Channels 5:3, 215-218; May/June 2011; © 2011 Landes Bioscience

The binding of cholera toxin to the periplasmic vestibule of the type II secretion channel

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The type II secretion system (T2SS) is a large macromolecular complex spanning the inner and outer membranes of many Gram-negative bacteria. The T2SS is responsible for the secretion of virulence factors such as cholera toxin (CT) and heat-labile enterotoxin (LT) from *Vibrio cholerae* and enterotoxigenic *Escherichia coli*, respectively. CT and LT are closely related AB_5 heterohexamers, composed of one A subunit and a B-pentamer. Both CT and LT are translocated, as folded protein complexes, from the periplasm across the outer membrane through the type II secretion channel, the secretin GspD. We recently published the 19 Å structure of the *V. cholerae* secretin (*Vc*GspD) in its closed state and showed by SPR measurements that the periplasmic domain of GspD interacts with the B-pentamer complex. Here we extend these studies by characterizing the binding of the cholera toxin B-pentamer to *Vc*GspD using electron microscopy of negatively stained preparations. Our studies indicate that the pentamer is captured within the large periplasmic vestibule of *Vc*GspD. These new results agree well with our previously published studies and are in accord with a piston-driven type II secretion mechanism.

Key words: secretin, GspD, electron cryomicroscopy, type II secretion system (T2SS), cholera toxin

Abbreviations: Gsp, general secretory pathway; cryoEM, electron cryomicroscopy; EM, electron microscopy; T2SS, type II secretion system

Submitted: 02/16/11

Revised: 02/23/11

Accepted: 02/23/11

DOI: 10.4161/chan.5.3.15268

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Addendum to: Reichow SL, Korotkov KV, Hol WG, Gonen T. Structure of the cholera toxin secretion channel in its closed state. Nat Struct Mol Biol 2010; 17:1226–32; PMID: 20852644; DOI: 10.1038/nsmb.1910.

Introduction

The type II secretion system (T2SS) is a major protein secretion pathway found in proteobacteria.[1,2] The T2SS is a multi-protein machinery that spans both the inner and outer membranes of bacteria and consists of three subassemblies: the inner membrane platform, periplasmic pseudopilus and outer membrane complex.[3,4] The inner membrane platform consists of a cytoplasmic secretion ATPase GspE that is associated with several copies of proteins GspL, GspM, GspF and GspC. The ATPase is thought to provide the energy for the secretion process. The pseudopilus is a fiber filament composed of pseudopilins GspG, GspH, GspI, GspJ and GspK. Multiple copies of major pseudopilin GspG form the body of the pseudopilus, whereas the four minor pseudopilins form the tip of the pseudopilus.[5,6] The pseudopilus has been proposed to act as a piston and/or plug during the secretion of exoproteins.[7-9] The outer membrane complex consists of the secretin GspD, one of the largest multimeric channels in the outer membrane of bacteria. The secretins are a diverse family of proteins, with homologs in other bacterial secretion systems such as the type III secretion system (T3SS), the type IV pilus biogenesis system and the filamentous phage assembly system.[10-15]

The T2SS is found in many pathogenic bacteria species, such as *Vibrio cholerae* and enterotoxigenic *Escherichia coli* (ETEC), where it secretes, among several other proteins, the major virulence factors cholera toxin (CT) and heat-labile enterotoxin (LT), respectively. CT and LT are closely related AB_5 hetero-hexamers composed of one A and five B subunits that are exported through the secretin channel from the periplasm to the extracellular space in their folded state.[16] Although the

