Structure and mechanism of bactericidal mammalian perforin-2, an ancient agent of innate immunity

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Perforin-2 (MPEG1) is thought to enable the killing of invading microbes engulfed by macrophages and other phagocytes, forming pores in their membranes. Loss of perforin-2 renders individual phagocytes and whole organisms significantly more susceptible to bacterial pathogens. Here, we reveal the mechanism of perforin-2 activation and activity using atomic structures of pre-pore and pore assemblies, high-speed atomic force microscopy, and functional assays. Perforin-2 forms a pre-pore assembly in which its pore-forming domain points in the opposite direction to its membrane-targeting domain. Acidification then triggers pore formation, via a 180° conformational change. This novel and unexpected mechanism prevents premature bactericidal attack and may have played a key role in the evolution of all perforin family proteins.

INTRODUCTION
Mammalian innate immunity is mediated by a range of factors including the membrane attack complex (MAC), which is deployed to kill invading microbes such as Neisseria species, while perforin-1 is used by cytotoxic lymphocytes to deliver granzymes into host cells targeted for destruction (1, 2). Both the MAC complex and perforin-1 are known to form large β-barrel pores using similar mechanisms (1–5). Perforin-2 (PFN2) has recently been identified as a novel and critically important component of innate immunity conserved throughout the animal kingdom (6–9) and evolutionarily close to the last common ancestor of the MAC and perforin-1 (7, 10). PFN2 is constitutively expressed in macrophages; however, its expression can be induced in many different cell types by interferons (α, β, and γ), bacterial infection, and pathogen-associated molecular patterns such as lipopolysaccharide (LPS) (10–14). Knockdown or deletion of PFN2 renders isolated cells and whole animals susceptible to infection, morbidity, and, in many cases, death (10, 12, 13). In human patients with pulmonary nontuberculous mycobacteria, four-point mutants of PFN2 have been identified (15), and macrophages expressing these mutant versions of PFN2 are unable to control Salmonella typhimurium or Staphylococcus aureus infection (15).

Because of the presence of a canonical MAC-perforin (MACPF) domain, PFN2 has been proposed to form functional pores in bacterial membranes, leading to bacterial death and control of infection (10–13, 16). However, unlike MAC components and perforin-1, PFN2 is initially synthesized as a type 1 transmembrane protein and found associated with the membranes of intracellular vesicles in which its large ectodomain (consisting mainly of the MACPF domain) and a unique P2 domain characteristic of PFN2 projects into the lumen. The PFN2 cytosolic tail, with a ubiquitin modification site, is then responsible for directing PFN2 to bacteria-encapsulating phagosomes (13) where it is thought to be further activated by proteolytic cleavage from its transmembrane anchor (12). In this study, we provide a detailed framework for understanding the role of PFN2 in defense against intracellular bacteria, showing that it first assembles into a pre-pore state as a ring of 16 subunits, the pore-forming transmembrane hairpins (TMHs) of which point in the opposite direction to their membrane-binding domains. A complete (180°) reconfiguration of the MACPF and membrane-binding P2 domains triggered by acidification then results in pore formation. The evolutionary closeness of PFN2 to the common origin of all MACPF/cholesterol-dependent cytolsin (CDC) proteins suggests that this previously unobserved mechanism of pre-pore–to–pore transition may have played an important role in the evolutionary adaptation of the entire family to which it belongs.

