Published in final edited form as:

Science. 2008 October 10; 322(5899): 265–268. doi:10.1126/science.1162403.

Small molecule-induced allosteric activation of the *V. cholerae* RTX cysteine protease domain

Patrick J. Lupardus^{1,4}, Aimee Shen^{3,4}, Matthew Bogyo^{3,*}, and K. Christopher Garcia^{1,2,*}

¹Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA 94305, USA

²Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA 94305, USA

³Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305, USA

Abstract

A eukaryotic-specific small molecule activates the protease domain of a *Vibrio cholerae* toxin, resulting in toxin autoprocessing and activation.

Vibrio cholerae RTX (Repeats-in-Toxin) is an actin-disrupting toxin that is autoprocessed by an internal cysteine protease domain (CPD). The RTX CPD is efficiently activated by the eukaryote-specific small molecule inositol hexakisphosphate (InsP₆) and we present the 2.1 angstrom structure of the RTX CPD in complex with InsP₆. InsP₆ binds to a conserved basic cleft that is distant from the protease active site. Biochemical and kinetic analyses of CPD mutants indicate that InsP₆ binding induces an allosteric switch that leads to autoprocessing and intracellular release of toxin effector domains.

Most secreted bacterial toxins are produced as inactive precursors that become proteolytically activated upon entering a eukaryotic cell (1). A select group of these toxins undergo autoproteolysis upon entry into the host cytosol, resulting in release of their effector domains (2, 3). The *Vibrio cholerae* RTX is a member of the Multifunctional Autoprocessing RTX (MARTX) family of toxins, which all contain a cysteine protease domain (CPD) predicted to mediate autoproteolytic activation of the secreted protoxin upon entry into the eukaryotic cytosol (4). Almost all clinical and environmental isolates of *V. cholerae* produce RTX (5), which enhances virulence and colonization in murine models of *V. cholerae* infection (6, 7). RTX autoprocessing is thought to release its actin disrupting effector domains from the target cell plasma membrane into the cytosol (4) (fig. S1). Although autoproteolysis is essential for RTX toxin function (3), the mechanism of RTX CPD activation is unclear.

It has recently been shown that the small molecules GTP and inositol hexakisphosphate (InsP₆) stimulate autoprocessing of the RTX CPD (3, 8), while InsP₆ activates a distantly related protease domain in Toxin B of *Clostridium difficile* (2, 9). We tested the ability of GTPγS, InsP₆, and InsP₆ metabolites to activate *V. cholerae* RTX CPD autocleavage in vitro. InsP₆ potently activated autocatalysis, with a half-maximal autocleavage concentration

* To whom correspondence should be addressed. mbogyo@stanford.edu (M.B.); kcgarcia@stanford.edu (K.C.G.).

⁴These authors contributed equally to this work

Summary: A eukaryotic-specific small molecule activates the protease domain of a *Vibrio cholerae* toxin, resulting in toxin autoprocessing and activation.

Supporting Online Material www.sciencemag.org/ Materials and Methods Figs. S1-S14 Tables S1-S3 References

(AC₅₀) of 0.9 nM versus 0.19 μ M, 0.72 μ M, and 240 μ M for InsP₆(1,4,5,6)₄, InsP₆(1,3,4,5,6)₅, and GTP γ S, respectively (Fig. 1A and fig. S3). We confirmed the interaction between InsP₆ and RTX CPD using surface plasmon resonance (SPR), which indicates InsP₆ binds to the RTX CPD with an equilibrium affinity constant (K_D) of 1.3 ± 0.2 μ M (Fig. 1B).

To gain structural insight into InsP₆-mediated activation of RTX CPD, we purified an autocleaved, minimal RTX CPD catalytic domain for co-crystallization with InsP₆ (10). We determined the structure of the RTX CPD at 2.1 Å resolution, consisting of amino acids 5 through 203 (with residue 1 being the P1' alanine and P1' referring to the residue C-terminal to the scissile bond). The protease domain is comprised of a seven-stranded beta sheet, with five central parallel strands and two antiparallel capping strands (Fig. 1C and D). Two helices flank one side of the sheet, lying diagonally in a groove created by a ~90° twist of the sheet, while a third helix caps the other side of the sheet. The Cys-His catalytic dyad (C140 and H91) lies at the C-terminal ends of the central D and E beta strands. The overall structure is reminiscent of the clan CD family of cysteine proteases, suggesting a common ancestor for this family of enzymes. Comparison with the two most closely related known protease structures, human caspase-7 (11) and *Porphyromonas gingivalis* gingipain-R (12) (fig. S4) reveals that two C-terminal helices have been replaced by a three-strand flap. This 'β-flap' structure forms a cleft in which we identified electron density for a single InsP₆ molecule (fig. S5).

