

Small Molecule–Induced Allosteric Activation of the *Vibrio cholerae* RTX Cysteine Protease Domain

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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 the 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 the release of their effector domains (2, 3). The *Vibrio cholerae* RTX (repeats in toxin) is a member of the multifunctional autoprocessing RTX (MARTX) family of toxins, which all contain a cysteine protease domain (CPD) predicted to mediate the 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's 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 guanosine 5'-triphosphate (GTP) and inositol hexakisphosphate (InsP₆) stimulate autoprocessing of the RTX CPD (3, 8); also, InsP₆ activates a distantly related protease domain in toxin B of *Clostridium difficile* (2, 9). We tested the ability of guanosine 5'-O-(3'-thiotriphosphate) (GTP-γ-S), InsP₆, and InsP₆ metabolites to ac-

tivate *V. cholerae* RTX CPD autocleavage in vitro. InsP₆ potently activated autocatalysis, with a half-maximal autocleavage concentration (AC₅₀) of 0.9 nM versus 0.19 μM, 0.72 μM, and 240 μM for InsP(1,4,5,6)₄, InsP(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 indicated that InsP₆ binds to the RTX CPD with an equilibrium binding affinity constant (K_D) of 1.3 ± 0.2 μM (Fig. 1B).

To gain structural insight into the InsP₆-mediated activation of RTX CPD, we purified an autocleaved minimal RTX CPD catalytic domain for cocrystallization with InsP₆ (10). We determined the structure of the RTX CPD at a resolution of 2.1 Å, 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 comprises a seven-stranded beta sheet, with five central parallel strands and two antiparallel capping strands (Fig. 1, C and D). Two helices flank one

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Fig. 1. InsP₆ activates the *V. cholerae* RTX CPD and the architecture of the InsP₆-CPD complex. (A) Activation of RTX cysteine protease domain autocleavage by InsP₆. Recombinant RTX CPD (amino acids 3391 to 3650) was incubated with the indicated concentrations of InsP₆ for 2 hours, and autocleavage was assessed by SDS–polyacrylamide gel electrophoresis (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 InsP₆. 50% of the wild-type CPD was autoprocessed (AC₅₀) at 0.85 ± 0.02 nM InsP₆ (mean ± SD). (B) SPR analysis of InsP₆ binding to wild-type biotinylated CPD immobilized on a streptavidin-coupled surface. Representative sensorgram (inset) shows the binding of InsP₆ to the CPD-bound surface over a concentration of 0.1 to 100 μM. Equilibrium binding analysis indicates a K_D of 1.3 ± 0.2 μM (SD). (C) Structure of the CPD-InsP₆ complex viewed from above the InsP₆-binding site. (D) A view of the structure rotated ~135° to show the active site. The InsP₆-binding and active sites are separated by a 22-amino acid β-hairpin structure (β-flap). InsP₆ and the side chains of the catalytic dyad (Cys140/His91) are shown as stick models.