RESULTS
Cryo-EM structure of the PFN2 pre-pore oligomer
To characterize the basis for PFN2 activity, we have determined the molecular structure of its ectodomain in the context of a pre-pore assembly formed in solution at endosomal pH 5.5 (Fig. 1, A and B; fig. S1; and table S1). Most of the oligomers observed were full rings containing 16 subunits, with a very small percentage (<2%) of incomplete rings (arcs) with similar curvature to the ring (fig. S1, A and B), as observed with other MACPF/CDC family proteins including perforin-1 (1, 2, 4, 5). We determined the near-atomic resolution structure of this pre-pore form at an overall resolution of 3.5 Å (fig. S1C), with local resolution varying from 3.3 to 4.75 Å (fig. S1D). The resolution of the peripheral P2 domain is relatively low in the cryo–electron microscopy (cryo-EM) structure, and in particular, an extended β-hairpin region was not well resolved. However, by determining a structure of the P2 domain in isolation by x-ray crystallography (at 2.05 Å resolution; Fig. 2, A and B, and table S2), we
were able to build an atomic model for the whole complex (Fig. 1, C and D) into the cryo-EM map (Fig. 1, A and B). This revealed the presence of the expected MACPF family domain at the protein's N terminus, followed by an epidermal growth factor (EGF)–like repeat and the P2 domain and finally a C-terminal tail (CTT) not present in other MACPF/CDC family proteins (Fig. 1, E to G).

As observed in other MACPF/CDC proteins, such as the Apicomplexan perforin–like protein TgPLP1 (17) and the pneumolysin prepore (18, 19), the MACPF domains make extensive complementary contacts with each other in the PFN2 prepore structure (Fig. 1H). However, the CTT region of PFN2 (A577-I642, colored orange in Fig. 1) threads back from the end of the globular region of the P2 domain, passes down between the MACPF and P2 domains, and folds into a two-stranded β sheet on the top of the MACPF domain before terminating in an α helix. This packs the CTT’s β strands against the hairpin of the P2 domain from an adjacent subunit to create a continuous four-stranded intermolecular β sheet (Fig. 1, D and I). The folding of the PFN2 polypeptide chain from one subunit onto the next generates an additional stable intersubunit interface (Fig. 1D) absent in other MACPF/CDC superfamily members.

The pre-pore of PFN2 has a distinctive hat-like appearance in profile (Fig. 1, A to D) with the MACPF and P2 domains arranged concentrically. The larger MACPF domain is located inside, and the smaller P2 domains toward the outside of the ring of subunits, linked via a truncated EGF-like domain (Fig. 1, E to G). The PFN2 MACPF domain is closely related to the equivalent region of perforin-1, with two sets of transmembrane hairpin (TMH1 and TMH2) α helices prominently displayed available to form a β sheet traversing a targeted membrane on pore formation (Fig. 1, J and K). Like perforin-1, the TMH regions of PFN2 are similar to each other in length, though shorter (40 and 45 for TMH1 and TMH2 in PFN2 compared to 53 and 57 residues in perforin-1, respectively). The MACPF domain also contains conserved glycosylation sites (Fig. 1J), in equivalent positions to those found in the MAC component C9 and perforin-1.

Construction of a structure-based phylogenetic tree for the PFN2 MACPF domain (fig. S2) places it at a position entirely consistent with sequence-based phylogenetic analyses, which indicated that perforin-1 derives from a PFN2 ancestral gene duplication (7, 9).

**The β hairpin of the P2 domain directs membrane recognition**

We determined structures of the P2 domain in isolation by x-ray crystallography in two crystal forms, with two crystallographically distinct copies in space group P2_1 at 2.05 Å and eight copies in space group P1 at 3.17 Å (table S2). In both crystal forms, the extended β hairpin of the P2 domain could be unambiguously modeled and extends for 45 Å (Fig. 2, fig. S3, and table S2). The P2 domain has pseudo-threefold symmetry (Fig. 2, A and B, and fig. S3B) with a topological arrangement of constituent modules not previously observed (fig. S3A) distinct from the membrane-binding APCβ domains of...
Apicomplexan perforin–like proteins (17). Each repeat contains three strands with a conserved interstrand disulfide bond [pairwise root mean square deviation (RMSD) = 0.76 to 1.00 Å calculated from the three repeats]. The first repeat is much more extended than the other two and gives rise to the long β-hairpin projection, which is braced by two additional disulfide bonds, and enriched in positively charged residues along its length (fig. S3, B and C) and with mainly hydrophobic residues (LKIF) forming its tip (fig. S3B). Overlay of the cryo-EM and 10 crystallographically distinct copies of the isolated P2 domain structure (see above, Fig. 2C) indicates flexibility in the β hairpin. The pore-forming conformation of the P2 hairpin echoes the crystal structures (see below), whereas the pre-pore positioning of the hairpin is distorted away from its ensemble of resting (crystallographic) conformations (Fig. 2C). The rigidifying disulfide bonds and the hydrophobic residues in the P2 domain tip are highly conserved across species, although the identities of the residues along the β hairpin mostly are not (fig. S4, dashed black box). The highly basic nature of this projection (fig. S3D) and functional analogy to other family members including TgPLP1, perforin-1, and the CDCs strongly suggest that the β hairpin will be involved in membrane targeting and binding by PFN2.

