

## Prepore for a breakthrough

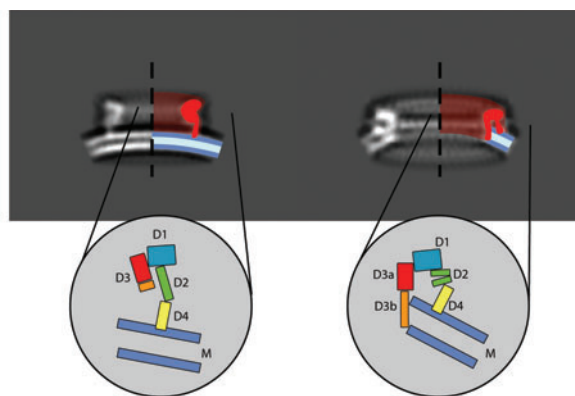
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**A key to understanding bacterial pathogenicity is the mechanism by which water-soluble protein toxins assemble on cell membranes to form oligomeric bilayer-spanning pores. The recent reconstruction from cryo-electron micrographs of three-dimensional pore and prepore structures of the cholesterol-dependent toxin pneumolysin shed new light on the later steps of the assembly of large toxin pores.**

Proteinaceous pore-forming toxins (PFTs) are secreted by many species of infectious bacteria. They may simply permeabilize or lyse mammalian cell targets causing nonspecific damage beneficial to the aggressor, such as the release of nutrients. Alternatively, they may behave more shrewdly and provide a pathway for the translocation of toxin subunits with enzymatic activity into cells, as in the case of anthrax toxin. Many pore-forming toxins break through the cell membrane by forming  $\beta$ -barrels comprising polypeptide strands contributed by a few<sup>1</sup> or many<sup>2</sup> subunits. Small pores are exemplified by staphylococcal  $\alpha$ -hemolysin with seven subunits and an internal diameter of  $\sim 20$  Å, and large pores by the cholesterol-dependent cytolysins (CDCs) with  $\sim 40$  subunits and an internal diameter of  $\sim 250$  Å. In a recent publication in *Cell*, Tilley and colleagues describe a structure, generated by cryo-EM at a resolution of 28 Å, of the 'prepore' intermediate in the assembly of pneumolysin, a member of the CDC family<sup>3</sup>. Together with a structure of the fully assembled pore, and a previous high-resolution crystal structure of the related perfringolysin O monomer<sup>4</sup>, the work yields the first detailed view of the conformational changes that occur during the formation of a large toxin pore.

Two distinct mechanisms for oligomeric pore formation have been considered. In the first, toxin monomers bind to the membrane surface, insert into the lipid bilayer and then oligomerize. For  $\beta$ -barrel-forming toxins, this

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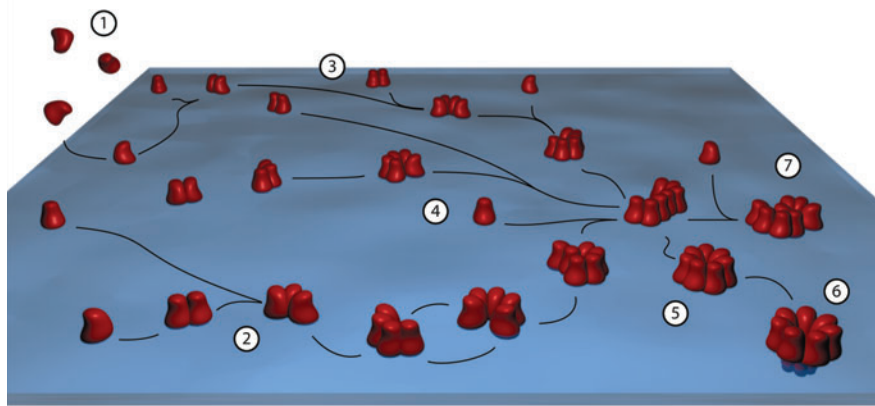
**Figure 1** Pore formation by CDCs. Left, section through the pneumolysin prepore based on cryo-EM<sup>3</sup>. The structure contains  $\sim 40$  subunits arranged in a ring. At bottom, a diagrammatic view of an individual cytolysin subunit within the prepore and its relationship to the lipid bilayer (M) is shown. Cryo-EM suggests that the conformation of the subunit is similar to that seen in the high-resolution crystal structure of the CDC perfringolysin O, which comprises four domains

