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Cooperative Assembly of β-Barrel Pore-Forming Toxins

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Bacterial β-barrel pore–forming toxins are secreted as water-soluble monomeric proteins and assemble into β-barrel–shaped pores/channels through membranes of target cells, causing cell death and lysis. The pore assemblies that undergo various intermediate stages are symbolized by the association of multi-subunit structures in cells. Crystal structures of water-soluble monomers and membrane-embedded oligomeric pores, and recent studies involving biochemical detection and direct visualization of the sequential assembly of the toxin monomers have solved the mystery of how the pores are formed. Here, we review the mechanism of the cooperative assembly of several toxins of interest to explain the nature of the activities of the toxins.

Key words: cooperative assembly, membrane binding, oligomerization, pore-forming toxins, single-molecule imaging.

Abbreviations: PFT, pore forming toxin; αHL, α-hemolysin; γHL, γ-hemolysin; Luk, leukocidin; PVL, Panton-Valentine leukocidin; PC, phosphatidylcholine; β-ME, β-mercaptoethanol.

Assembly of super-macromolecules is essential for cell function (*1*) Bacterial pore-forming toxins (PFTs) are powerful models with which to study the nature of the assembly of oligomeric molecules on target cell membranes because of the stability of recombinant monomers and their spontaneous assembly. PFTs have been shown to be lethal to human and animals, since they can damage the organs and tissues of the host (*2*). Hence, it is useful to understand the mechanism of assembly so that methods to inhibit critical intermediate stages can be bound, thereby preventing diseases. The PFT family is classified based on the structures of the transmembrane domains. PFTs have been grouped into α-helical and β-barrel transmembrane proteins. The β-barrel PFTs comprise the major group, which have been found in many cytolysins, *e.g.*, staphylococcal hemolysin and leukocidin, aerolysin, streptolysin, *Pseudomonas aeruginosa* cytotoxin, anthrax protective antigen, certain insecticidal δ-endotoxins, and cholesterol-dependent cytolysins (*2*, *3*).

The recent discovery of genes for and analyses of the crystal structures of the β-barrel PFTs, including the heptameric pore of staphyloccocal α-hemolysin (*4*), the monomers of LukF of leukocidin/γ-hemolysin (*5*), LukF-PV of Panton-Valentine leukocidin (*6*), pro-aerolysin (*7*), and perfringolysin O (*8*), and progress in elucidation of the mechanism of pore assembly have provided a general view of how bacterial toxins are formed. Here, we review the functions, structures, and mechanism of assembly of three hot-topic PFTs, including staphylococcal cytolysins (α- and γ-hemolysins, and leukocidin), aerolysin, and perfringolysin O. We also present recent success in the direct imaging of intermediates and pores of γ-hemolysin on erythrocyte membranes, in which the cooperative assembly of toxin components plays a critical role in the activities of the toxins under physiological conditions. This mechanism may be common to the β-barrel PFTs.

Functions and structural views

1. Staphylococcal hemolysin and leukocidin. *Staphylococcus aureus* secretes a number of PFTs, which include α-hemolysin (αHL), γ-hemolysin (γHL), leukocidin (Luk), and Panton-Valentine leukocidin (PVL). αHL targets rabbit erythrocytes. Both rabbit and human erythrocytes are highly sensitive to γHL, whereas white blood cells, including macrophages, neutrophiles, and monocytes, are sensitive to Luk and PVL. These toxins are secreted as ~30 kDa water-soluble monomers that bind strongly to susceptible cells to form small pores with 2 and 8 nm inner and outer diameters, respectively (*9*). While αHL monomers form homo-heptameric pores (*4*), γHL (LukF/Hlg2) and Luk (LukF/LukS) are bi-component proteins that share the common component LukF and oligomerise into hetero-oligomers comprising ~6–8 subunits that are reported to be hexamers (3:3), heptamers (3:4 and 4:3) or octamers (4:4) with equal numbers of each component (*9–11*). The sequence homology among staphylococcal αHL, γHL, and Luk is less than 32%, but the core amino acids in the basic structure of the proteins are rather conserved, suggesting that they share a similar structure (*12*). The crystal structure of the αHL pore shows a mushroom shape consisting of cap, rim, and stem domains composed of seven single protomers, that is, subunits of the heptameric pore (*4*). The structures of the water-soluble LukF monomer (*5*) (Fig. 1A) and LukF-PV of PVL (*6*) share a basic structure with the αHL protomer (Fig. 1B). The major differences between LukF and αHL protomers are the structures of the pre-stem domain. The LukF pre-stem domain folds back to the cap domain to cover the hydrophobic region, while, in αHL,

