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Structural Insights into the Type II Secretion Nanomachine

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Abstract

The Type II secretion nanomachine transports folded proteins across the outer membrane of Gram-negative bacteria. Recent x-ray crystallography, electron microscopy, and molecular modeling studies provide structural insights into three functionally and spatially connected units of this nanomachine: the cytoplasmic and inner membrane energy-harvesting complex, the periplasmic helical pseudopilus, and the outer membrane secretin. Key advances include cryo-EM reconstruction of the secretin and demonstration that it interacts with both secreted substrates and a crucial transmembrane clamp protein, plus a biochemical and structural explanation of the role of low-abundance pseudopilins in initiating pseudopilus growth. Combining structures and protein interactions, we synthesize a 3-D view of the complete complex consistent with a stepwise pathway in which secretin oligomerization defines sites of nanomachine biogenesis.

Keywords

Type II secretion; Type IV pili; inner membrane complex; secretin; secretion ATPase; pseudopilus

Introduction

Bacteria use complex nanomachines to transport macromolecules across the cell envelope. The Type II secretion system (T2SS) found in numerous Gram-negative bacteria transports folded proteins from the periplasm into the extracellular environment. T2SSs use ATP binding and hydrolysis cycles to assemble a periplasmic pseudopilus that pumps exoproteins through an outer membrane channel, proposed to serve as a piston to propel exoproteins through an outer membrane channel. The pseudopilus might push directly against exoproteins, facilitate opening of the channel, and/or block backward flow of exoproteins in the channel (for general reviews see [1], [2] and [3]).

At least twelve proteins are required for Type II secretion. High-resolution structures for most of these are now known, due in large part to the dedicated efforts of Professor Wim

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Hol and his colleagues. The T2SS proteins interact to form three substructures: the pseudopilus, the inner membrane (IM) platform, and the outer membrane (OM) secretin (Figure 1). Pseudopili are protein oligomers composed of pseudopilin monomers, which closely resemble the pilins that comprise Type IVa pili (T4P). In addition to multiple copies of the major subunit (GspG), three or four additional lower abundance (or “minor”) pseudopilins (GspH, GspI, GspJ, and GspK) form a tip complex that initiates pseudopilus formation [4–6] (Figure 2). All pseudopilins must be processed by a specific integral membrane protease (GspO) before they are competent for assembly. The IM assembly platform includes three integral membrane proteins (GspF, GspL, GspM) and a cytoplasmic, hexameric ATPase (GspE) [2,3]. This platform is coupled to the OM secretin (GspD) by the clamp protein (GspC) [2]. Key advances in the past few years have taken the field from a collection of individual domain structures and a cartoon model of the T2SS to an exciting new realm in which it is possible to synthesize from structural, biochemical and phenotypic data a hypothetical 3-D model of an entire trans-cell envelope T2SS nanomachine (Figure 1).

Important inferences for the T2SS can be drawn from studies of structurally similar T4P systems. The relatedness of T4P pilins and T2SS pseudopilins has long been appreciated, but diligent structural studies over the past decade have confirmed that other proteins within the T2SS have functional and structural analogs in the T4P system, even when sequence similarity between the analogs is absent or undetectable [7–15]. T2SS and T4P are members of a subclass of a larger family of nanomachines that assemble filamentous structures, including archaeal flagella, natural competence systems, and filamentous bacteriophage [2,3,16,17].

Pseudopilus formation requires an energy-transducing IM protein complex

The IM complex required for Type II secretion is formed by four conserved proteins: a cytoplasmic ATPase (GspE), two single-pass transmembrane proteins (GspL and GspM) and a polytopic transmembrane protein (GspF) [3]. Co-purification and a co-crystal structure have verified that the actin-like cytoplasmic domain of GspL interacts with the N-terminal domain of GspE [18]. Furthermore, the periplasmic domain of GspL has been cross-linked to GspG, the primary pseudopilin [19]. This interaction occurs both *in vitro* and *in vivo* and interestingly requires prior cleavage of the GspG leader peptide by GspO [19]. Cytoplasmic GspE-GspL and periplasmic GspL-pseudopilin interactions indicate that GspL itself serves to transmit conformational change generated by the GspE hexameric ATPase across the IM to facilitate pseudopilus assembly in the periplasm [19].

