Structure-Function Analysis of Inositol Hexakisphosphate-induced Autoprocessing of the Vibrio cholerae Multifunctional Autoprocessing RTX Toxin

Vibrio cholerae secretes a large virulence-associated multifunctional autoprocessing RTX toxin (MARTX<sub>vc</sub>). Autoprocessing of this toxin by an embedded cysteine protease domain (CPD) is essential for this toxin to induce actin depolymerization in a broad range of cell types. A homologous CPD is also present in the large clostridial toxin TcdB and recent studies showed that inositol hexakisphosphate (Ins(1,2,3,4,5,6)P<sub>6</sub> or InsP<sub>6</sub>) stimulates the autoprocessing of TcdB dependent upon the CPD (Egerer, M., Giesemann, T., Jank, T., Satchell, K. J., and Aktories, K. (2007) J. Biol. Chem. 282, 25314–25321). In this work, the autoprocessing activity of the CPD within MARTX<sub>vc</sub> is similarly found to be inducible by InsP<sub>6</sub>. The CPD is shown to bind InsP<sub>6</sub> (K<sub>d</sub> 0.6 μM), and InsP<sub>6</sub> is shown to stimulate intramolecular autoprocessing at both physiological concentrations and as low as 0.01 μM. Processed CPD did not bind InsP<sub>6</sub> indicating that, subsequent to cleavage, the activated CPD may shift to an inactive conformation. To further pursue the mechanism of autoprocessing, conserved residues among 24 identified CPDs were mutagenized. In addition to cysteine and histidine residues that form the catalytic site, 2 lysine residues essential for InsP<sub>6</sub> binding and 5 lysine and arginine residues resulting in loss of activity at low InsP<sub>6</sub> concentrations were identified. Overall, our data support a model in which basic residues located across the CPD structure form an InsP<sub>6</sub> binding pocket and that the binding of InsP<sub>6</sub> stimulates processing by altering the CPD to an activated conformation. After processing, InsP<sub>6</sub> is shown to be recycled, while the cleaved CPD becomes incapable of further binding of InsP<sub>6</sub>.

Vibrio cholerae is the etiologic agent of the acute intestinal infection cholera, that remains a world-wide problem with over 200,000 reported and an estimated 1 million actual cases each year (1, 2). To cause illness, V. cholerae colonizes the small intestine, where it secretes its major virulence factor, the ADP-ribosylating cholera toxin, which elicits massive fluid secretion resulting in the profuse diarrhea that is the hallmark of cholera infection. Nearly all O1, O139, and non-O1/non-O139 clinical isolates of V. cholerae produce another secreted toxin that is the founding member of a new family of bacterial protein toxins called the multifunctional autoprocessing repeats-in-toxins (MARTX<sup>2</sup> toxins (3–7)). In V. cholerae, this toxin has recently been shown to contribute to virulence in mice and is among three secreted factors associated with the ability of V. cholerae to establish an intestinal infection that persists beyond 24 h (8, 9). Hence, MARTX<sub>vc</sub> is proposed to function during the earliest stages of human exposure to V. cholerae either to modify the intestinal tract allowing colonization to occur or to reduce the functionality of innate immune cells preventing clearance. The broad distribution of MARTX<sub>vc</sub> among environmental isolates further suggests this toxin may have a role in extraintestinal survival (3–5–7).

MARTX<sub>vc</sub> is an unusually large protein of 4545 amino acids (aa) or >450 kDa. Most of its primary structure consists of 18–20 aa glycine-rich repeats that are proposed to form a translocation structure that facilitates transfer of the central portion of the toxin across the eukaryotic plasma membrane (4). All known activities of MARTX<sub>vc</sub> have been mapped to the central ~1700 aa of the protein. The RhGTPase-inactivation domain (RID) causes conversion of activated GTP-bound Rho, Rac, and CDC42 to the inactive GDP-bound forms resulting in depolymerization of actin (10). The adjacent actin cross-linking domain (ACD) catalyzes the covalent cross-linking of monomeric G-actin resulting in the irreversible destruction of the cytoskeleton (7, 11, 12). Together these domains rapidly round eukaryotic cells without causing cell lysis.

To access their respective substrates, it was postulated that the RID and ACD are released into the eukaryotic cytosol after translocation by a 25-kDa cysteine protease domain (CPD) that is embedded within the central portion of MARTX<sub>vc</sub>. Studies using recombinant CPD (rCPD) demonstrated that autoprocessing to the N-terminal side of rCPD is stimulated by a small molecule found in eukaryotic cell cytosol (13). Additional studies demonstrated that cleavage in vitro could be stimulated by binding of GTP, and particularly the non-hydrolyzable analog GTPγS. A mutation of the catalytic cysteine residue Cys-3568 in the holotoxin dramatically reduced both actin cross-linking

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2 The abbreviations used are: MARTX, multifunctional autoprocessing RTX toxin; RTX, repeats-in-toxins; CPD, cysteine protease domain; aa, amino acids; RID, RhGTPase-inactivation domain; Ins, inositol; DLS, dynamic light scattering; ITC, isothermal titration calorimetry; NEM, N-ethylmaleimide.
and Rho inactivation activities, demonstrating that autoprocessing activity by the CPD is necessary for the cytopathic effects of MARTX_{Vc} (13).