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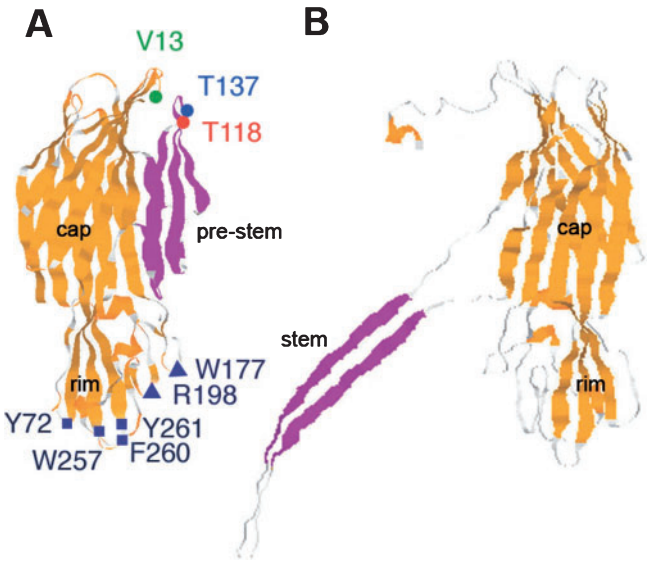

Fig. 1. **The water-soluble LukF monomer of γHL/Luk (A) and a single protomer of αHL (B).** The pre-stem region of LukF makes hydrophobic contact with the inner sheet of the β-sandwich (Cap) domain. The αHL protomer and LukF comparison is described in the text. Positions of the amino acid residues used to create double-cysteine mutations in LukF are shown in (A): V13 (green), T118 (cobalt), T137 (red). The violet ribbons represent the pre-stem/stem domains of (A) and (B), respectively. The two double-cysteine mutants are V13C-T137C (Cap–Stem) and T118C–T137C (Stem–Stem). The Y72, W257, F260 and Y261 residues at the bottom surface of the rim domain are essential residues for the initial binding on human red blood cells. The W177 and R198 residues are key residues not only for PC-binding but also for proper functional pore-formation with Hlg2. For further explanation, see Refs. *22* and *24*.

the pre-stem domain makes a long excursion into the membrane bilayer. The structures of the LukF monomer and αHL heptamer suggest the start and end of the pore assembly pathway in staphylococcal PFTs (*5*).

2. Aerolysin. Pro-aerolysin is a PFT from *Aeromonas hydrophyla* that is secreted as dimers of 52 kDa water-soluble proteins. After truncating a 40-residual

C-terminal peptide from pro-aerolysin, the activated aerolysin can bind and form pores 1.7 nm size to lyse several eukaryotic cell types, causing gastrointestinal diseases and severe wound infections in humans and animals (*13*, *14*). The structure of pro-aerolysin is rich in β-sheet (42%) and has significant α-helix (21%), including 4 functional domains (domains 1, 2, 3 and 4) and two disulfide bridges within domains 1 and 2 (*2*, *4*).

3. Perfringolysin O. Perfringolysin O is secreted by *Clostridium perfringens* and belongs to the cholesterol-dependent cytolysin (CDC) family. The sequence of perfringolysin O shares a high identity (40–80%) to that of other CDCs, suggesting that they all share a basic structure. Perfringolysin O has 4 functional domains and a core of β-sheet running through domains 1–3. Domain 3 is backed up to the central structure of the protein and harbors two α-helix regions that may cause the β-sheet to undergo conformational changes to insert through membranes. Domain 4 contains a tryptophan-rich region that is implicated in the binding of the toxin to cholesterol in membranes (*2*, *8*, *15*).