A hexameric ring structural model for GspE was created by superimposing the structure of the GspE monomer onto the asymmetric hexameric crystal structure of the T4P retraction ATPase PilT [20–22]. Based on this model, Sandkvist and colleagues proposed the six arginines close to the ATP binding site would be required for ATP binding and hydrolysis or for communication to a neighboring GspE subunit within the hexamer. Substitution of each Arg abolished ATPase activity and secretion but did not affect multimerization of GspE, association with the actin-like domain of GspL, or protein stability [20]. The authors concluded that GspE acts as a hexamer *in vivo*, with cycles of binding and hydrolysis of ATP leading to large conformational changes within the ring that are relayed across the membrane via GspL's transmembrane helix to other components of the T2SS. In this model, conformational changes in the GspE motor are driven by ATP binding, and these movements affect the conformation of the pseudopilus such that subunit addition is favored over subunit escape. While some hexameric ATPase motors are thought to harness energy for rotation (the flagellar rotor for example), there is no need to posit that GspE powers wholesale rotation of the T2SS IM complex within the membrane. Instead, ATP binding and

hydrolysis cycles by GspE promote pseudopilus extension by causing asymmetric structural rearrangements within the IM complex.

Many studies demonstrate GspL-GspM interactions in T2SS [3]. Genetic and biochemical studies and crystal structures of homologs of GspL and GspM from T4P systems are consistent with this conclusion [11,23]. Still to be ascertained, however, is the quaternary arrangement of GspL with GspM *in vivo*. Experiments with purified proteins indicated that the structurally homologous periplasmic ferredoxin-like domains of GspL and GspM can each form homodimers, which have been proposed to function in secretion [3,9,18]. Based on available evidence, we favor instead the possibility that GspL-M heterodimers are the functional unit in T2SSs. Firstly, two-hybrid analysis showed that GspM-GspM interactions require identical domains but are weaker than GspL-GspM interactions [24]. Secondly, comparing again to the T4P homologs of GspL (PilN) and GspM (PilO), PilN-PilO heterodimerization is preferred over homodimerization [11], and indeed analysis of stoichiometry *in vivo* suggests that homodimerization of PilO may inactivate the T4P biogenesis machinery [25].

The first structural study of a domain of the polytopic IM protein GspF suggests a homodimeric structure with four bundled cytoplasmic domains [14], consistent with EM data [26] and a crystal structure [15] of T4P homologs of GspF. The latter structure identifies a distinct monomer-monomer interface from that proposed in [14], however. Thus, the biologically functional arrangement of full-length GspF, and its interface and stoichiometry with other proteins of the IM platform remain unclear.

Conservation of (pseudo)pilin structure and in some cases secretion function imply a common mechanism underlying T2SS and T4P

In the T4P system, thousands of copies of the small single-pass membrane protein pilin assemble into long helical fibers. There is a strong structural similarity and therefore a likely ancestral relationship, between Type IV pilins and T2SS pseudopilins. All form a long α -helical backbone, exposed over the first ~25 hydrophobic amino acids (α 1-N) and partially covered over its C-terminal half (α 1-C) by a β -sheet to form a globular periplasmic head domain (Figure 2a). When the major pseudopilin (GspG) is over-expressed within a T2SS complex, T4P-like surface fibers known as hyper-pseudopili are formed [27,28]. Starting with the crystal structure of the soluble head domain of the *Klebsiella oxytoca* GspG (PulG), and STEM data from such hyper-pseudopili, Campos and colleagues applied distance restraints and refined a robust structural model of the T2SS hyper-pseudopilus. It shows a similar axial rise (~10.5 Å) and fiber diameter (~60–65 Å) to T4P [29,30] (Figure 2b). Conversely, T4P biogenesis systems secrete one or multiple proteins in a T2SS-like manner in *Vibrio cholerae*, - *Dichelobacter nodosus* and *Francisella tularensis* [31–33] despite maintaining a true pilin-specific fold rather than the smaller pseudopilin one [34] (Fig. 2a).

T4P pilins and T2SS pseudopilins are processed by specialized aspartic acid peptidases

Although delivered to the membrane via the canonical Sec pathway [35,36], pseudopilins are not cleaved C-terminal to the hydrophobic stretch in their signal sequences by signal peptidase but rather N-terminal to it by the integral inner membrane prepilin peptidase GspO (PilD in T4P). Thus the cleavage leaves the transmembrane helix attached to the pseudopilin and likely allows the N-terminus of the mature polypeptide to reside slightly inside the phospholipid head group layer based on energy minimization and steered molecular dynamics simulations of pilin in a bilayer [37]. Recently, the first structure of a member of this unique aspartic acid protease family was solved from the archaeal flagellar system of

Methanococcus maripaludis [38]. The preflagellin peptidase FlaK has a membrane-spanning helical bundle containing two conserved aspartic acid residues required for catalysis at the membrane-cytoplasm interface (Figure 2c). FlaK was crystallized in a presumed inactive conformation since the catalytic residues are 12 Å apart. Interactions with substrate could induce a conformational change, bringing the catalytic residues into close proximity for substrate cleavage.