To test this hypothesis, we made use of liposome sedimentation experiments with the P2 domain alone (Fig. 2, D to F). Liposomes containing phosphatidycholine (PC) and phosphatidylethanolamine (PE) showed no sedimentation of the P2 domain. When adding to these base lipids increasing concentrations of phosphatidylserine (PS) from 5 to 30%, the binding efficiency was gradually enhanced (Fig. 2D). Given that negatively charged lipids such as cardiolipin, Escherichia coli total lipid extract, and LPS from E. coli and Salmonella enterica are characteristic of major bacterial membrane lipid species, we also tested and confirmed preferential binding of the P2 domain to these lipids (Fig. 2E). In contrast, the P2 domain showed almost no binding to liposomes containing sphingomyelin, which is a neutrally charged lipid found in animal cell membranes (Fig. 2E). Truncating the β hairpin (residues Y427-V452) within the P2 domain (P2Δhairpin mutant) abolished its binding to PS, cardiolipin, and E. coli lipids, confirming the role of this region in membrane binding by PFN2 (Fig. 2F). Although the CTT domain contributes directly to the inter-subunit contacts with the P2 β hairpin and seems in the pre-pore assembly to be positioned such that it would lie along the top of the bilayer surface, truncation of the CTT domain (Δ606-652aa) within the ectodomain of mPFN2 showed no effect on the protein’s lipid binding activity (fig. S3E). Thus, membrane binding seems to be conferred exclusively by the tip region of the P2 domain β hairpin.

Dynamics of PFN2 pre-pore assembly on membranes
PFN2 pre-pore oligomeric assemblies were studied further using high-speed atomic force microscopy (HS-AFM) (Fig. 3). When PFN2 monomers were simply added directly to a freshly cleaved atomically flat mica surface (Fig. 3A and movie S1), HS-AFM found a wide distribution of arcs as well as rings of subunits with sizes ranging from 1 to 16 subunits (16 subunits corresponding to the complete ring) (Fig. 3C, green). In these experiments, only 7% of the assemblies overall formed complete rings (16 subunits). In contrast, on lipid bilayers (Fig. 3B), the overwhelming majority of membrane-bound oligomers formed complete rings (Fig. 3C, magenta). The complete rings were in total ≥80% of the assemblies found on lipid bilayers, uncompleted rings (arcs) only occurring on the competing borders of adjacent patches of hexagonal rings. While the assemblies displayed no long-range order on the mica, on the membrane, the rings arranged hexagonally (Fig. 3B). From this, we concluded that PFN2 existed as monomers or small-sized oligomers in solution and that their direct adsorption to the mica, where lateral diffusion is hindered, did not allow the protein to form complete rings. In contrast, when incubated on a membrane, where lateral two-dimensional (2D) diffusion is allowed, PFN2 formed stable rings 24 nm in diameter, which furthermore assembled into a hexagonal, i.e., the densest, molecular packing (Fig. 3C, inset, and movie S2). To further explore whether the oligomers observed using HS-AFM were membrane-bound PFN2 pre-pores, we performed a force-sweep experiment to clear them from the area of observation: The distribution of oligomeric assemblies recovered by diffusion within ~10 s after minimizing the force applied to the tip, as expected for membrane-associated structures (Fig. 3D and movie S3). The occasional observation of incomplete PFN2 rings on membranes allows the determination of subunit
association and dissociation rates, which were similar: 2.4 subunits·s⁻¹ and 2.3 subunits·s⁻¹, respectively (Fig. 3E).