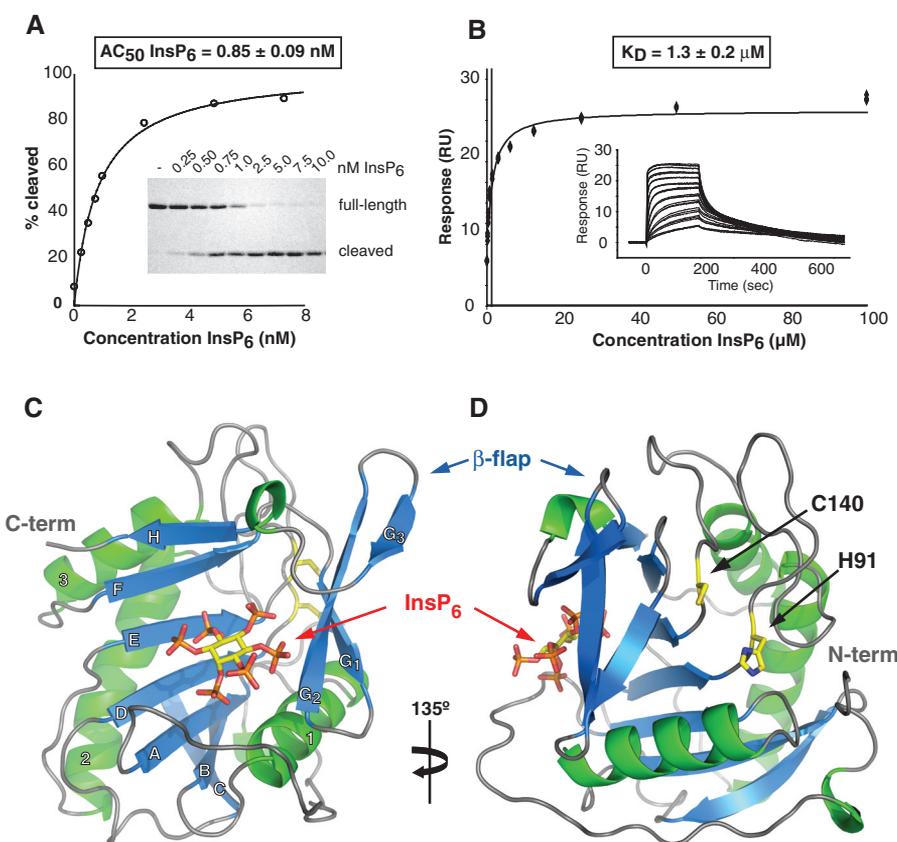


Fig. 2. The InsP_6 -binding and active sites. **(A)** Electrostatic surface potential of the CPD as viewed from above the InsP_6 -binding site. Blue denotes a positively charged surface; red denotes a negatively charged surface. InsP_6 is shown in the binding site as a stick model. **(B)** Close-up view of the InsP_6 -binding site. Side chains that directly interact with InsP_6 are labeled and shown as yellow sticks. The electron density for InsP_6 ($2F_{\text{obs}} - F_{\text{calc}}$) is contoured at 2σ . **(C)** Surface topology of the CPD active site. The P1 substrate pocket, C140, and H91 are highlighted in orange, 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 the 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. InsP_6 is shown as in (B) to demonstrate the position of the catalytic site with respect to the InsP_6 -binding site.