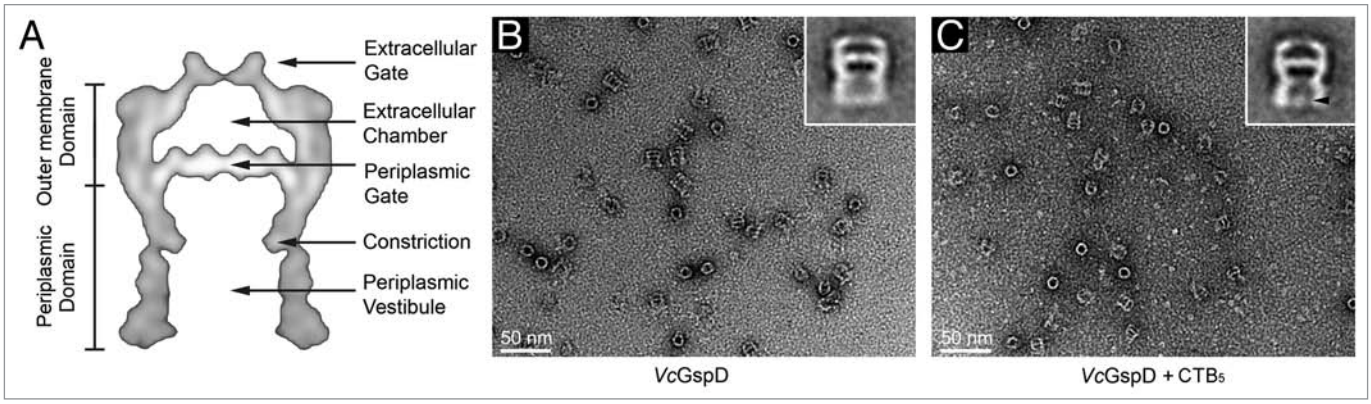

Figure 1. Negative stain electron microscopy of *Vc*GspD and the *Vc*GspD-CTB_5 complex. (A) Slice view of the *Vc*GspD cryoEM reconstruction (EMDB 1763).[21] The periplasmic and outer membrane domains are labeled. (B) Electron micrograph of negatively stained *Vc*GspD and (C) *Vc*GspD incubated with CTB_5. (B, inset) Representative reference-free class average of *Vc*GspD particles appears similar to the *Vc*GspD reconstruction with a large unobstructed periplasmic vestibule. (C, inset) Representative class average of *Vc*GspD mixed with CTB_5 contains an additional punctate density at the bottom of the periplasmic domain of *Vc*GspD (C, bottom inset arrow head). The punctate density is approximately 60 x 40 Å, which is consistent with the size of the CTB_5 complex.

secretion signals of proteins secreted by the T2SS are still unknown, in the case of both the CT and LT toxins the secretion signal resides in the B-pentamer and not in the A-subunit.[17] Interestingly, ETEC and *V. cholerae* are able to secrete both CT and LT.[18] Due to the apparent lack of a linear secretion signal sequence it has been suggested that the T2SS secretion signal is instead a structural motif present in the native or near-native protein structure.[18-20]

We recently described the 19 Å resolution structure of the T2SS secretin GspD from *V. cholerae* (*Vc*GspD) determined by electron cryomicroscopy (cryoEM) and single particle reconstructions.[21] The *V. cholerae* secretin forms a 200 Å tall dodecameric assembly that is ~0.9 MDa in molecular mass. The channel contains a large periplasmic vestibule with a constriction site that is believed to be important for substrate secretion. The periplasmic vestibule is separated from a large extracellular chamber by a periplasmic gate that is closed in our reconstruction. A second gate caps the extracellular chamber (**Fig. 1A**). The periplasmic domain of the secretin has been implicated in exoprotein recognition[7] and the crystal structure of a three-domain periplasmic fragment of ETEC GspD has been solved.[22] Surface plasmon resonance (SPR) studies have shown that the periplasmic domain of ETEC GspD interacts with the B-pentamer of heat-labile enterotoxin.[21] Here we used electron microscopy (EM) of negatively stained preparations to image the *Vc*GspD complex bound to the B-pentamer of cholera toxin (CTB_5). Our studies show that CTB_5 is captured within the large periplasmic vestibule of *Vc*GspD while the channel is in its closed state. We find that CTB_5 fits snuggly into the observed EM density compatible with a piston-driven mechanism for toxin secretion.

Results and Discussion

Electron microscopy of the *Vc*GspD-B_5 complex. Recombinant full-length *Vc*GspD and CTB_5 were each expressed in *E. coli* and purified to homogeneity for electron microscopy as described in reference 21. *Vc*GspD (50 μgml^{-1}) was either applied directly to a negatively charged carbon coated copper EM grid or first incubated with CTB_5 (7.5 μgml^{-1}) for 30 minutes at 25°C. Proteins were negatively stained with uranyl formate and visualized on a 100 kV transmission electron microscope (FEI Morgagni) as previously described in reference 23. Images were recorded on a 2k x 4k CCD camera (Gatan) at a nominal magnification of 44,000x at the specimen level (**Fig. 1B and C**). For image processing, micrographs were digitally binned twice to yield a final pixel size of 4.1 $Åpixel^{-1}$. Particles were picked manually in Ximdisp[24] and processed in SPIDER[25] to generate two-dimensional (2D) reference free class averages (**Fig. 1B and C**, insets).

The class averages of *Vc*GspD particles oriented perpendicular to the channel axis (side view) reveals a protein density that is consistent with the features observed in our cryoEM reconstruction (compare **Fig. 1A and B**, inset). The periplasmic domain is 125 Å long and forms a large unobstructed periplasmic vestibule with a constriction located just below the periplasmic gate. The periplasmic gate appears in a closed conformation, effectively separating the periplasmic vestibule from the extracellular chamber. The extracellular chamber is capped at the top by a weak protein density corresponding to the extracellular gate that was observed in the *Vc*GspD cryoEM reconstruction.[21] The identification of these features suggests the channel architecture is well preserved under the conditions used for EM of negatively stained specimens.