Recently, it has been reported that Clostridium difficile Toxin B (TcdB) also undergoes autocatalytic cleavage and that processing depends on a region of the toxin with sequence similarity to MARTX_{Vc} CPD. The autoprocessing activity of TcdB was activated by dithiothreitol and inositol phosphate compounds with inositol hexakisphosphate (Ins(1,2,3,4,5,6)P_6) or InsP_6 functioning as the best stimulatory molecule (14, 15).

Inositol phosphates (InsP_n) are commonly present in eukaryotic cells. Inositol triphosphate (Ins(1,4,5)P_3) plays an important role in signal transduction in eukaryotic cells. However, the highly phosphorylated inositol phosphates are the major inositol phosphates present in the cells (16) where the intracellular concentration of InsP_6 reaches up to 40 – 60 μM in several cell types (17–19). This range of concentrations was able to activate the cleavage of TcdB (14, 15).

In this work, we focused on the molecular basis of MARTX_{Vc} CPD autoproteolysis. We showed that InsP_6 is able to bind and activate the autoprocessing activity of MARTX_{Vc} CPD. We have identified crucial amino acid residues within CPD that are involved in InsP_6-induced processing of CPD. We also provide evidence for a molecular mechanism of InsP_6-activated autoprocessing. Because of the high sequence similarity among the CPDs from a group of 24 known and putative bacterial toxins, it is likely that other proteins use a similar mechanism for autoprocessing.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Growth Conditions, and Reagents—Escherichia coli DH5a, used for all DNA manipulations, and E. coli BL21(ADE3), used for production of recombinant proteins, were routinely grown with shaking in Luria-Bertani (LB) medium supplemented with 100 μg/ml of ampicillin at 37 °C. myo-Inositol and sodium or potassium salts of phosphorylated d-myo-inositol compounds were obtained from Axxora or Sigma.

**Site-directed Mutagenesis**—Amino acid substitutions were introduced into the pHisCPD overexpression vector (13) using the Quikchange II XL mutagenesis kit (Stratagene, La Jolla, CA). The mutations were generated with the suitable sense and antisense primers. Plasmid DNA was then prepared using the Quikchange II XL mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol for pET30-XA-LIC cloning (Novagen, Madison, WI) using modified vector pMCSG7 (20) as was done previously for pHisCPD overexpression vector (13). The protein expressed from this plasmid, rCPDΔ51, represents CPD from the cleavage site with 21 amino acids of plasmid-derived sequence and N-terminal His tag.

**Production and Purification of Proteins**—For the initial screening of mutant protein activities, proteins were purified from 50-ml cultures using TALON affinity chromatography (Clontech, Mountain View, CA) according to the manufacturer’s recommendations. For InsP_6 binding assays, proteins were purified from 500-ml cultures by the method previously described (13) using a HisTrap HP column on an AKTA purifier FPLC system (GE Healthcare). All proteins were eluted in buffer containing 250 mM imidazole, dialyzed into 20 mM Tris, 500 mM NaCl, pH 7.4, and after the addition of 10% glycerol were stored at −80 °C. Protein concentration was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE), using extinction coefficients calculated by ProtParam.

**In Vitro Cleavage Assay**—Purified proteins were diluted into appropriate concentrations (as indicated in the figures) in a buffer of 20 mM Tris, 60 mM NaCl, 250 mM sucrose, 3 mM imidazole, pH 7.5. Reaction was initiated by addition of InsP_6 at concentrations indicated and incubated at 37 °C for the time indicated. Reactions were stopped by the addition of SDS-PAGE loading buffer, and boiled for 1 min. Samples were separated by 15% SDS-PAGE gels and stained with Coomassie Blue R250. For semiquantitative determination of percent cleavage, gels were scanned, and the digital images were analyzed using NIH ImageJ 1.38 software. The percent cleavage was calculated using Equation 1,

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\frac{[P \times 100/(P + FL)] - [P_0 \times 100/(P_0 + FL_0)]}{P_0}
\]

where P is the signal intensity of processed protein, and FL is the signal intensity of unprocessed protein. P_0 and FL_0 are the corresponding signal intensities of the control sample for the experiment. Fourier transform mass spectrometry of cleavage products was performed as previously described (13).

**InsP_6 Binding Assay**—Isothermal Titration Calorimetry (ITC) was performed with rCPD variants and InsP_6 using an MSC-ITC Calorimeter (Microcal, Inc.) at the Northwestern Keck Biophysics Facility. Purified proteins were dialyzed into 20 mM Tris, 150 mM NaCl, 1.5% glycerol, pH 7.5, and diluted to final concentrations of 15 μM in the same buffer. The same buffer was used to dilute InsP_6 to a final concentration of 1.4 mM. Titrations were performed at 37 °C by injecting 17 × 2 μl of ligand into the protein sample in the ITC cell. Resulting data were plotted with a nonlinear least-squares algorithm using Origin7 software (Microcal) and a model for a single class of binding. The binding constant (K_b = 1/K_d) was calculated from the curve.