Cooperative assembly and pore opening

Although the sequences of β-PFTs are diverse, they may share a common pore assembly pathway because they are related in terms of structure with functional domains for binding, oligomerization, and especially a β-strand transmembrane to form β-barrel channels.

1. Binding to target cells. For single-component toxins, the membrane binding stage is simple. αHL selectively binds to rabbit erythrocytes with K_d ~ 2 nM. Aerolysin binds to several cell lines, such as baby hamster kidney cells, with K_d ~ 20 nM. Perfringolysin prefers to bind to membranes containing cholesterol (*2*). But for the bi-component toxins γHL and Luk, this stage is more complicated, since each component binds to the target cell membranes with a different affinity. For example in γHL, LukF (K_d ~ 500 pM) has a higher binding constant to erythrocyte membranes than Hlg2 [K_d ~ 10 nM (*12*)], whereas in the case of Luk, LukS (K_d ~ 1 nM) has stronger affinity for leukocytes than LukF (K_d ~ 20 nM) (Nguyen, T.V. and Kamio, Y., unpublished data). Although each component can bind individually to membranes, it seems that the binding of LukF to erythrocytes is prerequisite for the binding of Hlg2 in γHL, and that the binding of LukS allows the sequential binding of LukF in the case of Luk (*16*). This is because the components with the higher binding constant enhance the membrane association of the components with the lower constant by further oligomerization reactions that switch the association-dissociation balance to the association direction. In particular, Hlg2 binds approximately 4 times more than it can without LukF (*17*). The high specific binding of toxin to target cells suggests that the membrane-binding is regulated by receptors. However, such receptors remain to be identified, including those for staphylococcal αHL, γHL, Luk and PVL. Even in the case of perfringolysin O and other CDCs, cholesterols are only primary factors, but not adequate alone for toxin binding on sensitive cells (*2*). A glycosylphosphatidyl inositol anchored protein (GPI) has been found in mammalian cells as a receptor for aerolysin, because GPI-deficient cells are resistant to aerolysin. Recently, it has been reported that lipid rafts in membranes are essential for aerolysin to bind and concentrate for further oligomerization (*18*).

2. Oligomerization of individual subunits: cooperative stages. Little is known about oligomerization, because researchers have been unable to observe the process in real-time. To date, oligomers have been detected as steady-states at the end of a process, or occasionally at intermediate stages by using impair mutants that can not proceed to the final stage. Hence, the kinetics of oligomerization and the structural changes involved remain unclear.

Stoichiometric assembly and critical amino acid residues for oligomerization have been well studied for a number of toxins. The crystal structure of the αHL pore shows 7 subunits arranged in a mushroom shape in a side-view and a ring shape in a down-view. At lower resolution, aerolysin pores are estimated to be heptamers. In γHL, the analyses of LukF and Hlg2 populations, and LukF and LukS molecules in γHL and Luk, respectively, have implied that the single pore complexes of γHL and Luk may be hexamers consisting of three molecules, each