The InsP₆ binding pocket is lined with basic residues, burying approximately 890 Å² of surface area (Fig. 2A). Nine of the twelve residues that directly interact with InsP₆ via hydrogen bond contacts are positively charged, with a core of six residues (K54, R85, S136, R171, K183, and K200) forming the bottom of the pocket and six others surrounding the InsP₆ molecule (T28, R29, H55, S169, and R182) (Fig. 2B and fig. S6). K195 covers the top of the InsP₆ molecule, interacting with the C1, C5 and C6 phosphate groups. The protein–InsP₆ interface is further stabilized by a network of water molecules between the InsP₆ and β-flap. On the opposite side of the β-flap is the catalytic dyad and a large hydrophobic pocket for the P1 amino acid (the residue N-terminal to the scissile bond) (Fig. 2C). The β-flap contributes three of the twelve hydrophobic amino acids that line the base of the P1 pocket, while helix 1 and two central beta sheets (D and E) contribute the remainder (Fig. 2D). The surface properties and size of the P1 substrate pocket are consistent with the cleavage of RTX CPD after an N-terminal leucine (3). The final N-terminal residue observed in our structure is I5 (the P5' position) (Fig. 2C), which lies ~14 Å from the catalytic cysteine. No electron density was observed for the P1' through P4' positions, suggesting that they do not make strong contacts with the protease and may minimally contribute to substrate specificity.

The InsP₆ binding site is structurally segregated from the active site, clearly indicating that InsP₆ does not act as a co-factor for catalysis. Indeed, kinetic analyses revealed that InsP₆ binding is independent of substrate binding to the active site and that the concentration of InsP₆ does not alter the affinity of the InsP₆-bound enzyme for its substrate (fig. S7). Since RTX CPD activity was strictly dependent on InsP₆ in these analyses, we hypothesized that InsP₆ binding may regulate exposure of the active site. We therefore tested the ability of a fluorescent maleimide derivative to alkylate the catalytic cysteine of wildtype CPD and a mutant CPD lacking two InsP₆ interacting residues (R182Q/K183N). Whereas weak fluorescent labeling was observed for both wildtype and mutant CPD in the absence of InsP₆, dose-dependent labeling of the active site cysteine was observed in the presence of InsP₆ only for the wildtype CPD (fig. S8). Thus, productive binding of InsP₆ is required to expose the active site cysteine of the protease to substrates and inhibitors. Consistent with this observation, pre-treatment of wildtype CPD immobilized on an SPR chip with N-

ethylmaleimide (NEM) alone failed to block InsP₆-induced autocleavage of the CPD from the chip (fig. S9). Simultaneous treatment of the CPD with InsP₆ and NEM, however, inhibited InsP₆-induced autocleavage, indicating that NEM can only react with the active site cysteine in the presence of InsP₆. These results strongly suggest an allosteric mechanism of activation in which the active site is disordered or occluded in the absence of InsP₆, a mode of regulation that likely protects the protease active site sulfhydryl until the toxin enters a eukaryotic cell.

Inspection of the structure suggested that the β -flap, which lines the side of the InsP₆ binding cleft closest to the catalytic site, may contribute to enzyme activation by properly ordering the P1 pocket and active site. Among the many side chains that coordinate InsP₆, the β -flap contains three interacting residues, R182, K183, and K195, that form a three-pronged 'clamp' above and below InsP₆ (Fig. 2A and 3A). We tested the effect of the mutations R182Q, K183N, and K195N on both InsP₆ binding (fig. S10) and catalytic activity (fig. S11) relative to wildtype CPD (Fig. 3A). Mutation of K183 and K195 abrogated both InsP₆ binding and autocleavage activity (Fig. 3A, left table), while mutation of R182 only moderately reduced InsP₆ binding but decreased autocatalysis by 340-fold relative to wildtype. Notably, R182 not only binds InsP₆, it also engages in structurally-stabilizing hydrogen bonding interactions with D24 (fig. S12). This may explain the more significant impact of the R182Q mutation on catalysis rather than InsP₆ binding, since R182 may primarily fine-tune the structure of the β -flap and contribute nominally to binding InsP₆. Thus, diminution of InsP₆ binding on one side of the flap clearly reduces catalysis mediated by residues on the opposite side of the flap.