Roles of minor pseudopilins in pseudopilus initiation and substrate recognition

The pilin fold is shared by the four minor T2SS pseudopilins, though each has unique features revealed by crystallography (Figure 2a). GspI is the central component in a complex of the four minor pseudopilins [5], which initiate the assembly of the T2SS pseudopilus. The largest of the T2SS minor pseudopilins (GspK) contains a second periplasmic domain inserted within the canonical fold. This domain forms a bulky cap that would prevent other pseudopilins from assembling above it [4]. Current biochemical experiments and molecular simulations suggest that GspI and GspJ initiate pseudopilus assembly by forming a staggered IM heterodimer, which is pulled toward the periplasm by its interaction with GspK [4,6,39]. An exciting idea that follows from these simulations is that lifting of the initiation complex in the membrane could tug other IM platform proteins further into the bilayer, and could thus activate the ATPase activity of GspE, which is stimulated by phospholipids [6,40].

The minor pseudopilin complex can interact in at least some cases with secreted substrates and the secretin, suggesting that minor pseudopilins play roles in substrate selection and secretin opening [41,42].

Outer membrane secretins are coupled to the IM complex via the channel clamp and both interact with exoproteins

Several recent EM and crystallography experiments have improved our understanding of the dodecameric OM secretin ring [13,42–45]. GspD consists of an extracellular cap, a conserved OM-embedded, trypsin- and detergent- resistant ~155 Å diameter torus and an N-terminal region that protrudes into the periplasmic space to form a collar of stacked rings that surround a periplasmic vestibule [42,43] (Figure 1). A constriction separates this vestibule from a periplasmic chamber, which itself is closed off from an extracellular chamber by a periplasmic gate. The periplasmic collar of GspD includes four small domains, designated N0–N3 [17]. Utilizing a camelid antibody, Korotkov *et al.* stabilized the first three of these for crystal structure determination [45]. While N1 and N2 (as well as N3 based on sequence conservation) are structurally homologous KH domains separated by flexible linkers, N0 has the fold of the signaling domain of TonB-dependent OM receptors. 12-fold cylindrical models of the N0–N1, N2 and N3 domains were constructed and fit remarkably well into the 19 Å resolution 3D cryo-EM reconstruction of the complete secretin [14,42]. The size and shape of the resulting vestibule accommodate the heterohexameric cholera toxin substrate, which is consistent with additional EM results showing the toxin resides within the vestibule *in vitro* [44] as it presumably would *in vivo* prior to export. The *P. aeruginosa* T2SS substrate elastase was also shown to interact directly with the N0–N1 domain of its cognate secretin by surface plasmon resonance (SPR) [41]. These recent data corroborate previous observations of interactions between the secretins and exoprotein substrates [2,3].

The channel clamp (GspC) has been proposed to traverse the periplasm and bind to the secretin [2,3]. New results from three GspC-GspD pairs verify a direct interaction

[13,41,46]. The “homology region” of the channel clamp (GspC-HR) and the N0 domain of the secretin interact in co-crystal structures of GspC-HR with N0–N1 or N0–N1–N2 fragments of GspD [13]. Amino acid changes to the interface disrupt secretion *in vivo* [13]. In the *P. aeruginosa* T2SS, SPR was used to demonstrate a GspC–GspD interaction dependent on GspD–N3 [41]. Taken together, these results suggest multiple interactions between GspC and GspD. There is now strong support for a model in which GspC and GspD form an unbroken connection from the IM through the periplasm to the outside of the cell.

The SPR and pull-down experiments of Douzi *et al.* [41] provide evidence of additional specific interaction partners for the GspC channel clamp protein, namely the secreted substrates themselves. Thus the minor pseudopilin tip complex, the channel clamp protein, and the N0–N1 domain of the secretin are all demonstrated to interact with exoproteins.