When *Vc*GspD was mixed with CTB_5 we observed two distinct groups of particles in the class averages. The first group (corresponding to ~35% of all side views) appeared similar to class averages of free *Vc*GspD, in that they contained an unobstructed periplasmic vestibule (**Fig. 1B** and inset). However, a second group of class averages (corresponding to ~45% of all side views) clearly contained an additional punctate density at the entrance of the periplasmic vestibule (**Fig. 1C** and inset arrow head). This density has approximate dimensions 60 x 40 Å that correspond well to the size of the B-pentamer. Therefore,

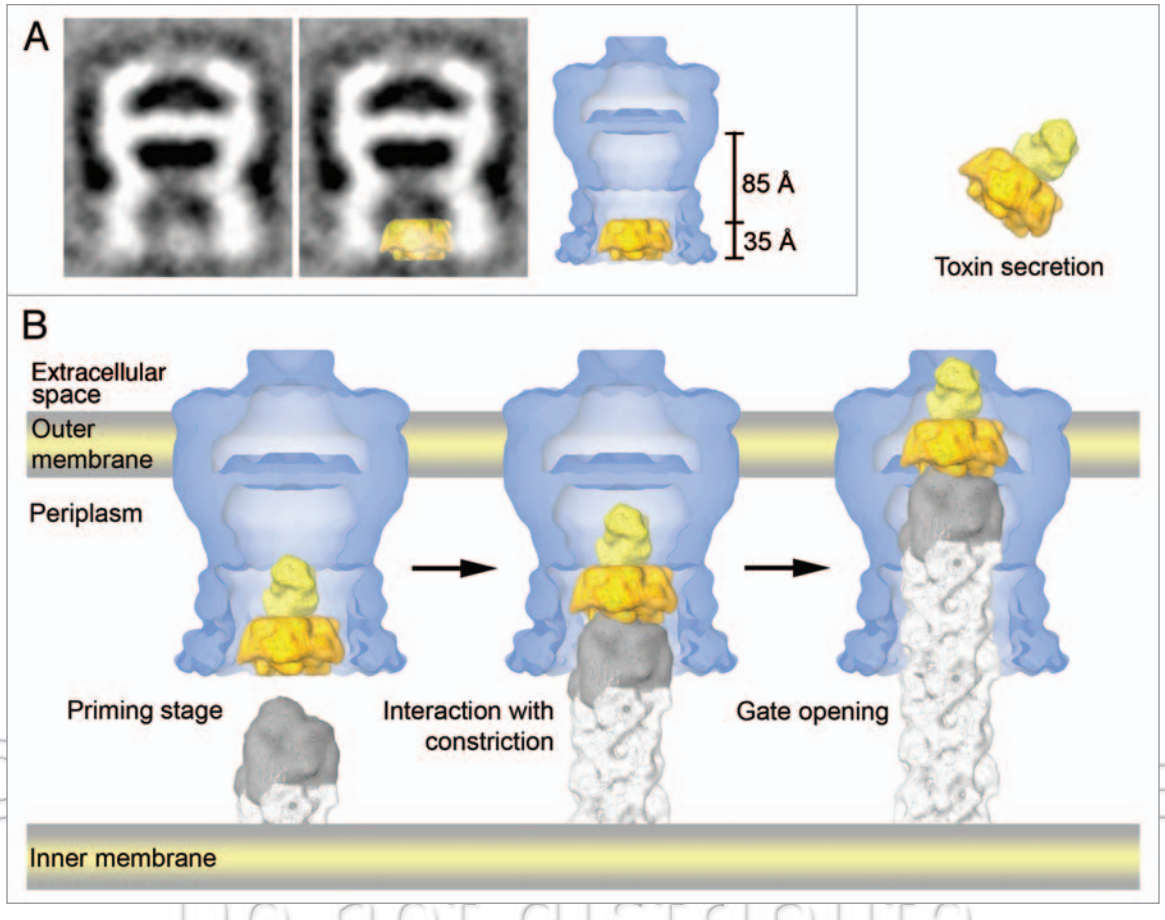

Figure 2. Model of the *Vc*GspD-toxin complex and implications for the T2SS toxin secretion mechanism. (A) The atomic structure of CTB_5 (gold, PDB ID 1S5E),[26] was fit into the CTB_5-bound *Vc*GspD EM density. The CTB_5 structure overlays well with the punctate density located at the entrance of the *Vc*GspD periplasmic vestibule seen in projection averages (left). The overlay was used to guide the placement of CTB_5 into the three-dimensional (3D) cryoEM reconstruction of *Vc*GspD (EMDB 1763),[21] placing the pentamer 85 Å below the periplasmic gate. Scale bar shown corresponds to both left and right panels. The CTB_5 structure fits snuggly within the large periplasmic vestibule, just below the channel constriction (right). (B) Key structural steps in the proposed piston driven mechanism for toxins through the T2SS.[7,21] (B, left) The secretin is loaded or primed by binding large exoproteins such as the AB_5 cholera holotoxin (gold) while the channel is in its closed state. (B, middle) The pseudopilus tip (grey, PDB ID 3CI0),[5] then binds the periplasmic domain sealing the channel entrance. As the pseudopilus (white, a pseudopilus model based on the GspG monomer structure (PDB ID 3FU1),[29] and a type IV pilus structure (PDB ID 2HIL),[30]) extends via an ATP-dependent process, it would act like a piston to force the toxin into the secretin constriction. (B, right) This interaction of the toxin with the constriction could trigger a conformational change in the secretin to open the periplasmic gate, allowing the toxin to enter the extracellular chamber and be secreted. The figure was adapted from Reichow et al.[21]

our EM analysis suggests that CTB_5 binds within the *Vc*GspD periplasmic vestibule. The distribution of free and CTB_5-bound *Vc*GspD particles is consistent with the relatively weak binding affinity observed between CTB_5 and the periplasmic domain of GspD in solution (K_D 15.5 ± 1.7 μM),[21] and the low protein concentrations required for EM sample preparation.

Molecular model of the secretin-toxin complex. The class averages obtained from the CTB_5-bound *Vc*GspD particles were used to guide the placement of the complete cholera toxin AB_5 heterohexamer (PDB ID 1S5E),[26] within the cryoEM reconstruction of *Vc*GspD to obtain further structural insight into the secretion mechanism. First, the atomic structure of CTB_5 was placed into the EM density obtained from the class averages of the *Vc*GspD-CTB_5 complex (**Fig. 2A**). The CTB_5 structure overlays well with the punctate density located within the periplasmic vestibule of *Vc*GspD. This placement positions