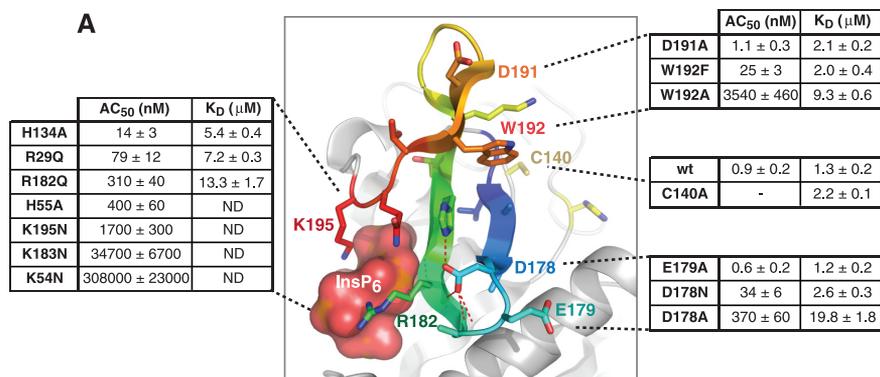
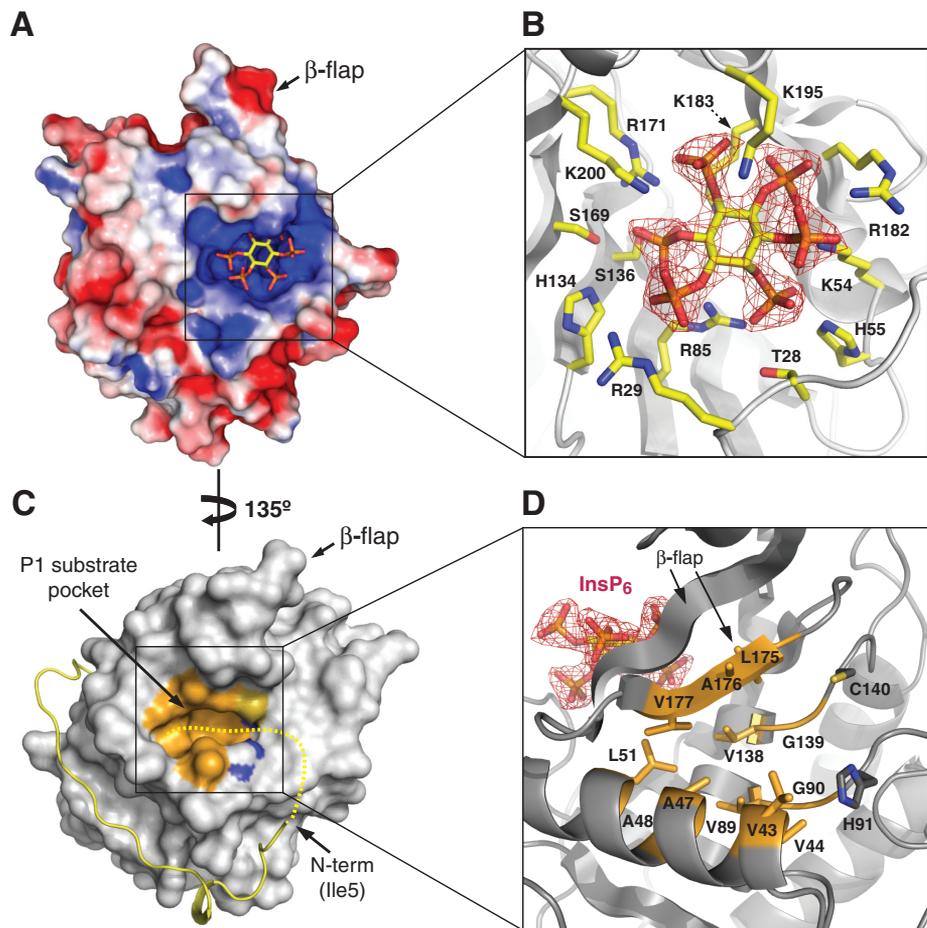
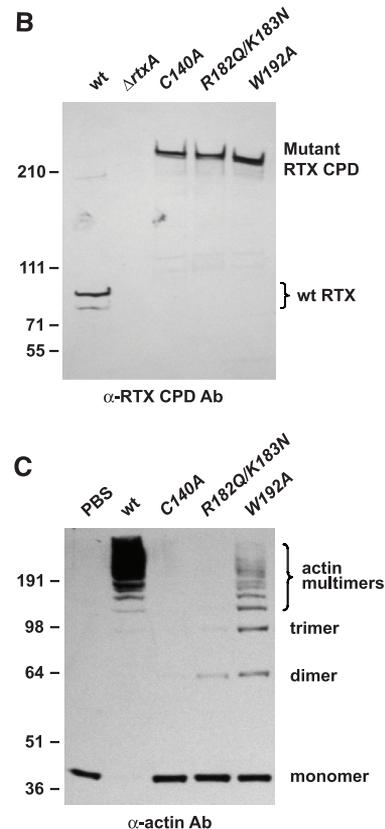


Fig. 3. β -Flap mutations decouple CPD autocatalysis and RTX activity from InsP_6 binding. **(A)** Comparison of autocleavage efficiency (AC_{50}) versus InsP_6 binding (K_D) measured by SPR for mutations in the InsP_6 -binding site (left table) and β -flap (right tables, top and bottom). The β -flap region of the CPD is rainbow-colored, starting with blue at the N-terminal end. The β -flap, catalytic site, and visible InsP_6 -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* (ΔrtxA), or point mutations in the region encoding the CPD domain of RTX (C140A is catalytic-dead; R182Q/K183N is mutated at two InsP_6 -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 the HFF cells were lysed. Actin crosslinking was visualized by SDS-PAGE and Western blotting by using an actin-specific antibody. The crosslinked forms of actin are labeled to the right.