The dimensions of the pre-pores measured by AFM [~24 nm outer diameter with an upper protrusion (top ring) diameter of ~11 nm; Fig. 3C, inset] provide further evidence of the orientation of the pre-pore oligomer with respect to the membrane. Consulting the PFN2 pre-pore structure, an upper protrusion of ~11 nm diameter arises when the TMH regions of the MACPF domain are accessible to the HS-AFM tip, orienting the P2 domain β-hairpin toward the lipid bilayer. If the oligomer were bound the other way up, then the diameter of the upper surface of the membrane-bound pre-pore would be ~15 nm. Thus, HS-AFM shows dynamic pre-pore formation as a lateral diffusion-dependent process leading to ring-shaped assemblies with ~24 nm outer diameter. The pre-pores can adhere to the bilayer via their β-hairpin exposing face, in agreement with the structural charge distribution of the pre-pore (fig. S3D) and lipid-binding experiments (Fig. 2). In this orientation, the membrane-inserting regions of the MACPF domain would point directly away from the targeted membrane.

Acidic pH triggers PFN2 pre-pore–to–pore transition

We next examined the membrane-perforating activity of mPFN2 using several complementary approaches. First, a sulforhodamine B–based leakage assay revealed PFN2 concentration–dependent dye leakage from pre-formed liposomes (PC/PS/E. coli total lipid extracts, 5:1:4) at pH 5.5, whereas no dye leakage was observed at pH 7.5 (Fig. 4A and fig. S5, A and B). At acidic pH 4.0 and 3.0, mPFN2 showed increased dye leakage compared to that at pH 5.5 (Fig. 4B and fig. S5C). Furthermore, negative-stain electron microscopy revealed that most of the membrane-bound mPFN2 oligomers were pre-pores with occasional pores at pH 5.5 (Fig. 4C and figs. S5D, red box, and S6A). Side views of these pre-pores displayed similar profiles to those of
measurements revealed the formation of discrete pores in membranes targeted by PFN2 with a range of sizes and conductance noise—in line with observations made for perforin-1 pores (4)—but with a consistent pattern of pores opening in discrete steps due to a defined pre-pore–to–pore transition (Fig. 4G).

Last, we performed direct pre-pore–to–pore activation experiments via HS-AFM when coupled to a buffer exchange system (20). Upon exposure to a low pH shock, the PFN2 pre-pore rings began to form pores after 14 s and all ~70 being imaged transited to the pore state within 3 s (Fig. 5 and movie S4). The resulting structures were higher (raised by ~4 nm) compared to the pre-pore state, in agreement with the pores imaged by negative-stain electron microscopy (Fig. 4D).

Overall, these results show that acidic pH triggers pre-pore–to–pore transformation, which is achieved by a 180° rotation of the MACPF and P2 domains with respect to each other so that membrane insertion can occur. This would require at least two significant conformational rearrangements: Rotation at the MACPF/P2 domain interface and unfurling of the TMHs into β hairpins. To test this hypothesis, we made use of two PFN2 disulfide bond mutants, following a strategy successfully implemented with perforin-1 and other MACPF/CDC family members (2). PFN2G568C/T594C locks the CTT to the P2 domain, and PFN2K251C/G286C prevents the deployment of the TMH2 region from the MACPF domain (fig. S7, A to C): Neither PFN2G568C/T594C nor PFN2K251C/G286C was active to form pores on liposomes when the engineered disulfide bridge was present at pH 5.5.
main TMHs, for pore formation to occur. To allow a 180° rotation, and then the unfurling of the MACPF do-
the interdomain lock between the MACPF domain and P2 domain
(fig. S7G). Together, these results demonstrate the need to release
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interface (fig. S7G). Together, these results demonstrate the need to release the interdomain lock between the MACPF domain and P2 domain to allow a 180° rotation, and then the unfurling of the MACPF domain TMHs, for pore formation to occur.