**Dynamic Light Scattering (DLS)**—600 μl of 0.5 mg/ml rCPD mutant proteins in 20 mM Tris, 150 mM NaCl, 1.5% glycerol, pH 8.0 (without InsP_6) were monitored for particle size in six measurements over 25 min at 25 °C using a Zetasizer NanoS (Malvern Instruments, Westborough, MA).

**RESULTS**

Physiological Concentrations of InsP_6 Activate the Autoproteolytic Activity of rCPD—Recent studies showed that InsP_6 is a potent stimulator of autoprocessing of TcdB dependent upon a
domain homologous to the MARTXVc CPD (15). Based on this observation, it was tested whether InsP₆ is also a stimulator of the CPD from MARTXVc. For in vitro experiments, rCPD, which represents the CPD of MARTXVc plus 75-aa sequence upstream of the identified cleavage site with 6×His tags at both the N and C terminus (13), was used. As shown in Fig. 1, addition of 100 μM InsP₆ to 2 μg (2.9 μM) rCPD stimulated the autoprocessing activity of rCPD. A cleavage product of about 25 kDa was observed, which corresponds to the size of the rCPD autoprocessing activity of rCPD. A cleavage product of about 25 kDa was observed, which corresponds to the size of rCPD autoprocessing activity of rCPD. A cleavage product of about 25 kDa was observed, which corresponds to the size of rCPD autoprocessing activity of rCPD. A cleavage product of about 25 kDa was observed, which corresponds to the size of rCPD autoprocessing activity of rCPD. A cleavage product of about 25 kDa was observed, which corresponds to the size of rCPD autoprocessing activity of rCPD. A cleavage product of about 25 kDa was observed, which corresponds to the size of rCPD autoprocessing activity of rCPD. A cleavage product of about 25 kDa was observed, which corresponds to the size of rCPD autoprocessing activity of rCPD. A cleavage product of about 25 kDa was observed, which corresponds to the size of rCPD autoprocessing activity of rCPD.

InsP₆-induced Autoprocessing of MARTXVc

To further characterize the cleavage reaction induced by InsP₆, the kinetics of rCPD autoprocessing were investigated. As shown in Fig. 2, addition of 100 μM InsP₆ resulted in autoprocessing with a reaction half-time of about 30 min under the given conditions and cleavage of nearly 100% was observed within 3 h. At 2 h, a wide range of concentrations of InsP₆ was able to activate the rCPD cleavage (Fig. 3). Even concentrations as low as 0.01 μM were able to induce 35% cleavage by 2 h. By comparing the amount of InsP₆ added to the percent of rCPD cleaved, our data indicate that 1 molecule of InsP₆ was able to initiate the cleavage of ~100 molecules of rCPD indicating that InsP₆ is a potent stimulator of autoprocessing.

TABLE 1

Inositol compounds as stimulators of rCPD autoprocessing

| InsP₆          | Percent rCPD processed by indicated concentration of inositol compound |
|---------------|---------------------------------------------------------------|
|               | 1 μM | 10 μM | 100 μM | 1000 μM   |
| myo-Inositol  | <5   | <5    | <5     | <5        |
| Ins(1,4,5)P₃  | <5   | <5    | 5       | 29        |
| Ins(1,3,4,5)P₄| <5   | <5    | 18      | 29        |
| Ins(1,4,5,6)P₄| <5   | <5    | 28      | 49        |
| Ins(1,3,4,5,6)P₅| 10   | 46    | 59      | 52        |
| Ins(1,2,3,4,5,6)P₆| 71   | 71    | 70      | 57        |

MARTXVc CPD is specifically stimulated by InsP₆ dependent upon the catalytic cysteine residue.

Characterization of the InsP₆-induced Autoprocessing of rCPD—To further characterize the cleavage reaction induced by InsP₆, the kinetics of rCPD autoprocessing were investigated. As shown in Fig. 2, addition of 100 μM InsP₆ resulted in autoprocessing with a reaction half-time of about 30 min under the given conditions and cleavage of nearly 100% was observed within 3 h. At 2 h, a wide range of concentrations of InsP₆ was able to activate the rCPD cleavage (Fig. 3). Even concentrations as low as 0.01 μM were able to induce 35% cleavage by 2 h. By comparing the amount of InsP₆ added to the percent of rCPD cleaved, our data indicate that 1 molecule of InsP₆ was able to initiate the cleavage of ~100 molecules of rCPD indicating that InsP₆ is a potent stimulator of autoprocessing.

rCPD Cleaves in cis—Our observation that only one molecule of InsP₆ is able to stimulate autoprocessing of ~100 molecules of rCPD could be consistent with an in trans activation model wherein one activated CPD processes numerous other CPD proteins. To test a possible in trans cleavage model, we followed the approach of Reinke et al. (1-4) and used increasing concentrations of protein and limiting concentrations of InsP₆. If cleavage occurs by an intermolecular mechanism, efficiency of processing should increase with increasing concentration of protein. If cleavage occurs by an intramolecular mechanism, then cleavage should remain constant with increasing protein concentration. As a positive control, a saturating InsP₆ concentration of 100 μM InsP₆ was used to demonstrate maximum processing under these conditions at all dilutions (Fig. 4). At lower InsP₆ concentrations, the recovered cleavage product remained constant with increasing protein concentration (Fig. 4).
InsP$_{6}$-induced Autoprocessing of MARTX$_{Vc}$

This result indicated that InsP$_{6}$ binds to rCPD at either the same or an overlapping site as GTP$_{\gamma_S}$.