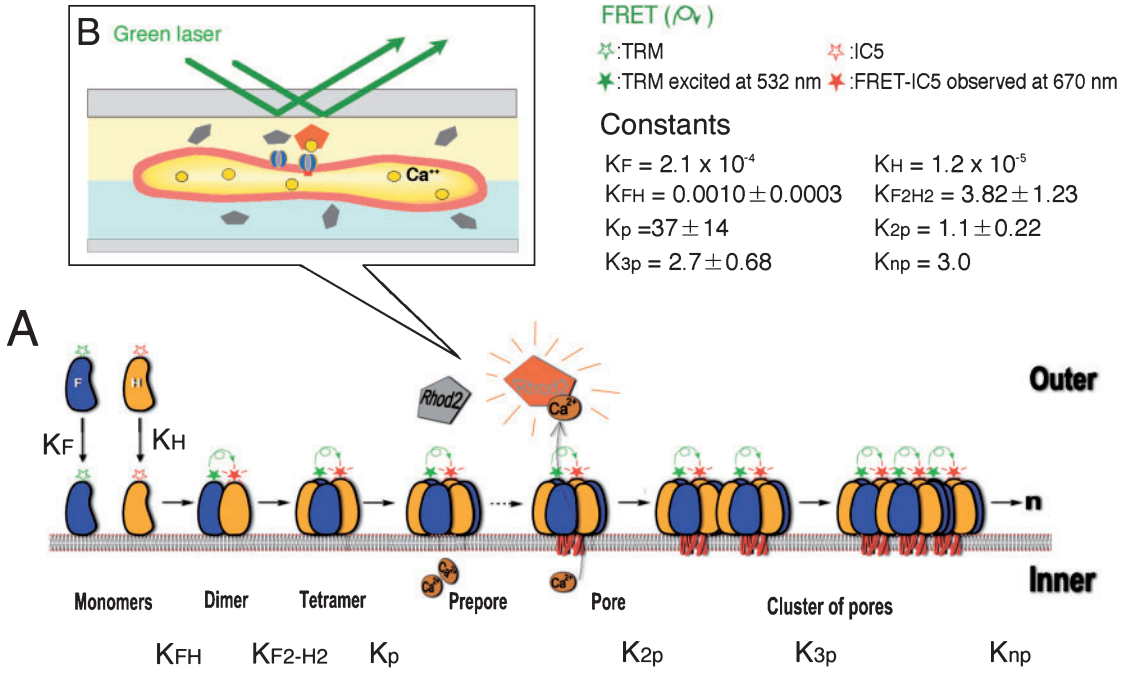

Fig. 2. **Cartoon model of the pore assembly of LukF and Hlg2 in hetero-oligomeric pore formation (A) and the observation of the opening of pores formed by the β-ME–treated Cap-Stem or Stem–Stem mutants of LukF.** Water-soluble LukF-TMR (F; blue) and Hlg2-IC5 (H; yellow) monomers bind to putative binding sites on the membranes. Sequentially, the membrane-bound monomers assemble into small oligomers (*e.g.* dimers and tetramers), then into single pores and clusters of pores. FRET signals of Hlg2-IC5 were observed at 670 nm when LukF-TMR was excited at 530 nm. The numbers indicate equilibrium binding and association constants (μm^{-2}). The red lines indicate the transmembrane domains inserting through lipid bilayers upon pore formation. (B) Ghost cells containing pre-pores (blue) consisting of the Cap-Stem or Stem–Stem mutants of LukF and wild-type Hlg were loaded with 10 mM Ca^{2+} (yellow), adhered onto slide glasses by L-polylysine, and put in a bath buffer containing 10 nM Rhod2 (gray), a Ca^{2+}-sensitive fluorescence indicator. Pore opening was triggered by the injection of additional bath buffer containing 20 mM β-ME and 10 nM Rhod2. Using total internal reflection fluorescence microscopy (enclosed in the square)., signals of Rhod2 near the basal membrane of ghost cells within a depth of about 100 nm of the evanescent field were monitored. During pore-formation, LukF undergoes a large conformational change to insert into the membrane, lysing the cell. LukF and its double-cysteine mutants, in which disulfide bonds are reduced by β-ME, can process this step. For details, see Refs. *17* and *22*.

with two components. Later, Ferreras *et al.* reported that γHL and Luk assemble into oligomers on phosphatidylcholine (PC)-cholesterol liposomes, and that hexamerization of the toxins is best fitted to the kinetics of vesicle permeabilization by γHL and Luk (*19*). Thereafter, Olson *et al*. constructed a model of heptameric pores of the two-component cytolysins using the αHL heptamer as a template (*5*). Miles *et al.* reported that LukF and LukS of Luk form an octameric transmembrane pore (*11*). Sugawara *et al*. isolated transmembrane pore complexes of γHL from human erythrocyte membranes and analyzed them electron microscopically and biochemically, and demonstrated that LukF and Hlg2 assemble in a stochastic manner to form alternate complexes with subunit stoichiometries of 3:4 and 4:3 (*10*). By contrast with staphylococcal PFTs, perfringolysin O can oligomerize into significantly larger pores with flexible sizes (24–48 nm) ranging from 10–50 subunits (*2*).