(D1–D4)<sup>4</sup>. D4 provides the initial interaction with target membranes<sup>24</sup> and remains a point of contact in the prepore<sup>18</sup>. Right, section through the fully assembly pore<sup>3</sup>. The ring has dropped 30 Å into the bilayer and the cytolysin subunits have undergone a remarkable conformational change involving the collapse of D2 and the insertion of a refolded portion of D3 into the bilayer<sup>3,18</sup>. The inserted region, D3b, consists of two  $\beta$ -hairpins that contribute four strands to an  $\sim 160$ -strand membrane-spanning  $\beta$ -barrel<sup>2</sup>. The role of cholesterol in pore formation is still under debate. It was long thought that the primary function of cholesterol is to act as a receptor for CDCs. However, at least in some cases, attachment may involve protein receptors<sup>24</sup>. In keeping with a role for cholesterol in the prepore to pore conversion<sup>25</sup>, the lipid bilayer becomes deformed in the vicinity of the pore and it is proposed that cholesterol may be present at this site<sup>3</sup>.

mechanism is problematic because the backbone amides of individual transmembrane hairpins would prefer an aqueous environment where they can form hydrogen bonds at their edges. By contrast,  $\alpha$ -helices are content within a bilayer provided they are made up of largely hydrophobic amino acids. In the second mechanism, the monomers oligomerize on the membrane surface to form a prepore. The formation of the  $\beta$ -barrel follows by a concerted but otherwise unspecified mechanism. Accumulating experimental evidence supports the latter model, both in the case of PFTs that form small pores (staphylococcal  $\alpha$ -hemolysin<sup>5,6</sup>, aerolysin<sup>7</sup>, *Clostridium septicum*  $\alpha$ -toxin<sup>8</sup>, anthrax protective antigen<sup>9</sup> and leu-

kocidin<sup>10</sup>) and in the case of the CDCs, which form large pores (perfringolysin O<sup>11</sup>).

The concept of a prepore intermediate was first proposed on the basis of experiments with truncation mutants of staphylococcal  $\alpha$ -hemolysin<sup>5</sup>. There was however a danger that the mutants diverged from the normal assembly pathway to form irrelevant structures. Crucially, soon afterwards, a kinetically competent prepore intermediate was demonstrated by using an  $\alpha$ -hemolysin mutant that could be arrested in assembly with  $Zn^{2+}$  ions and released to complete assembly with a chelating agent<sup>6</sup>. Crystallographic studies of the related leukocidin F and S monomers and the fully assembled heptameric  $\alpha$ -hemolysin pore



**Figure 2** A general pathway for the assembly of PFTs that form  $\beta$ -barrels. Water-soluble monomers bind to membranes as individual molecules (1). Binding may or may not involve a specific membrane receptor, such as an oligosaccharide or a protein. Lateral diffusion and interprotomer interactions lead the way to prepore formation. During oligomerization, individual monomers might be added at the end of a growing chain (pathway 2), until it reaches the proper length for circularization to the prepore (5), perhaps occasionally overshooting to form oligomers with too many subunits to close stably (7). In another possible pathway (see 3 and 4) monomers first form dimers and small oligomers, and random encounters between them lead to prepore formation. These alternative pathways (sequential and random) have not yet been clearly distinguished for any PFT. Prepore formation is most probably irreversible<sup>6</sup>. Prepore-to-pore conversion follows and is slow and, except at very low monomer concentrations, rate-limiting (6 and Fig. 1)<sup>6,18</sup>. The small area of enclosed bilayer is presumably destabilized and disperses, leaving an open pore.

later defined the initial and final structures in the assembly pathway and allowed speculation about particulars of the associated conformational changes<sup>1,12–14</sup>. Atomic force microscopy (AFM) experiments demonstrated that the  $\alpha$ -hemolysin prepore sits on the membrane with its seven-fold axis perpendicular to the surface<sup>15</sup>. Mutants arrested at the prepore stage, often stapled up with cleavable disulfide bonds, were later used to provide evidence for prepores for other PFTs<sup>10,11,16,17</sup>.