To further address binding of InsP$_{6}$ to rCPD, ITC was used to establish InsP$_{6}$ binding properties. ITC monitors and quantifies the heat absorption or release that accompanies binding of a ligand to a protein and allows the direct determination of a binding constant ($K_d$). A typical ITC experiment was designed wherein InsP$_{6}$ was titrated against a solution of uncleavable rCPD C-S in the ITC cell at 37 °C. For these studies, the rCPD C-S mutant was used to prevent measurement of the heat change upon breaking of the peptide bond during processing in wild-type rCPD. The same buffer and temperature were used as in the previously established binding assay for GTP$_{\gamma_S}$-BODIPY and rCPD C-S (13) because a similar type of interaction was expected.

From the measured heat changes at each addition of InsP$_{6}$ (Fig. 5), a dissociation constant was calculated, $K_d$ of $6 \times 10^{-7}$ M (0.6 μM). When compared with the dissociation constant for GTP$_{\gamma_S}$, $K_d$ of $1.3 \times 10^{-5}$ M (130 μM), the binding affinity of rCPD C-S for InsP$_{6}$ is much stronger. Importantly, the dissociation constant correlates to a concentration ~100-fold below the physiological InsP$_{6}$ concentration.

**Processed rCPD No Longer Binds InsP$_{6}$**—To determine if rCPD can still bind InsP$_{6}$ after processing has occurred, 15 μM rCPD was treated for 5 h with an InsP$_{6}$ concentration corresponding the first injection in an ITC experiment (~2.15 μM). Full cleavage of rCPD was confirmed by separating a portion of the reaction by SDS-PAGE (data not shown). The remaining cleaved rCPD was then measured by ITC for InsP$_{6}$ binding. As shown in Fig. 5, cleaved rCPD does not bind InsP$_{6}$. To show that the 5-h incubation at 37 °C was not the cause for the loss of binding, the same 5-h treatment was applied to rCPD C-S, and then ITC was done to test InsP$_{6}$ binding. The measured binding was the same as previously observed for rCPD C-S binding to InsP$_{6}$ except without the first InsP$_{6}$ injection (data not shown). These data suggest that after InsP$_{6}$ binds rCPD and activates cleavage, the protein undergoes a conformational change, which causes the release of InsP$_{6}$ molecule.

**rCPDΔ51 Binds InsP$_{6}$**—The observation that the binding properties of rCPD to InsP$_{6}$ change after the cleavage raises the question as to whether the sequence of rCPD upstream of the cleavage site is involved in InsP$_{6}$ binding. The sequence upstream of rCPD cleavage site consists of 24 non-MARTX$_{Vc}$ aa from the cloning vector followed by 51 aa of MARTX$_{Vc}$ sequence. In the protein rCPDΔ51, the 51-aa part of MARTX$_{Vc}$ sequence is absent leaving only the plasmid sequence upstream of the rCPD cleavage site. Under the same reaction conditions, rCPDΔ51 was processed after addition of InsP$_{6}$ (data not shown) indicating that InsP$_{6}$-induced processing does not require upstream MARTX$_{Vc}$ sequence. To confirm the binding properties remain the same when the 51-aa part of MARTX$_{Vc}$ sequence is absent, the C3568S mutation was introduced into rCPDΔ51 and binding of InsP$_{6}$ was measured by ITC. The same binding curve was measured giving the same $K_d$ as rCPD C-S (data not shown). These data showed that the MARTX$_{Vc}$ aa upstream of the Leu-3428 to Ala-3429 processing site do not contribute to InsP$_{6}$ binding or to the autoprocessing activity of rCPD. Thus, the function of InsP$_{6}$ binding that induces cleavage...
followed by structural changes is found solely within the CPD itself.

Site-directed Mutagenesis of Residues Involved in rCPD Autoprocessing—A previously performed bioinformatics analysis revealed 19 CPDs found in large bacterial proteins (13). A more recent search identified 24 CPDs (alignment shown in supplemental Fig. S1). From the sequence alignment, key residues were identified that included known and putative catalytic residues, positively charged residues that might interact with the negatively charged InsP$_6$, and strongly conserved hydrophobic residues. Altogether, 23 residues were identified as potentially important for InsP$_6$-induced autoprocessing (Fig. 6A). Site-directed mutagenesis was used to alter the identified codons on the rCPD overexpression vector pHisCPD (13), and then mutant proteins were purified and tested for autoprocessing at 37 °C after addition of 0.1 mM InsP$_6$ for 1 h (Fig. 6B). This concentration of InsP$_6$ under the experimental conditions stimulated the cleavage of ~60% of wild-type rCPD (Fig. 6B). This limiting concentration of InsP$_6$ thus allowed detection of mutants that were either totally or partially defective in autoprocessing activity.