The complete ring-shaped oligomeric states that can quickly transit to functional pores are called pre-pores. Pre-pores were first detected for αHL (*20*), then for aerolysin (*21*), perfringolysin O (*15*) and γHL (*22*) using mutants that spontaneously arrest the pre-stem domain. Several amino acids locating at oligomerization domains function as keys for oligomerization. The H35 (*4*, *5*), S33, and T29 residues (*17*) of αHL, LukF, and Hlg2, respectively, participate critically in the interface side-by-side interaction. In aerolysin, a mutation at the H132 residue in domain 2 dramatically inhibits oligomerization (*14*).

Using single-molecule imaging of fluorescent-labeling proteins, we have directly observed the sequential assembly of LukF and Hlg2 monomers into oligomers on erythrocyte membranes for the first time (*17*). Single-cysteine LukF and Hlg2 mutants were specifically labeled with donor (trimethyl rodamine; TMR) and acceptor (IC5) dyes, respectively, so that multiple species of intermediate oligomers could be distinguished based on fluorescence resonance energy transfer (FRET) between the donor and acceptor. From the calculated association constants, we demonstrated that the cooperative stages play key roles in increasing the total number of pores at low physiological concentrations of toxin components. These stages include tetramerization, single pore formation, and finally the formation of clusters of more than 2 pores (Fig. 2A). The model of cooperative oligomerization for γHL could be applied to other β-barrel PFTs.

3. Pore formation. The formation of functional pores has been extensively studied for αHL, aerolysin, and perfringolysin O using biochemical detection methods, but not by direct observation. For αHL, single-cysteine muta-

tions and labeling with acrylodan at residues 118–140 of the stem domain suggest the movement of this domain from water to the hydrophobic membranes at this stage (*23*). The transition from pre-pore to pore was expected to be a cooperative process, since a combination of inactive mutants and wild-type suppressed the membrane-penetration of the active subunits (*2*). In aerolysin, upon channel formation, a number of cell signaling responses, such as release of calcium from ER and activation of protein G, occurred. The membrane insertion domain of aerolysin has not been unidentified, but is believed to be generated from α-helix hairpins during the oligomerization process (*7*, *13*, *14*). In perfringolysin O, domain 3 is expected to associate with the other domains upon pore formation so that the base of domain 4 may interact with the lipid bilayer (*8*, *15*). Interestingly, the β-barrel structural pores of those toxins are stable even in SDS at room temperature (*2*). For γHL, we have successfully observed a real-time pore opening event as indicated by Ca^{2+} efflux through the pores (Fig. 2B) (*22*). We designed double-cysteine mutants of LukF, in which single disulfide bonds connect either the pre-stem domain to the Cap domain (V13C-T137C, Cap–Stem), or two β-strands within the pre-stem domain (T118C–T137C, Stem–Stem) to control the pore assembly of γHL at the intermediate stages, whereby the pre-stem domain is trapped by internal covalent disulfide bond formation, so that several intermediates are arrested and then released to proceed to pore formation upon exposure to a reducing reagent. Ca^{2+} efflux is detected using a Ca^{2+} sensitive fluorescent dye, Rhod2, that is cell impermeant, surrounding the cells as the signals of individual Rhod2 flames. Unlocking the disulfide bonds by exposure to β-mercaptoethanol (β-ME) converts the pre-pores of both mutants to pores. This indicates the need for conformational changes around the key residues T118 and T137 in the LukF pre-stem domain during pore formation. Taken together, these data indicate that pre-pores are legitimate intermediates during γHL pore assembly, and that conformational changes around residues T118 and T137 in the pre-stem domain are essential for pore formation. Recently, we demonstrated using single-molecule imaging technique that a W177T/R198T mutant of LukF, which has no binding activity toward PC, forms intermediate oligomers with Hlg2, including dimers, tetramers, and hexamer/heptamers on HRBC. But, the mutant does not form functional pores. Hence, we conclude that W177 and R198 residues are essential for proper pore-formation by staphylococcal γHL (*24*). The data suggest that interaction between W177 and R198 residues and PC on the membranes is a key to the formation of functional pores.