side of the sheet, lying diagonally in a groove created by a $\sim 90^\circ$ twist of the sheet, and 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 β strands. The overall structure is reminiscent of the clan CD family of cysteine proteases, which suggests 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 five others surrounding the InsP₆ molecule (T28, R29, H55, H134, S169, and R182) (Fig. 2B and fig. S6) (13). 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 InsP₆ and the 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 one side of the P1 pocket, and 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 15 (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, which clearly indicates that InsP₆ does not act as a cofactor 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). Because 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 wild-type CPD and a mutant CPD lacking two InsP₆-interacting residues (R182Q/K183N) (14). Although weak fluorescent labeling was observed for both wild-type 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 wild-type 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, pretreatment of wild-type 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, which indicated 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 residues (R182, K183, and K195) that form a three-pronged “clamp” above and below InsP₆ (Figs. 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 wild-type CPD (Fig. 3A). Mutation of K183 and K195 abrogated both InsP₆ binding and autocleavage activity (Fig. 3A, left table), whereas mutation of R182 only moderately reduced InsP₆ binding but dramatically decreased autocatalysis as compared with the wild type. R182 not only binds InsP₆, it also engages in structurally stabilizing hydrogen-bonding interactions with D24 (fig. S12). This may explain the more considerable impact of the R182Q mutation on catalysis rather than InsP₆ binding, because R182 may primarily fine-tune the structure of the β -flap and may 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 the 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 and D178N) and more potent (W192A and D178A) mutations; as controls, we also mutated nearby solvent-facing residues D191 and E179 (D191A and E179A). The conservative mutations (W192F and D178N) minimally altered InsP₆-binding affinity yet considerably reduced autocatalysis relative to the wild type and controls D191A and E179A (Fig. 3A, right). Fur-

thermore, 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 InsP₆ binding and catalytic activity similar to those of 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 ± 0.6 μ 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 because 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 although most wild-type RTX was autoprocessed during the growth of *V. cholerae* in LB medium, 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 medium, 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 cross-linking induced by the mutant strains R182Q/K183N and W192A was severely reduced relative to the wild type, whereas C140A failed to induce actin cross-linking (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), which strongly suggests a shared mechanism for toxin activation in Gram-negative bacteria. Because InsP₆ is exclusive to eukaryotes (15) and is present at cytosolic concentrations >10 μ M (16), 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.

References and Notes

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13. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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17. We thank S. Juo for assistance with data collection and structure determination, E. D. Sandoval for assistance with kinetic analyses and helpful discussion, M. Blokech 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 NIH Keck Foundation and the Howard Hughes Medical Institute. M.B. is supported by the Burroughs Wellcome Foundation, the Searle Scholars Program, and the NIH National Technology Center for Networks and Pathways (grant U54RR020843). 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 3EEB.

Supporting Online Material

www.sciencemag.org/cgi/content/full/322/5899/265/DC1
Materials and Methods
Figs. S1 to S14
Tables S1 to S3
References

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Noncytotoxic Lytic Granule–Mediated CD8⁺ T Cell Inhibition of HSV-1 Reactivation from Neuronal Latency

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Reactivation of herpes simplex virus type 1 (HSV-1) from neuronal latency is a common and potentially devastating cause of disease worldwide. CD8⁺ T cells can completely inhibit HSV reactivation in mice, with interferon- γ affording a portion of this protection. We found that CD8⁺ T cell lytic granules are also required for the maintenance of neuronal latency both in vivo and in ex vivo ganglia cultures and that their directed release to the junction with neurons in latently infected ganglia did not induce neuronal apoptosis. Here, we describe a nonlethal mechanism of viral inactivation in which the lytic granule component, granzyme B, degrades the HSV-1 immediate early protein, ICP4, which is essential for further viral gene expression.