Of the 23 residues targeted, 12 mutants showed no defects for in vitro autoprocessing activated by 0.1 μM InsP$_6$ (Fig. 6B). The 11 defective mutants were then tested for autoprocessing with 10-fold increasing concentrations of InsP$_6$. Of these, only one mutant, the catalytic cysteine mutant, rCPD C-S, was fully defective at the highest InsP$_6$ concentration of 100 μM. All other mutants showed varying levels of partial defects. Four of these mutants were deemed significantly defective with at least 50% loss of function at 100 μM InsP$_6$ compared with wild-type rCPD (Table 2). In addition, two proteins rCPD R3521A and R3593A showed increased cleavage with 100% processing at 0.1 μM InsP$_6$ and were nearly fully cleaved in concentrations of InsP$_6$ as low as 10 nM. Analysis of individual mutants is described in detail below.

Analysis of Putative Catalytic Site—Previously, it was shown that Cys-3568 and His-3519 residues were essential for CPD-EGFP autoprocessing in vivo after transient expression in epi-
TABLE 2
Autoprocessing efficiency of rCPD mutants in varying concentrations of InsP6

| rCPD mutation | Percent rCPD processed by indicated concentration of InsP6 | Wild-type | C3568S H3519A | E3467A/D3469A |
|---------------|----------------------------------------------------------|-----------|----------------|--------------|
|               | Percent rCPD processed by indicated concentration of InsP6 | 0.1 μM | 1.0 μM | 10 μM | 100 μM |
| Wild-type     | 60 | 63 | 63 | 63 | 63 |
| Putative catalytic site mutants | | | | | |
| C3568S       | <5 | <5 | <5 | <5 | 5 |
| H3519A       | <5 | <5 | 9 | 11 | 11 |
| E3467A/D3469A| <5 | 8 | 28 | 45 | 45 |
| Strongly defective mutants | | | | | |
| L3479D       | <5 | 14 | 23 | 30 | 30 |
| K3482A       | <5 | <5 | <5 | 22 | 22 |
| K3611A       | <5 | 11 | 12 | 19 | 19 |
| Partially defective mutants | | | | | |
| R2457A       | <5 | 57 | 63 | 63 | 63 |
| A3475I       | <5 | 14 | 58 | 58 | 58 |
| R3513A       | <5 | <5 | 12 | 51 | 51 |
| R3593A       | <5 | 22 | 57 | 64 | 64 |
| R3610A       | <5 | 37 | 52 | 52 | 52 |
| K3628A       | <5 | 10 | 37 | 47 | 47 |

*Mutant protein rCPD L3479D was shown by dynamic light scattering to be aggregated.*

Putative InsP6 Binding Residues—Because InsP6 is a highly negatively charged molecule, strong basic CPD residues could participate in binding of InsP6. In total, 11 arginine or lysine residues including the 100% conserved Lys-3482 were changed to alanine and tested for a defect in autoprocessing. Of 11 mutant proteins tested, seven were defective for autoprocessing at 0.1 μM InsP6 (Fig. 6B) and two of these mutants, rCPD K3482A and K3611A, were also at least 50% defective at 100 μM InsP6 (Table 2).

rCPD Residues Defective for InsP6 Binding—The four residues critical for autoprocessing activity of rCPD, His-3519, Leu-3479, Lys-3482, and Lys-3611, and also the combination of Glu-3467/Asp-3469 were tested for InsP6 binding. As shown in Fig. 5, rCPD K3482A was unable to bind InsP6. rCPD K3611A and L3479D, as well as these three mutations combined with a C3568S mutation, were also unable to bind InsP6 (data not shown). Analysis of the rCPD L3479D protein by light scattering revealed this protein was aggregated in the absence of InsP6 likely accounting for its loss of binding activity. By contrast, rCPD K3611A and K3482A were identical in size to rCPD C-S suggesting these proteins have a stable conformation and thus these two lysine residues could form the basis of binding pocket formed by two distant parts of CPD (data not shown). Other arginine and lysine residues that have reduced processing efficiency at low concentrations of InsP6 may also assist in formation of this pocket.

The two putative catalytic site mutants, rCPD E3467A/D3469A/C3568S (E3467A/D3469A mutations in combination with C3568S mutation to prevent cleavage) and rCPD H3519A were also found to have decreased binding of InsP6 (Fig. 5). These proteins were not aggregated in the absence of InsP6 as determined by light scattering although mutant E3467A/D3469A/C3568S showed some drift in particle size over 25 min suggesting slight changes to conformation over time. At the concentration of protein used in the ITC, the KD for these mutants was outside the quantitative range of the assay and could not be determined although extrapolation of the data suggested the dissociation constant would be in the millimolar range.