Model for assembly and function of the T2SS complex

Structural, genetic, and biochemical data contribute to a stepwise model of T2SS assembly (Figure 3). *In vivo* fluorescence microscopy studies indicate that secretins promote focal localization of other T2SS proteins [47,48]. Secretin monomers must pass through the peptidoglycan during transfer to the OM, in a process that may be independent of the classic OM β -barrel assembly machinery [49]. In some T2SS, secretins are localized by virtue of their interaction with a lipid-modified chaperone that is targeted to the OM by the LOL pathway [50,51]. Specific peptidoglycan-remodeling enzymes are required for OM localization and oligomerization of some secretins [52,53]. The secretin oligomer interacts with the channel clamp, which likely recruits and stabilizes the IM complex to form a connection between the IM complex and the OM secretin [2,3,41]. Pseudopilus formation is initiated by the minor pseudopilins (GspHIJK) [6], with polymerization of the major pseudopilin GspG powered by ATP-dependent conformational changes of the GspE motor acting on GspL.

GspC and/or the minor pseudopilin complex bind specifically albeit transiently (based on μ molar affinities) to substrates to recruit them to the T2SS [41,42]. The substrate is moved via a low affinity hand-off to the GspD–N0–N1 domain and thus into the periplasmic vestibule of the secretin. An exposed β -strand in the GspD–N0 domain might interact transiently with an exposed strand in a minor pseudopilin (Figure 2), GspC, or the substrate to encourage this exchange [45]. Given that substrate can lodge in the secretin vestibule without the involvement of other T2SS components *in vitro* [44], the observed affinity for the pseudopilin tip complex could instead play a role during the latter stages of secretion as pseudopilus elongation pushes the substrate further into the secretin. Likely each T2SS has co-evolved with its substrate(s) so that it is difficult to predict for a particular system which protein-protein interactions are most critical for substrate recognition. The molecular details of the last steps in secretion are unknown, but lead to opening of the secretin gates and release of the exoprotein from the cell.

Outstanding Questions

Several aspects of T2SS function remain open for structural investigation. The stoichiometry of proteins in the complex remains elusive as do the structural details of the IM transmembrane helices. Do six GspC clamp monomers traverse the periplasm to dock with 12 secretin subunits [13]? What is the stoichiometric ratio between the multipass IM protein (GspF) and the other IM platform proteins, and is GspF indeed the central member of the membrane complex as it is often depicted [2,3] (Figure 1)? Intriguingly, although the GspF homolog is required for secretion and pseudopilus formation, its over-expression in *P. aeruginosa* blocks hyper-pseudopilus formation [28].

The role of GspH in assembling the T2SS is somewhat unsettled, although it is certainly required for secretion under normal cellular concentrations of the T2SS proteins. The *P. aeruginosa* GspH homolog is part of a stable tetramer of the periplasmic domains of minor pseudopilins *in vitro*. It is thus proposed to form an adapter to link the major subunits to the tip complex [5,41]. On the other hand, in *K. oxytoca*, GspH is dispensable for hyperpseudopilus formation, and indeed for secretion if the rest of the complex is over-expressed [6].

Finally, although tantalizing clues are beginning to emerge there is still no complete model of how substrates are recognized by the T2SS complex or how they traverse the secretin. It is also unclear how many GspG pseudopilin monomers must polymerize to push the substrate through the periplasmic gate of the secretin. Once a substrate has passed the N3 constriction and arrived in the periplasmic chamber, the periplasmic and/or extracellular gates might open without any additional force from the pseudopilus and the substrate would be drawn out by diffusion. This model suggests as few as four but not more than ten pseudopilin monomers might be needed per secretion event (Figure 1).

The application of a wide variety of methods for structure determination will continue to be important in advancing the T2SS field. Crystal structures of soluble domains have provided a patchwork picture of a T2SS, but exciting developments and a richer understanding have come with the integration of these structures with results from non-crystallographic methods such as EM, small-angle X-ray scattering, nuclear magnetic resonance, and computational modeling to reveal membrane-associated structures and define the dynamic protein-protein interactions required for T2SS function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Type II secretion systems move folded proteins across the bacterial outer membrane.

3D structures of all major soluble domains in secretion system proteins have been solved.

A gated outer membrane secretin allows for passage of secreted substrates.

The secretin is connected to an energy-harvesting complex by a clamp protein.

Specialized subunits initiate assembly of a pilus-like filament required for secretion.