Several lines of evidence support a role for CD8⁺ T cells in controlling herpes simplex virus type 1 (HSV-1) latency. CD8⁺ T cells, many expressing granzyme B (GrB), are found juxtaposed to HSV-1 latently infected sensory neurons of both humans (1–4) and mice (5–8). In C57BL/6 mice, CD8⁺ T cells specific for the immunodominant HSV-1 glycoprotein B_{498–505} epitope (gB-CD8) polarize their T cell receptor (TCR) to junctions with neurons in situ forming apparent immunologic synapses (9). Murine gB-CD8 can block HSV-1 reactivation from latency in vivo and in ex vivo ganglia cultures in a major histocompatibility complex–dependent manner (9–11). Because HSV-1 estab-

lishes latency solely within ganglionic neurons (12, 13), we hypothesize that some latently infected neurons directly present viral antigens to HSV-specific CD8⁺ T cells during attempted reactivation, which is subsequently quelled by CD8⁺ T cell effector functions.

CD8⁺ T cells can use interferon- γ (IFN- γ) to block HSV-1 reactivation in some, but not all, latently infected sensory neurons (14, 15). HSV-1 reactivation is suppressed by CD8⁺ T cells in neurons that are refractory to IFN- γ through an as yet undefined mechanism. Lytic granules represent an important CD8⁺ T cell effector mechanism, but their use is generally lethal to targeted cells. GrB-expressing gB-CD8 from latently infected trigeminal ganglia (TG) polarized and released their lytic granules toward susceptible fibroblasts, leading to apoptosis (fig. S1) (16). Thus, we investigated whether gB-CD8 used lytic granules during immunosurveillance of latently infected neurons and also whether they induced neuronal apoptosis.

GrB⁺ gB-CD8 expanded from latently infected TG of wild-type (WT) mice (fig. S2) were added to cultures of dispersed TG in which reactivated HSV-1 had spread to the surrounding fibroblasts. Most fibroblasts targeted by gB-CD8 showed active caspase staining in punctate, multifocal, or diffuse patterns (fig. S3, A and C), which is consistent with early, intermediate, and late stages of apoptosis, respectively (17). Conversely, none of the gB-CD8–targeted neurons showed

caspase activation (fig. S3, B and C). Thus, either CD8⁺ T cells do not release lytic granules toward neurons, or lytic granule release does not activate the caspase system of neurons.

To distinguish between these possibilities, we first documented CD8⁺ T cell polarization of GrB toward junctions with neurons in latently infected TG in situ (Fig. 1A) and ex vivo (Fig. 1B), suggesting ongoing use of directed lytic granule release by CD8⁺ T cells during immunosurveillance of latently infected ganglia. Histologic studies of HSV-1 latently infected human (1–4) and murine (5–8) ganglia have failed to detect morphologic signs of apoptosis in neurons in direct contact with activated CD8⁺ T cells. To directly investigate whether neurons are refractory to lytic granule-mediated apoptosis, WT GrB-expressing gB-CD8 were added to dispersed latently infected TG directly ex vivo when HSV-1 is confined to neurons. Of 13 documented neuron/CD8⁺ T cell interactions exhibiting lytic granule release, none of the targeted neurons exhibited activated caspases (Fig. 1C), whereas neuronal caspases could be activated by ethanol treatment (Fig. 1D). CD8⁺ T cells contacting nonneuronal cells or not contacting any cell showed no lytic granule release (Fig. 1E). The selective resistance of neurons to apoptosis induction by CD8⁺ T cell lytic granules might be

Table 1. Pfn^{-/-} and GrB^{-/-} gB-CD8 are less efficient than WT gB-CD8 at inhibiting HSV-1 reactivation in a common pool of latently infected neurons. HSV-1 reactivation detected by plaque assay of supernatants from dispersed 34- to 44-dpi WT TG depleted of endogenous CD8⁺ T cells and cultured at one-fifth TG per well with the indicated number and type of gB-CD8 from culture initiation. +, reactivation detected; –, no reactivation detected; x, condition not tested. Data were pooled from four independent experiments (n = 15 to 35 cultures per condition).

Number of gB-CD8 per well	Type of gB-CD8		
	WT	Pfn ^{-/-}	GrB ^{-/-}
2 × 10 ³	+	x	x
2 × 10 ⁴	–	+	+
4 × 10 ⁴	–	+	+
5 × 10 ⁴	–	+	–
1 × 10 ⁵	–	+	–

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