The ability of proteins rCPD R3521A/C3568S and R3593A/C3568S to bind InsP6 was examined to determine if their increase in processing efficiency was related to a lowered binding affinity. It was found that the binding constant was not identifiable as a suitable candidate for the formation of an oxanion hole. Despite the fact that Gln-3465 is 100% conserved among all 24 putative CPDs (suppl. Fig. S1), rCPD Q3465A was not defective for autoprocessing. A double mutation of nearby Q3461A in combination with Q3465A also showed no defect in autoprocessing (data not shown). Thus, if this protein requires an oxanion hole, the essential residue is located elsewhere.

Putative InsP6. While Cys-3568 was shown to be absolutely necessary for the expression of N-terminal fragment of TcdB (15). Surprisingly, the sequence of MARTXVc CPD. However, the single mutation of nearby Q3461A in combination with Q3465A also showed no defect in autoprocessing (data not shown). Thus, if this protein requires an oxanion hole, the essential residue is located elsewhere.

The ability of proteins rCPD R3521A/C3568S and R3593A/C3568S to bind InsP6 was examined to determine if their increase in processing efficiency was related to a lowered binding affinity. It was found that the binding constant was not
100% processed when InsP$_6$ is present in excess (Fig. 7C, lanes 7 and 9).

Because inhibition of processing of both rCPD and rCPD$\Delta$51 by rCPD C-S was observed at only 6-fold below the rCPD C-S dissociation constant, our results are consistent with the wild-type rCPD dissociation constant being close or the same as that measured for rCPD C-S. If the dissociation constant for the wild-type protein was significantly lower than for rCPD C-S, inhibition would not have been observed as InsP$_6$ would have preferentially bound to wild-type rCPD or rCPD$\Delta$51 and rCPD C-S would thus fail to inhibit. Consistent with these conclusions, mutant rCPD K3482A, which does not bind InsP$_6$ (Fig. 5), does not compete with wild-type rCPD or rCPD$\Delta$51 for InsP$_6$ resulting in no inhibition of rCPD or rCPD$\Delta$51 cleavage (Fig. 7, A, lane 8 and C, lane 8).

**DISCUSSION**

Recent studies have recognized a growing number of large bacterial protein toxins that are processed after translocation [13, 21, 22]. These toxins fall into two families: the MARTX family toxins, including MARTX$_{Vc}$ (13) and MARTX$_{Vv}$ (22), and the clostridial glucosyltransferase toxins (CGTs), including TcdA, TcdB, TcsL, and Tcno (14). All of these toxins have recently been shown to undergo an inducible autoprocessing event. For both TcdB and MARTX$_{Vc}$, it is known that autoprocessing is essential for these toxins to induce cell rounding [13, 15, 23].

The signal to initiate autoprocessing and the catalytic domain responsible for the autoprocessing of TcdB has been the subject of much controversy. Rupnik et al. (24) originally proposed that the peptidase was a eukaryotic protease, but this model was negated when it was shown that protein-free cell extracts were able to stimulate processing *in vitro* (14). It was then found that inositol phosphates, in particular InsP$_6$, were stimulatory factors for a protease activity embedded within the toxin itself. This group suggested the mechanism of processing involved an aspartyl protease located downstream of the TcdB hydrophobic region (14). About the same time, we published that MARTX$_{Vc}$ is autoprocessed, dependent upon a cysteine residue within the CPD (13). Egerer et al. (15) quickly corrected the processing model for TcdB and showed that InsP$_6$-induced processing involved not an aspartyl protease, but a CPD homologous to the MARTX$_{Vc}$ CPD, and this CPD is similar located adjacent to the processing site. Although the mechanisms seemed similar, we had shown that GTP and particularly the non-hydrolyzable analog GTP$\gamma$S, was a low affinity stimulator molecule for CPD activation (13). The poor efficiency of processing and binding that did not exactly correlate with the addition of eukaryotic cell cytosol suggested that MARTX$_{Vc}$ CPD could use an alternative activator *in vivo* or required a cytosolic co-activator for efficient stimulation of autoprocessing. Thus, we investigated whether MARTX$_{Vc}$ would respond to InsP$_6$.

Here we show, that InsP$_6$ is able to activate the CPD of MARTX$_{Vc}$. Moreover, very small concentrations as low as 10 nM InsP$_6$ were able to activate the CPD, whereas lesser phosphorylated inositol compounds required concentrations from 10 $\mu$M to 1 mM to stimulate processing (Table 1). By comparing the binding properties and activation kinetics of InsP$_6$ with those of