We sought to determine if mutation of non-InsP₆ interacting residues in the β -flap might 'decouple' InsP₆ binding affinity from the autocatalytic activity of the enzyme. We focused on W192 in strand G₃, which makes van der Waals contacts with strands G₁ and G₂ (Fig. 3A), and D178, which stabilizes the G₁-G₂ β -hairpin by hydrogen bonding with three backbone amide nitrogens and helps anchor the center of the G₂ strand by forming a salt bridge with the side chain of H184 (Fig. 3A). We made conservative (W192F, D178N) and more potent (W192A, D178A) mutations; as controls, we also mutated nearby solvent-facing residues D191 and E179 (D191A, E179A). The conservative mutations (W192F and D178N) minimally altered InsP₆ binding affinity yet considerably reduced autocatalysis relative to wildtype and the controls D191A and E179A (Fig. 3A, right panels). Furthermore, the more potent W192A and D178A mutations induced only a modest drop in InsP₆ binding affinity yet a more severe defect in catalysis. The D178A mutant exhibited similar InsP₆ binding and catalytic activity as the R182Q mutant, consistent with their similar positions in the β -flap. The W192A mutant was most dramatically affected: despite having moderate affinity for InsP₆ ($9.3 \pm 0.6 \mu\text{M}$), W192A had a catalytic defect that was twice as strong as that of the InsP₆-binding mutant K195N. Thus, mutation of residues that apparently communicate InsP₆ binding to the active site through structural rearrangement of the β -flap can decouple InsP₆ binding from enzyme activation. The proposed structural rearrangement however appears to be subtle, as the circular dichroism (CD) spectra of the free- and InsP₆-bound enzymes were nearly superimposable (fig. S13).

To assess the biological importance of InsP₆ binding and β -flap integrity on RTX CPD function in vivo, we constructed *V. cholerae* strains containing CPD point mutations C140A (catalytic-dead), R182Q/K183N (InsP₆-binding defective), and W192A (β -flap transition defective). Western detection of the CPD (Fig. 3B) showed that while most wildtype RTX was autoprocessed during growth of *V. cholerae* in LB media, RTX containing catalytic dead (C140A), InsP₆-binding (R182Q/K183N), or β -flap (W192A) mutations were unprocessed (Fig. 3B). Thus, even in the presence of high levels of InsP₆ in LB media, all three mutations prevented CPD activation in the native RTX. When *V. cholerae* mutant

strains or supernatants were incubated with human foreskin fibroblast (HFF) cells, intracellular actin crosslinking induced by mutant strains R182Q/K183N and W192A was severely reduced relative to wildtype, while C140A failed to induce actin crosslinking (Fig. 3C and fig. S14). Thus, InsP₆ binding and an intact β -flap are required for RTX autocleavage and effector function.

The InsP₆-interacting residues in related MARTX cysteine protease domains are almost invariably conserved (fig. S2), strongly suggesting a shared mechanism for toxin activation in Gram-negative bacteria. Since InsP₆ is exclusive to eukaryotes (13) and is present at cytosolic concentrations >10 μ M (14), the responsiveness of the MARTX family to InsP₆ through the evolution of a proteolytic biosensor seems an ingenious strategy for regulating the function of a secreted toxin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank S. Juo for assistance with data collection and structure determination, E. D. Sandoval for assistance with kinetic analyses and helpful discussion, M. Bloech and G. Schoolnik for help with *V. cholerae* strain construction, and A. Guzzetta for intact mass analysis of SeMet-labeled CPD. P.J.L. is a Damon Runyon Fellow, supported by the Damon Runyon Cancer Research Foundation. K.C.G. is supported by the Keck Foundation and the Howard Hughes Medical Institute. M.B. is supported by the Burroughs Wellcome Foundation and the Searle Scholars Program. P.J.L., A.S., M.B., and K.C.G. are listed as inventors on a patent application related to use of the *V. cholerae* RTX CPD for biotechnical applications. Coordinates and structure factors have been deposited in the Protein Data Bank (www.rcsb.org) under accession number XXXX.

References and notes

1. Gordon VM, Leppla SH. Infect. Immun. 1994; 62:333. [PubMed: 8300195]
2. Reineke J, et al. Nature. 2007; 446:415. [PubMed: 17334356]
3. Sheahan KL, Cordero CL, Satchell KJ. EMBO J. 2007; 26:2552. [PubMed: 17464284]
4. Satchell KJ. Infect. Immun. 2007; 75:5079. [PubMed: 17646359]
5. Cordero CL, Sozhamannan S, Satchell KJ. J. Clin. Microbiol. 2007; 45:2289. [PubMed: 17522276]
6. Olivier V, Haines GK 3rd, Tan Y, Satchell KJ. Infect. Immun. 2007; 75:5035. [PubMed: 17698573]
7. Olivier V, Salzman NH, Satchell KJ. Infect. Immun. 2007; 75:5043. [PubMed: 17698571]
8. Prochazkova K, Satchell KJ. J. Biol. Chem. 2008
9. Egerer M, Gieseemann T, Jank T, Satchell KJ, Aktories K. J. Biol. Chem. 2007; 282:25314. [PubMed: 17591770]
10. Materials and methods are available as supporting material at Science Online.
11. Hardy JA, Lam J, Nguyen JT, O'Brien T, Wells JA. Proc. Natl. Acad. Sci. U.S.A. 2004; 101:12461. [PubMed: 15314233]
12. Eichinger A, et al. EMBO J. 1999; 18:5453. [PubMed: 10523290]
13. Michell RH. Nat. Rev. Mol. Cell Biol. 2008; 9:151. [PubMed: 18216771]
14. Irvine RF, Schell MJ. Nat. Rev. Mol. Cell Biol. 2001; 2:327. [PubMed: 11331907]

