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 AB5 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.

**Introduction**

The type II secretion system (T2SS) is a major protein secretion pathway found in proteobacteria. 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. 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, GspL, 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. The pseudopilus has been proposed to act as a piston and/or plug during the secretion of exoproteins. 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.

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, 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. Although the...
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.67 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.19-20

We recently described the 19 Å resolution structure of the T2SS secretin GspD from V. cholerae (VcGspD) 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 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 chamber of VcGspD (Fig. 1A and inset). The identification of these features suggests the channel architecture is well preserved under the conditions used for EM of negatively stained specimens.

When VcGspD was mixed with CTB, 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 VcGspD, 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,

Results and Discussion

Electron microscopy of the VcGspD-B5 complex. Recombinant full-length VcGspD and CTB5 were each expressed in E. coli and purified to homogeneity for electron microscopy as described in reference 21. VcGspD, in that they contained an unobstructed periplasmic vestibule (Fig. 1B and inset).
our EM analysis suggests that CTB₅ binds within the V₃GspD periplasmic vestibule. The distribution of free and CTB₅-bound V₃GspD particles is consistent with the relatively weak binding affinity observed between CTB₅ and the periplasmic domain of GspD in solution (Kᵣ 15.5 ± 1.7 μM), and the low protein concentrations required for EM sample preparation.

**Molecular model of the secretin-toxin complex.** The class averages obtained from the CTB₅-bound V₃GspD particles were used to guide the placement of the complete cholera toxin AB₅ heterohexamer (PDB ID 1S5E), within the cryoEM reconstruction of V₃GspD to obtain further structural insight into the secretion mechanism. First, the atomic structure of CTB₅ was placed into the EM density obtained from the class averages of the V₃GspD-CTB₅ complex (Fig. 2A). The CTB₅ structure overlays well with the punctate density located within the periplasmic vestibule of V₃GspD. This placement positions the CTB₅ complex at the entrance of the V₃GspD periplasmic vestibule, roughly 85 Å below the periplasmic gate. Based on this placement, we modeled the complete AB₅ cholera toxin molecule into the periplasmic vestibule of the V₃GspD cryoEM reconstruction (Fig. 2B, left). The enormous 55.4 x 10⁴ Å³ periplasmic vestibule is capable of housing the 9.3 x 10⁴ Å³ AB₅ toxin. The resulting model places the cholera toxin just below the V₃GspD 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. 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 V₃GspD-toxin complex that we observed in this study corresponds to the priming stage in obtaining further structural insight into the secretion mechanism.
the piston-driven secretion mechanism (Fig. 2B). 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 plaque 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. 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. As the pseudopilus extends further—probably through continuing ATP-driven polymerization—it would act as a piston forcing the toxin to interact with the VgspD constriction (Fig. 2B, middle). This interaction with the secretin constriction could provide the trigger that causes a conformational 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. With the periplasmic gate open, the toxin may pass into the extracellular vestibule for secretion (Fig. 2B, 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.

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