**InsP$_6$-induced Autoprocessing of MARTX$_{Vc}$**

**FIGURE 7. Competitive inhibition of rCPD autoprocessing by addition of rCPD mutants.** All processing reactions were performed with indicated concentrations of protein and InsP$_6$, for 2 h at 37°C. In all gels, samples were separated by SDS-PAGE and stained with Coomassie Blue R250 to detect full-length (FL) or processed (P) proteins. A, 2 $\mu$g of rCPD (wt) and/or rCPD C-S (or rCPD K3482A) were incubated in the absence or presence of 0.1 $\mu$M InsP$_6$. Indicated concentrations of rCPD and/or rCPD C-S were incubated with or without indicated concentrations of InsP$_6$. For both A and B, FL indicates full-length unprocessed protein of both wt rCPD and/or mutant variants, while P indicates processed proteins. C, 2 $\mu$g of wt rCPD$\Delta$51 (wt$\Delta$51) and/or rCPD C-S or rCPD$\Delta$51 variants were mixed and processing was activated by addition of the indicated concentration of InsP$_6$. FL indicates the full-length of rCPD C-S or rCPD K3482A, while FL$\Delta$51 marks the full-length size of rCPD$\Delta$51 and P$\Delta$51 marks the processed size of rCPD$\Delta$51. As indicated, the processed form of rCPD C-S or rCPD K3482A was expected to migrate similar to full-length rCPD$\Delta$51. However, it is clear this processing does not occur as there is no decrease in the amount of FL rCPD C-S or rCPD K3482A.

significantly different than for rCPD C-S indicating that the enhancement to processing of these mutant is not related to an altered binding capacity of InsP$_6$ (data not shown).

**Competitive Inhibition of rCPD Autoprocessing—** To confirm all the results presented above, we designed a competitive processing experiment. rCPD was mixed with rCPD C-S, which binds InsP$_6$ or with rCPD K3482A, which does not bind InsP$_6$. Then 0.1 $\mu$M InsP$_6$ (6-fold below the $K_d$) was added to initiate autoprocessing, and the mixture was incubated for 2 h. In the mixed reactions, the amounts of processed products were analyzed by SDS-PAGE.

In the rCPD/rCPD C-S mixed reaction (Fig. 7A, lane 7), the amount of processed product was not equivalent to that of the reaction with rCPD alone (lane 4) as would be expected from an *in trans* cleavage reaction, where rCPD would be able to cleave rCPD C-S. This result confirms our finding of an intramolecular processing mechanism. Indeed, rCPD C-S inhibited the autoprocessing of rCPD resulting in a lesser recovery of processed product than in rCPD alone (Fig. 7A, lanes 4 and 7). The inhibition of rCPD processing by rCPD C-S was overcome by increasing concentrations of InsP$_6$ (Fig. 7B), indicating that the basis for the inhibition is rCPD C-S competing for binding of a limiting amount of InsP$_6$.

Similar results were obtained when rCPD C-S was mixed with the shorter protein rCPD$\Delta$51, a reaction wherein the wild-type and mutant proteins can be distinguished by size. It was found that only rCPD$\Delta$51 was processed, and this processing was inhibited by rCPD C-S at low InsP$_6$ concentrations, but was
GTPγS, we propose that InsP₆ is the physiologically relevant compound. First, InsP₆ is widely present at relatively high concentrations in eukaryotic cells (up to 60 μM) (17–19) but is not present in the extracellular spaces or the bacterial cytoplasm. Thus, toxin processing would only occur after translocation of the CPD to the eukaryotic cytosol and would not occur either within bacteria or before binding to a target cell. This model is consistent with our observation that rCPD can be purified from E. coli at its full, unprocessed size despite high concentrations of GTP in the bacterial cytoplasm. Second, the observed kinetics for the processing of rCPD induced by eukaryotic cell cytosol (13) closely follows that of InsP₆ (Fig. 2). Finally, we found that the affinity of InsP₆ for rCPD C-S is much stronger (Kₐ 6 × 10⁻⁷ M) than was described for GTPγS (Kₐ 1.3 × 10⁻⁴ M). Moreover, the presence of 1 mM GTPγS did not change the Kₐ of InsP₆ (data not shown), although preincubation with InsP₆ does inhibit binding of GTPγS-BODIPY. Under physiological conditions, GTPγS did not function as a co-activator with InsP₆ and a synergistic activity was not observed. An additive effect was observed only when a limiting concentration of 0.005 μM InsP₆ was used (data not shown). A similar additive effect was observed with TcdB in presence of dithiothreitol and limiting concentrations of InsP₆ (15). Dithiothreitol has no effect on rCPD processing, likely because the protein has only one cysteine residue so no intramolecular disulfide bridges can be formed (data not shown). Overall, we conclude that InsP₆ is a potent in vivo stimulator of processing for both MARTX and CGT toxins and likely for all related bacterial toxins and large secreted proteins that contain a CPD (supplemental Fig. S1).

GTPγS and GTP are likely successful mimics of InsP₆ and can interact with rCPD possibly through its negatively charged phosphate groups to activate processing when added at high concentration.