Cryo-EM structure of the PFN2 pore
To generate a sample of pores for structure determination, we prepared pre-pores at pH 5.5 as for the structure reported above and then lowered the pH to 3.6-4.0 in the presence of Cymal-6 (fig. S8, A and B). The cryo-EM structure of the pore was determined to an overall resolution of ~5 Å (Fig. 6, A and B; fig. S8, C and D; and table S1). The resolution for the central MACPF region of the cryo-EM map is ~4.5 Å, whereas the peripheral regions have lower resolutions ranging from 5.0 to 9.2 Å (fig. S8, C and D). We were able to construct a model of the pore fitted to its cryo-EM density guided by the distinctive features of each PFN2 domain and their connectivity and the conserved two glycans visible in both structures. We were unable to resolve the CTT domain due to the absence of density for the CTT in the PFN2 pore map, suggesting that it may have become intrinsically disordered on pore formation. Our structure confirms the rotation between the MACPF and P2 domains by 180° and reveals other conformational changes accompanying membrane insertion including the deployment of TMH1 and TMH2 to generate a 64-stranded β-barrel (Fig. 6, B to D). In the pore form, the MACPF domain has rotated with respect to the P2 domain by ~180° when viewed from the side and by ~90° when viewed from above (looking down toward the membrane surface) compared to its position in the pre-pore, resulting in a compound screw rotation (Fig. 6D). The pore-forming oligomer structure is entirely consistent with the pores imaged directly on liposome membranes and is 206 Å in diameter and 140 Å tall from the top of the MACPF domain to the tip of the β-barrel (Fig. 6, A and B) (1, 2, 5, 21, 22).

DISCUSSION
Our structures of mPFN2 pre-pore and pore forms provide intriguing insights into its mechanism of pore formation. Depending on whether PFN2 is released to the phagosome lumen by proteolysis (12), two models can be proposed for the mechanism underlining pore formation by PFN2 (Fig. 6E). In model 1, monomeric PFN2 is released into the phagosome lumen after its cleavage from the phagosome membrane, binding to a target bacterial surface and oligomerizing upon it into a pre-pore assembly. Upon activation by acidification of the phagosome, the MACPF domain rotates by 180° with respect to the membrane-bound P2 domain, followed by the unfurling of its TMHs (Fig. 6E, left, model 1). If considered as a rigid body movement, the steric clash between MACPF domains will not allow rings of subunits to make this transition intact, and we hypothesize that pore formation begins with the opening of the pre-pore oligomer between two subunits, allowing the conversion to the pore state to begin. In an alternative model (Fig. 6E, right, model 2), PFN2 remains membrane-anchored in its pre-pore form and close approach of the phagosome membrane and bacterium is essential for the binding of the pre-pore to the bacterial surface via a 180° rotation of the P2 domain. Subsequently, the MACPF domain TMHs would be unfurled and inserted into the bacterial surface; in this model, the CTT would act as an interdomain lock by interacting with an adjacent subunit’s P2 domain β hairpin, as in the pre-pore structure. But upon pore formation on a bacterial membrane, the CTT might then become a flexible linker between the phagosome and bacterial membranes. However, in this model, the oligomerization of PFN2 into a pre-pore occurs before membrane binding, whereas in all other MACPF/CDC proteins membrane binding precedes oligomerization. In addition, rotation of the P2 domain with respect to the MACPF, as in model 2, also results in a clash between subunits. Which model outlined is more correct—i.e., whether the P2 domain binds the target membrane before or after pre-pore formation—and the exact conformational pathway taken in each case will require further experimentation and, most likely, a combination of high-resolution real-time AFM imaging with structural characterization of pore formation intermediates.