Fig. 1. InsP_6 activates the *V. cholerae* RTX cysteine protease domain (CPD) and the architecture of InsP_6 -CPD complex. **(A)** Activation of RTX cysteine protease domain autocleavage by InsP_6 . Recombinant RTX CPD (amino acids 3391-3650) was incubated with the indicated concentrations of InsP_6 for 2 h, and autocleavage was assessed by SDS-PAGE and Coomassie staining (representative gel inset). Reactions were performed in triplicate, and the amount of autocleaved protein relative to the total protein amount was analyzed by densitometry, averaged and plotted versus concentration of InsP_6 . Fifty-percent of wildtype CPD was autoprocessed (AC_{50}) at $0.85 \pm 0.02 \text{ nM}$ InsP_6 (mean $\pm$ SD). **(B)** SPR analysis of InsP_6 binding to wildtype biotinylated CPD immobilized on a streptavidin-coupled surface. Representative sensorgram (inset) shows binding of InsP_6 to the CPD-bound surface over a concentration of 0.1 to 100 μM . Equilibrium binding analysis indicates a dissociation constant (K_D) of $1.3 \pm 0.2 \mu\text{M}$ (SD). **(C)** Structure of the CPD- InsP_6 complex viewed from above the InsP_6 -binding site. **(D)** A view of the structure rotated $\sim 135^\circ$ to show the active site. The InsP_6 binding site and active site are separated by a 22 amino acid β -hairpin structure (labeled ' β -flap'). InsP_6 and the side chains of the catalytic dyad (Cys140/His91) are shown as stick models.

Fig. 2.

The InsP₆ binding and active sites. (A) Electrostatic surface potential of the CPD as viewed from above the InsP₆ binding site. Blue denotes positively charged surface; red denotes negatively charged surface. InsP₆ is shown in the binding site as a stick model. (B) Close-up view of the InsP₆ binding site. Side chains that directly interact with InsP₆ are labeled and shown as yellow sticks. The electron density for InsP₆ (2Fo-Fc) is contoured at 2 σ . (C) Surface topology of the CPD active site. The P1 substrate pocket is highlighted in orange, and C140 and H91 in yellow and blue respectively. The N-terminus is shown as a yellow ribbon, terminating at Ile5 and highlighting the threading of this region along the surface of the core domain. The remaining residues not visible at the N-terminus are depicted as a yellow dashed line to illustrate approximate positioning of the chain during catalysis. (D) Close-up view of the P1 substrate pocket. Amino acids that line the pocket are labeled and colored orange. InsP₆ is shown as in (B) to demonstrate the position of the catalytic site with respect to the InsP₆ binding site.

Fig. 3.

β -flap mutations decouple CPD autocatalysis and RTX activity from InsP₆ binding. **(A)** Comparison of autocleavage efficiency (AC₅₀) versus InsP₆ binding (K_D) measured by SPR for mutations in the InsP₆ binding site (left table) and β -flap (right tables, top and bottom). The β -flap region of the CPD is rainbow-colored starting with blue at the N-terminal end. β -flap, catalytic site, and visible InsP₆-interacting side chains are shown as sticks. Data are expressed as mean $\pm$ SD. ND, not determinable. **(B)** Western blot analysis of RTX in supernatant harvested from log-phase *V. cholerae* cultures. Supernatants from *V. cholerae* strains harboring either an intact *rtxA* gene (wt), a null mutation in *rtxA* (Δ rtxA), or point mutations in the region encoding the CPD domain of RTX (C140A is catalytic-dead; R182Q/K183N is mutated at two InsP₆-binding residues; and W192A is a β -flap mutation) were blotted using an anti-CPD antibody. **(C)** Actin crosslinking induced upon incubation of *V. cholerae* with HFF cells. *V. cholerae* strains used in (A) were incubated with HFFs for 90 min, then HFF cells were lysed. Actin crosslinking was visualized by SDS-PAGE and Western blotting using an anti-actin antibody. The cross-linked forms of actin are labeled to the right.