To study rCPD residues involved in InsP₆-induced autoprocessing, scanning mutagenesis was performed targeting residues highly conserved among 24 putative CPDs. Over half of the mutations had no defect in processing when screened at low concentration of InsP₆ indicating they are not essential for processing. Concerning the catalytic site, we confirmed that Cys-3568 and His-3519 are necessary residues involved in autoprocessing. Surprisingly, while rCPD C3568S retains the ability to bind InsP₆ and indeed was used as a catalytically inactive mutant to solve the binding constant, rCPD H3519A had a dramatic loss in InsP₆ binding. This could indicate that the mutation causes a significant conformational change compared with the wild-type uninduced structure. Notably, a mutation of the nearby residues Arg-3521 and Arg-3593 to Ala resulted in an increased cleavage activity (Fig. 6B) although the binding affinity for InsP₆ was not significantly increased (data not shown). Thus, we suggest that rather than affecting binding per se, this region is important for the conformation shift induced by binding of InsP₆, which results in activation of the protease to initiate autoprocessing. Mutagenesis of the histidine could lock the protein in an open non-binding conformation while mutation of the arginines might set the protein closer to the activated conformation. This region may also be important for a second shift to a third conformation upon processing and release of the InsP₆ as the protein becomes inactive to further binding of InsP₆.

Our further study of other residues that would contribute to the protease catalytic site conflicts with studies on the mechanism of TcdB processing. Egerer et al. (15) found that the Asp residue of TcdB equivalent to Asp-3469 was essential for processing. We found that both Asp-3469 and the nearby Glu-3467 needed to be mutated to block activity. Glu-3467 is not conserved in TcdB, which could explain why the single mutant was defective for TcdB. Interestingly, the CPD of a putative RTX toxin in Y. pseudotuberculosis has only the Glu-3467 residue and Asp-3469 is not conserved suggesting that in this protein, the glutamate might be absolutely essential for processing because the aspartate is absent. However, in MARTX Vc rCPD, processing of even the double mutant was lost only at low InsP₆ concentrations and light scattering measurements showed a slight drift in particle size of this protein over time. These observations suggest these residues may affect either protein structure leading to the loss of InsP₆ binding or the loss of the ability to transition to an active conformation except at high InsP₆ concentrations that stabilize the structure. Hence, if a third catalytic residue is essential for CPD enzyme action, it may require analysis of a crystal structure to identify the location of this residue. Furthermore, the location of residues that contribute to formation of an oxygen hole may also require a tertiary structure for proper identification.

The site-directed mutagenesis also identified rCPD residues important for InsP₆ binding. The most highly conserved positively charged residues, Lys-3482 and Lys-3611, were identified as significantly defective for processing and defective for InsP₆ binding. An additional five lysine or arginine residues spread across the protein were also identified that are partially defective. Thus, it is proposed that in the folded structure, the peptide chains at both ends of the molecule interact to form a positively charged binding pocket for binding of the highly negatively charged InsP₆. Analysis of other proteins that bind InsP₆ similarly showed that these proteins form a binding pocket of mostly lysine and arginine residues located throughout the protein (25, 26).

Finally, to further understand the mechanism of rCPD autoprocessing, we needed to explain two observations. First, the minimal concentration of InsP₆ able to stimulate the autoprocessing was found to be 0.01 μM, which is 60-fold below the determined dissociation constant (Kₐ 6 × 10⁻⁷ M). Second, we calculated that 1 molecule of InsP₆ activated the processing of up to 100 molecules of rCPD despite the fact that processing occurs by an intramolecular, not intermolecular, mechanism. During the course of a reaction, although occupancy of the InsP₆ binding pocket would be low under limiting concentrations of InsP₆ proteins that do successfully bind InsP₆ would be processed but then become effectively inert and no longer compete with uncleaved protein for binding of InsP₆. This suggestion was confirmed in the ITC binding assay wherein no binding of InsP₆ to the cleaved molecule was observed. Thus, the reaction could proceed, albeit more slowly, at concentrations below the binding constant and below a 1:1 ratio of protein to InsP₆ because the stimulator molecule can be recycled. This model is supported by our finding that a catalytic rCPD C-S
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mutant can inhibit processing of either wild-type rCPD or rCPDΔ51 by competing for low InsP₆ concentrations, but rCPD K3482A that cannot bind InsP₆ has no effect.

Based on the data obtained, we can propose a preliminary working model for the molecular mechanism of InsP₆ activated autoprocessing of the MARTXᵥc holotoxin. After translocation into the cytosol, the CPD in its native conformation can bind InsP₆ with a Kᵅ well below the physiological concentrations of InsP₆ into a binding pocket consisting of residues arranged across the entire length of the CPD. InsP₆ binding either induces proper protein folding or a conformation shift, possibly near the catalytic histidine, that then allows the catalytic site to form and/or the cleavage site to be inserted within the catalytic site. Alternatively, InsP₆ itself might help form part of the catalytic site. Once activated, the molecule undergoes autoprocessing. Following processing, the CPD portion of MARTXᵥc is altered to a third conformation such that it releases InsP₆ and is no longer capable of binding InsP₆. These observations suggest that the protease is inactivated after successful cleavage and thus does not likely go on to target other proteins within the eukaryotic cell.

Overall, in this study we examined the activation step for the autoprocessing activity of the MARTXᵥc toxin of *V. cholerae*. The domain corresponding to this activity, CPD, is conserved in other large bacterial toxins. Therefore, the obtained information could contribute to the understanding of the mechanism of activity of virulence factors of other pathogenic bacteria, such as *C. difficile* or *Yersinia* sp.

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