Applications and conclusion

Studying the mechanism of the cooperative assembly of toxins in terms of structural changes and the kinetics of reactions at intermediate stages will be useful for understanding at the molecular biological level the folding and insertion of β-barrel membrane aqua channels, and of how single molecules are brought together into macromolecular complexes in cells. The mechanism of assembly of the three toxins discussed here could be extended to other PFTs. Specific binding allows toxins to target host cells, thereby increasing the number of toxins bound to the membranes to form pores. The cooperative oligomerization of single pores and the formation of more than two pores may be a common pathway for β-barrel PFTs, which enhance their total number of pores at low physiological concentrations. The SDS-stable channels of functional pores function like biological weapons in cells, ensuring a stable current and flux of water, ion, and other macromolecules to lyse the cells.

In terms of applications in medical fields, understanding the mechanism of pore oligomerization may lead to the discovery of chemical reagents and mutations to stop reactions at the intermediate state to prevent disease. For example in staphylococcal toxins and aerolysin, single mutations at residue H35 of αHL, residue S33 of LukF, residue T29 of Hlg2, or residue H132 of aerolysin can stop the assembly pathways at the dimerization stages of these toxins. Such mutations may be used as alternative methods to rescue patients who have acquired bacteria resistant to antibiotics, such as MRSA.

Concerning the architecture of the pore, αHL is the most studied for applications in the field of protein engineering, including drug delivery systems. The Bayley group has designed biochannels whose current is regulated by heavy metals using mutations of the glycine-rich region in the stem domain to pentahistidine. The current is turned on and off by the addition of Zn^{2+} and EDTA (*25*). Recently, this group has noncovalenty linked the pore with adapter molecules that mediate the blocking of the channel by small molecule analytes, such as β-cyclodextrin, to make the pore act as a stochastic sensor (*26*). This sensor could be applied to the detection analytes of many different types and sizes.

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REFERENCES

1. Alberts, B., Alexander, J., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002) Molecular Biology of the Cell, 4th ed., Garland Science, NewYork
2. van der Goot, G. (2001) Pore-Forming Toxins, Springer-Verlag, Berlin, Heidelberg, Germany
3. Parker, W.M. (2003) Cryptic clues as to how water-soluble protein toxins form pores in membranes. *Toxicon* **42**, 1–6
4. Song, L., Hobaugh, M.R, Shustak, C., Cheyley, A., Bayley, H., and Gouaux, E. (1996) Structure of staphylococcal α-hemolysin, a heptameric transmembrane pore. *Science* **274**, 1859–1866
5. Olson, R., Nariya, H., Yokota, K., Kamio, Y., and Gouaux, E. (1999) Crystal structure of staphylococcal LukF delineates conformational changes accompanying formation of a transmembrane channel. *Nat. Struct. Biol.* **6**, 134–140
6. Pedelacq, J.D., Maveyraud, L., Prevost, G., Baba-Moussa, L., Gonzalez, A., Courcelle, E., Shepard, W., Monteil, H., Samama, J.P., and Mourey, L. (1999) The structure of a *Staphylococcus aureus* leucocidin component (LukF-PV) reveals the fold of the water-soluble species of a family of transmembrane pore-forming toxins. *Struct. Fold Des.* **7**, 277–287
7. Parker, M.W., Buckley, J.T., Postma, J.P., Tucker, A.D., Leonard, K., Pattus, F., and Tsernoglou, D. (1994) Structure of the *Aeromonas* toxin proaerolysin in its water-soluble and membrane-channel states. *Nature* **367**, 292–295
8. Rossjohn, J., Feil, S.C., McKinstry, W.J., Tweten, R.K., and Parker, M.W. (1997) Structure of a cholesterol-binding, thiol-

