. In addition, we have eliminated an indirect model in which the RTX toxin activates tissue TGase, the only known eukaryotic enzyme with actin cross-linking activity, suggesting that the ACD catalyzes the cross-linking reaction.

For proof of a direct model of RTX action, it was necessary to determine that the ACD has actin cross-linking activity in vitro in the absence of other host proteins. We have demonstrated that LF\textsubscript{N}ACD cross-links actin in vitro and that the reaction requires only Mg\textsuperscript{2+} and ATP. In subsequent studies, a His\textsubscript{6}-ACD fusion protein was also shown to have actin cross-linking activity in the presence of Mg\textsuperscript{2+} and ATP, demonstrating that LF\textsubscript{N} does not enhance the enzymatic function of the ACD.\(^5\)

Taken together, these data indicate that the ACD can bind actin and directly introduce a covalent linkage between monomers. Although we have shown that the ACD is sufficient to cross-link actin, the chemical nature of the covalent bond remains elusive. Extensive efforts have been made to purify cross-linked actin from cells treated with *V. cholerae* bacteria producing the RTX toxin to identify the cross-linked peptide by mass spectrometry. However, the presence of eukaryotic protein contaminants complicated our analysis. The development of an in vitro actin cross-linking assay using only purified actin, LF\textsubscript{N}ACD, Mg\textsuperscript{2+}, and ATP has simplified the generation of cross-linked actin proteins. Yet only 80% of predicted actin peptides were detected by liquid chromatography/tandem mass spectrometry after in-gel trypsin digestion, and there were no peptides unique to the dimer, trimer, tetramer, and pentamer actin species (data not shown). These results suggest that cross-linked actin may need to be purified and digested in solution to isolate the covalently cross-linked actin peptide. The time-dependent actin cross-linking reaction can be manipulated to increase the overall concentration of dimer peptides, and the peptides generated from in-solution proteolytic digestion of the purified dimer would then be analyzed by mass spectrometry.

Data from the cytoskeletal inhibitor study with LatB and Dol11 suggest that G-actin is the substrate of the actin cross-linking reaction in vivo. Although these results confirm the pre-

\(^5\) C. L. Cordero, D. S. Kudryashov, E. Reisler, and K. J. Satchell, unpublished data.
rious finding with cytochalasin D (9), the possibility that actin oligomers or even F-actin can also serve as a substrate of the ACD cannot be fully excluded. Doll1 inhibits actin depolymerization by binding to F-actin between two long-pitch strands of diagonal monomer subunits (36). This interaction may prevent access to actin protomers in the filament that would otherwise be cross-linked by the ACD. In future studies, synthetic actin substrates and our in vitro actin cross-linking assay will be used to fully clarify the substrate of the cross-linking reaction.

The requirement for Mg$^{2+}$ and ATP in actin cross-linking in vitro indicates that the initiation of actin polymerization is necessary for the ACD-catalyzed reaction. Actin polymerization is dependent upon a thermodynamically unfavorable nucleation step that results from the self-association of actin monomers into a noncovalent trimer complex (26). It is possible that Mg$^{2+}$ and ATP are important for the actin cross-linking reaction to promote the formation of nucleated actin and that the ACD can either catalyze isopeptide linkages between actin monomers in the process of nucleating or bind to the trimer complex and covalently attach additional monomers during the elongation phase of polymerization.

Although plausible, this model contrasts with our in vivo identification of G-actin as a substrate. Furthermore, we found that neither cross-linked actin nor LFNACD could serve to nucleate actin either in the presence or absence of LFNACD. Hence, it is unlikely that LFNACD functions at the point of actin elongation. Instead, we favor the possibility that Mg$^{2+}$ and ATP are essential for the enzymatic activity of the ACD rather than for the regulation of actin dynamics. Tissue TGase is a bifunctional enzyme that covalently cross-links actin, among other protein substrates, and has also been shown to bind and hydrolyze GTP and ATP in the presence of Mg$^{2+}$ (37). Although we have established that tissue TGase is not involved in the ACD-dependent cross-linking reaction, the requirement for Mg$^{2+}$ and ATP in our in vitro cross-linking assay indicates that the cross-linking activity of the ACD is energy-consuming and coupled to ATPase activity. Sequence analysis of the ACD revealed a putative ATP-binding site at amino acids 2120−2127. The sequence SXXAXGKT conforms to the consensus ATP-binding Walker type A consensus sequence GXGXGK(T/S) (38), assuming conservative Ser and Ala changes for the Gly residues. A linker insertion mutant near this site disrupted the function of the ACD when expressed as a transgene, indicating that this region is required for cross-linking activity. The presence of this site suggests that the ACD is capable of binding and hydrolyzing ATP. Ongoing experiments are focused on the role of Mg$^{2+}$ and ATP in the ACD-catalyzed actin cross-linking reaction.

We suggest, as a mechanism, that the covalent cross-linking of cellular actin leads to the disassembly of actin filaments because of the irreversible chemical modification of the actin monomer and its subsequent inability to undergo polymerization. Depletion of the G-actin pool leads to a shift in the critical concentration of actin within the cell and ultimately results in the depolymerization of actin stress fibers. It has been shown that microinjection of covalently cross-linked actin proteins into PtK2 cells disrupts the steady state between G-actin and F-actin, resulting in disassembly of the endogenous actin filament network (39). We hypothesize that a recently observed second cell rounding activity of the multifunctional RTX toxin also increases the overall amount of G-actin in the cell by an as yet uncharacterized mechanism (7), which subsequently provides more substrate for the actin cross-linking reaction catalyzed by the ACD.

The ACD sequence is 59% identical to the C-terminal portion of VgrG-1, also known as VC1416, a V. cholerae protein that we have shown previously to cross-link actin when transiently expressed in mammalian cells (7). Recently, Pukatzki et al. (40) reported that VgrG-1 is a putative effector protein of the newly identified type VI secretion system, which is predicted to translocate potential virulence factors into target cells. Our investigation of the mechanism of actin cross-linking by the ACD may contribute to the understanding of VgrG-1 activity, as the strong sequence conservation suggests that VgrG-1 has a cross-linking mechanism similar to that of the ACD for its function in the rounding of J774 macrophage cells.

We have established that the ACD of the V. cholerae RTX toxin is a cross-linking enzyme that catalyzes the formation of covalent linkages between actin monomers. An ongoing study of the actin cross-linking reaction will further determine the enzymatic properties of the ACD and allow us to pursue its structure-function interactions with two molecules of actin. As a whole, this work will advance our knowledge of this unique mechanism of actin depolymerization and clarify the role of the RTX toxin in the pathogenesis of cholera disease.

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