What new perspectives do Tilley and colleagues<sup>3</sup> bring to the prepore story? First, they provide spectacular new views of the pneumolysin pore and prepore, which are massive structures compared with the  $\alpha$ -hemolysin heptamer (Fig. 1). Second, they fit the four-domain 2.7-Å crystal structure of monomeric perfringolysin O to both structures. The prepore comprises a ring of subunits on the surface of the membrane that contains largely unperturbed monomers. By contrast, the subunits in the prepore have undergone a striking reorganization, resulting in a structure with several unusual features, including a hollow rim, vertical  $\beta$ -strands and a distorted lipid bilayer. Third, the availability of the two structures allows the authors to speculate about the interrelated conformational changes in the pneumolysin subunits that are associated with membrane insertion. Fourth, the ability to visualize the bilayer shows that the top of the ring of subunits moves 30 Å closer to the bilayer upon insertion (Fig. 1). Based on these

observations, Tilley and colleagues<sup>3</sup> propose an assembly mechanism for the CDCs similar to that thought to occur with the PFTs that form smaller pores. This raises the question of whether there is a common mechanism of assembly shared by all  $\beta$ -barrel PFTs.

What is lacking in the *Cell* paper<sup>3</sup> is proof that the supposed prepore is actually a kinetically competent assembly intermediate (the different number of subunits in the prepore ( $31 \pm 3$ ) and pore ( $38 \pm 3$ ;  $44 \pm 3$ ) is a worry in this regard) and supporting evidence on the assignment of the structural domains. Fortunately, such evidence was provided earlier by Czajkowsky and co-workers, who used AFM to monitor the prepore to pore transition in perfringolysin O<sup>18</sup>. These workers used mutants arrested as prepores to obtain views of this structure. Importantly, they were able to follow the prepore-to-pore transition, notably the movement toward the bilayer, by ‘time-lapse’ AFM after releasing prepores by the reduction of subunits stapled with disulfides. Finally, they confirmed the orientation of the protein in the membrane by visualizing an antibody and streptavidin bound to specific sites on the polypeptide chain. By using these data, and evidence provided by environmentally sensitive fluorescence probes attached at specific sites, the authors formulated a mechanism for insertion that is strikingly similar to that in the Tilley paper. Together then, the two papers provide firm support for the existence of a prepore in the case of the CDCs and its

dramatic descent into the bilayer upon pore formation. Further, the structural basis for membrane insertion is accounted for, in at least a provisional manner.

Although the evidence is thinner, it seems unlikely that  $\alpha$ -hemolysin undergoes such a large movement toward the bilayer upon insertion. Is this a fundamental difference between small and large pore formation? Perhaps not. Domain D2 of the CDCs, which is proposed to undergo the largest change in structure when the pore is formed, connects the pore-forming domains, D1–D3, to the membrane (receptor) binding domain D4 (Fig. 1). Certain homologs of  $\alpha$ -hemolysin carry what are likely to be receptor-binding domains; for example, the *Vibrio cholerae* cytotoxin VCC has two lectin domains fused at the C terminus<sup>19</sup>. It is not clear how these domains are oriented in the VCC prepore or whether they are released from the membrane surface before insertion to form the pore.

What more is there to learn about the assembly of PFTs? Certainly, more structural information would be valuable. In the case of the CDCs, high-resolution structures of both the prepore and pore are lacking. In both cases, this is likely to await purification of monodisperse fractions with precise numbers of subunits for examination by X-ray crystallography. The pathway to the prepore is also not well understood. In the case of the CDCs, there is some evidence for the sequential addition of subunits<sup>20</sup>. But for the most part, mechanisms involving sequential and random addition of subunits have not been distinguished (Fig. 2). The observation of the assembly of individual oligomers by single-molecule techniques holds great promise in this area<sup>21</sup>. The mechanism by which the  $\beta$ -barrel forms and the bilayer is penetrated is also far from clear. Folding studies on membrane proteins lag behind those of soluble proteins, but considerable progress has been made in the case of  $\beta$ -barrels<sup>22</sup>. Membrane insertion by PFTs is essentially a refolding problem, which is simplified by the structural constraints placed on the system by the prepore framework.

The structures provided by Tilley and colleagues<sup>3</sup> provide a solid basis for tackling these important details. A more comprehensive understanding of the structure and assembly of toxin pores and their assembly pathways will pave the way to rationally designed therapeutic agents that block the pores or prevent their assembly<sup>23</sup>.

[AU: See queries in refs. 3,16]

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