activated cytolysin and a model of its membrane form. *Cell* **30**, 685–692
9. Tomita, T. and Kamio, Y. (1997) Molecular biology of the pore-forming cytolysins from *Staphylococcus aureus* α-hemolysin and γ-hemolysins and leukocidin. *Biosci. Biotechnol. Biochem.* **61**, 565–572
10. Sugawara-Tomita, N., Tomita, T., and Kamio, Y. (2002) Stochastic assembly of two-component staphylococcal γ-hemolysin into heteroheptameric transmembrane pores with alternate subunit arrangements in ratios of 3:4 and 4:3. *J. Bacteriol.* **184**, 4747–4756
11. Miles, G., Movileanu, L., and Bayley, H. (2002) Subunit composition of a bicomponent toxin: Staphylococcal leukocidin forms an octameric transmembrane pore. *Protein Sci.* **11**, 894–902
12. Gouaux, E., Hobaugh, M.R., and Song, L. (1997) α-Hemolysin, γ-hemolysin, and leukocidin from *Staphylococcus aureus*: distant in sequence but similar in structure. *Protein Sci.* **6**, 2631–2635
13. Parker, M., van der Goot, F.G., and Buckley, J.T. (1998) Aerolysin–the ins and outs of a model channel-forming toxin. *Mol. Microbiol.* **19**, 205–212
14. Fivaz, M., Abrami, L., Tsitrin, Y., and van der Goot, F.G. (2001) Not as simple as just punching a hole. *Toxicon* **39**, 1637–1645
15. Shepard, L.A., Shatursky, O., Johnson, A.E., and Tweten, R.K. (2000) The mechanism of pore assembly for a cholesterol-dependent cytolysin: formation of a large prepore complex precedes the insertion of the transmembrane beta-hairpins. *Biochemistry* **39**, 10284–10293
16. Kaneko, J., Ozawa, T., Tomita, T., and Kamio, Y. (1997) Sequential binding of staphylococcal γ-hemolysin to human erythrocytes and complex formation of the hemolysin on the cell surface. *Biosci. Biotechnol. Biochem.* **61**, 846–851
17. Nguyen, T.V., Kamio, Y., and Higuchi, H. (2003) Single-molecule imaging of cooperative assembly of γ-hemolysin on erythrocyte membranes. *EMBO J.* **19**, 4968–4979
18. Abrami, L. and van der Goot, F.G. (1999) Plasma membrane microdomains act as concentration platforms to facilitate intoxication by aerolysin. *J. Cell Biol.* **147**, 175–184
19. Ferreras, M., Frank, H., Serra, M.D., Colin, D.A., Prevost, G., and Menestrina, G. (1998) The interaction of *Staphylococcus aureus* bi-component γ-hemolysins and leucocidins with cells and lipid membranes. *Biochim. Biophys. Acta* **1414**, 108–126
20. Walker, B., Krishnasastry, M., Zorn, L., and Bayley, H. (1992) Assembly of the oligomeric membrane pore formed by Staphylococcal alpha-hemolysin examined by truncation mutagenesis. *J. Biol. Chem.* **267**, 21782–21786
21. Rossjohn, J., Raja, S.M., Nelson, K.L., Feil, S.C., van der Goot, F.G., Parker, M.W., and Buckley, J.T. (1998) Movement of a loop in domain 3 of aerolysin is required for channel formation. *Biochemistry* **37**, 741–746
22. Nguyen, T.V., Higuchi, H., and Kamio, Y. (2002) Controlling pore assembly of staphylococcal γ-hemolysin by low temperature and by disulfide bond formation in double-cysteine LukF mutants. *Mol. Microbiol.* **45**, 1485–1498
23. Valeva, A., Weisser, A., Walker, B., Kehoe, M., Bayley, H., Bhakdi, S., and Palmer, M. (1996) Molecular architecture of a toxin pore: a 15-residue sequence lines the transmembrane channel of staphylococcal alpha-toxin. *EMBO J.* **15**, 1857–1864
24. Monma, N., Nguyen, T.V., Kaneko, J., Higuchi, H., and Kamio. Y. (2004) Essential W177 and R198 residues of LukF for phosphatidylcholine-binding and pore-formation of Staphylococcal γ-hemolysin on human erythrocyte membranes. *J. Biochem.* **136**, 427–431
25. Walker, B., Braha, O., Cheley, S., and Bayley, H. (1995) An intermediate in the assembly of a pore-forming protein trapped with a genetically-engineered switch. *Chem. Biol.* **2**, 99–105
26. Gu, L.Q., Braha, O., Conlan, S., Cheley, S., and Bayley, H. (1999) Stochastic sensing of organic analytes by a pore-forming protein containing a molecular adapter. *Nature* **398**, 686–690