the CTB_5 complex at the entrance of the *Vc*GspD periplasmic vestibule, roughly 85 Å below the periplasmic gate. Based on this placement, we modeled the complete AB_5 cholera toxin molecule into the periplasmic vestibule of the *Vc*GspD cryoEM reconstruction (**Fig. 2B**, left). The enormous 55.4 x 10^4 $Å^3$ periplasmic vestibule is capable of housing the 9.3 x 10^4 $Å^3$ AB_5 toxin. The resulting model places the cholera toxin just below the *Vc*GspD periplasmic constriction. This placement is almost identical to what we proposed previously based on the steric consideration of the constriction diameter, which narrows the channel to 55 Å.[21] Indeed, the B-pentamer is 65 Å wide, suggesting that the toxin could not pass through the constriction without major conformational changes occurring in the channel.

We propose that the *Vc*GspD-toxin complex that we observed in this study corresponds to the priming stage in

the piston-driven secretion mechanism (**Fig. 2B**).[7,21] In this model, the toxin is bound within the periplasmic vestibule of the secretin with the channel gate in a closed conformation, forming a substrate loaded or primed state (**Fig. 2B**, left). Then, following this priming stage, a signal is somehow transferred via the inner membrane platform to the secretion ATPase GspE in the cytoplasm, leading to ATP hydrolysis and polymerization of pseudopilins and resulting in formation or extension of the pseudopilus.[27,28] Eventually, the pseudopilus tip complex GspK-GspI-GspJ reaches the secretin and possibly interacts with the periplasmic domain of the channel, sealing the channel's entrance. We have previously shown by SPR that the trimeric pseudopilus tip complex interacts with the periplasmic domain of secretin.[21] As the pseudopilus extends further—probably through continuing ATP-driven polymerization—it would act as a piston forcing the toxin to interact with the *Vc*GspD constriction (**Fig. 2B**, middle). This interaction with the secretin constriction could provide the trigger that causes a conformation change in the channel to open the periplasmic gate. This conformational change may resemble the conformational change that was observed in the T3SS secretin following needle attachment.[10,11,21] With the periplasmic gate open, the toxin may pass into the extracellular vestibule for secretion (**Fig. 2**B, right). Once the toxin is secreted, the pseudopilus may transiently remain extended, acting as a plug to prevent channel leakage. By an as yet unknown mechanism, the GspD periplasmic gate closes, and the pseudopilus retracts and/or disassembles partially or fully to reset the system. Whereas this model is in line with our current structural knowledge of the T2SS, a complete understanding of this mechanism will require future high-resolution structural studies of T2SS assembly and secretion.

Acknowledgements

We thank the Murdock Charitable Trust and the Washington Research Foundation for generous support of our electron cryomicroscopy laboratory. This research is supported by the National Institutes of Health grant AI34501. Tamir Gonen is a Howard Hughes Medical Institute Early Career Scientist. The authors declare no financial interest related to this work.

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