Figure 1. Molecular model of the Type II secretion nanomachine

(a) GspE hexamer (green) was modeled by aligning *V. cholerae* EpsE domains (1P9R, [21]) onto the homologous domains of the *P. aeruginosa* PilT hexamer (3JVV, [22]). GspF dimer (pink) was created with two *V. cholerae* EpsF domain-1 homodimers (3C1Q, [14]). One each of GspL (dark blue) and of GspM (light blue) periplasmic domains (2W7V, [9]; 1UV7, [10]) were aligned to the PilO homodimer (2RJZ, [11]) to create the GspL-GspM heterodimer (surface from PilO dimer.) Minor pseudopilins (GspK, dark green; GspHII, light greens) were manually added by grafting α_1 -N helices onto *V. cholerae* EpsH (2QV8, [54]) and ETEC GspIJK (3C1O, [4]) structures and fitting these onto the tip of the *K. oxytoca* PulG pseudopilus computational model [29], shown with four monomers of GspG (gray). The twelve-fold ring model of the ETEC GspD N-terminal domains $\times$ (dark red cartoons) (3EZJ, [45]) is positioned within the *V. cholerae* GspD EM reconstruction (red surface) (3EZJ, [45] and EMD 1763, [42]). ETEC GspC HR domain (orange) was aligned to GspD twelve-fold ring using the GspC-GspD co-crystal structure (3OSS, [13]). *V. cholerae* GspC PDZ domain (2I4S, [55]) is positioned near the GspD N3 domain, just under the OM. Stoichiometry and relative arrangement of inner membrane proteins is currently unknown, although a 6:6:6:6:12 assembly of GspE:L:M:C:D is consistent with available data. For model viewing, one GspF dimer, two GspL-M heterodimers, and two GspC chains are shown. (b) In a transverse view at the periplasmic side of the IM, six GspL-M periplasmic domain heterodimers pack well around the pseudopilus. (c) In a transverse view at the cytoplasmic side of the IM, six-fold arrangement of GspL cytoplasmic actin-like domains around GspF dimer gives a ring with similar diameter. (d) A pseudopilus modeled into a cutaway of the GspD EM reconstruction shows that ~ten copies of the major pseudopilin along with one of each of the four minor pseudopilins would be sufficient to extend the pseudopilus into the periplasmic vestibule as far as the GspD N3 constriction. GspL/M hexameric rings were constructed from domain structures using a local python script and screened manually for best packing. Protein structural domains were aligned and proteins,

membrane grids, and model transmembrane helices were arranged in PyMOL. Linkers were added and final figure was constructed using Adobe Illustrator. See Suppl. Table 1 for details additional structures not represented.

Figure 2. Conserved architecture and assembly of pseudopilins

(a) Representative structures of the major and minor pseudopilins. N-terminal α_1 -N helix (dark gray) and β -sheet domain (light gray) are conserved, while each minor pseudopilin also has unique features (greens as in Figure 1). Full-length α_1 helices are shown for PilE and FimA, whereas all other structures are from soluble periplasmic domains of pseudopilins, missing the α_1 -C region of the N-terminal helix. Abbreviations: *K.o.*, *K. oxytoca*; *V.c.*, *V. cholerae*; *V.v.*, *V. vulnificus*; *P.a.*, *P. aeruginosa*; ETEC, enterotoxigenic *E. coli*. Subscripts denote species-specific names that deviate from standard T2SS nomenclature. (Structures include 2QV8, [54]; 2RET, [56]; 3NJE, [57]; 3CI0, [4]; 1T92, [58]; 3FU1, [59]; 2KEP, [60]; 3SOK, [34]; 1AY2, [61]. See Suppl. Table 1 for details and additional pseudopilin structures not represented.) **(b)** Type IV pilus (left, 2HIL [62]) and T2SS pseudopilus (right, computational model [30]) have nearly identical diameter and axial rise per subunit. Eighteen pilin and eight pseudopilin subunits are depicted as cartoons in each filament, with semi-transparent surfaces. Only the α_1 -helices are shown in the lower halves of each structure to highlight packing. The ETEC GspIJK soluble domain co-crystal structure (3CI0, [4]) is shown above the pseudopilus suggestive of its position *in vivo*. **(c)** *M. maripaludis* FlaK (3S0X, [38]), a member of the prepilin aspartic acid protease family. Catalytic aspartic acid side chains (spheres) and GxGD sequence (red) are invariant. Central helices (gold) are well-conserved throughout this protein family while the C-terminal cytoplasmic subdomain (silver) is unique to archaeal preflagellin peptidases. GspO is predicted to include the central (gold) and peripheral (bronze) transmembrane helices as well as two additional N-terminal transmembrane helices and a distinct cytoplasmic domain.

Figure 3. Stepwise assembly model for T2SS (Upper panel)

Formation of the envelope-spanning channel. The outer membrane (OM), inner membrane (IM), and peptidoglycan layer (PG) are shown, with dimensions from [63]. **(Lower panel)** Assembly and function of the pseudopilus. For clarity, the panel focuses on the IM complex, and the C-terminal domains of the secretin and channel clamp are not shown. For relevant references, see details in text.