CONCLUSION
Together, we have used cryo-EM to determine structures for the pre-pore and pore forms of PFN2, x-ray crystallography to determine multiple conformations of its membrane-binding P2 domain, high-speed AFM to image pre-pore and pore formation in real time, functional assays of PFN2 based on fluorescence imaging and spectroscopy and conductance measurements, and site-directed mutagenesis to test hypotheses arising from the structural and dynamic data. The outstanding insight from our study is that the TMH membrane-inserting regions of the pore-forming MACPF domain are oriented...
The other domains out of which PFN2 is built are found in similar forms in many other MACPF/CDC family proteins, such as perforin-1 and components of the complement MAC as well as non–pore-forming family members like astrotactin-2 (23, 28–30), and a related set of domains is found in the bacterial CDCs (31). So why are the domains arranged with the MACPF and P2 domains pointing in opposite directions in PFN2? Given the pH activation of pore formation, we suggest that this pre-pore arrangement holds PFN2 in an inactive state until the correct endosomal pH is achieved to facilitate effective killing of bacteria by membrane perforation. Given the antiquity of the PFN2 gene [see above and (7, 9)], we further suggest that the configuration of all other MACPF/CDC proteins described with pore-forming and membrane-binding regions pointing in the same direction in the pre-pore state is an adaptation of the opposite configuration found in PFN2. This may have been facilitated by the loss of the CTT from ancestral PFN2 on duplication to initiate the evolution of perforin-1, because it is not found in other family members.

**MATERIALS AND METHODS**

**Construct preparation, protein expression, and purification**

The P2 domain of murine PFN2 (mPFN2; UniProt: A1L314) was cloned into the pHLsec vector (32) with Age I and Kpn I restriction sites. The P2 domain of murine PFN2 (mPFN2; UniProt: A1L314) was cloned into the pHLsec vector (32) with Age I and Kpn I restriction sites.
cutting sites, resulting in a signal peptide in the N terminus of the proteins and a KTHHHHHH tag in the C terminus. The signal peptide was cleaved during protein secretion into the medium, leaving an additional three residues Glu-Thr-Gly at the N terminus of the proteins. Full-length mPFN2 ectodomain (residues 20 to 652) was cloned into pHLsec-1D4, replacing the His6 tag with a rhodopsin 1D4 tag (33). Overlapping polymerase chain reaction was performed to introduce site-directed mutagenesis.

All the proteins in this study were produced recombinantly from mammalian human embryonic kidney (HEK) 293T cells or HEK293S cells using a transient transfection and expression protocol as described (17, 30). To express the P2 domain in HEK293T cells for crystallization, 6 mg of purified DNA vector was transfected into 3 liters of HEK293T cells in the presence of the glycosylation inhibitor kifunensine (final concentration, 5 μM) with 12 mg of polyethyleneimine. The transfected cells were harvested 4 to 5 days after transfection. Three liters of medium containing secreted proteins was harvested by centrifugation at 5000g for 45 min to remove the cell debris, filtered through a 0.22-μm filtration membrane, and dialyzed against 100× volume of phosphate-buffered saline [10 mM phosphate (pH 7.5) and 300 mM NaCl] overnight at 4°C. The dialyzed medium was supplemented with 20 mM imidazole and loaded onto a HisTrap HP (GE Healthcare) column overnight at room temperature and eluted with a linear imidazole gradient (20 to 500 mM imidazole) in 25 mM tris (pH 7.5) and 500 mM NaCl. The eluted proteins were pooled together and deglycosylated with Endo F1 overnight at 4°C, concentrated, and applied to a size-exclusion chromatography column (Superdex 75 16/600), and a peak corresponding to the homogeneous oligomers was pooled together and concentrated to ~0.5 mg/ml. Tween 20 was added to the protein solution at a final concentration of 0.01% and incubated for 45 min to remove the cell debris, filtered through a 0.22-μm filtration membrane, and dialyzed against 100× volume of phosphate-buffered saline [10 mM phosphate (pH 7.5) and 300 mM NaCl] overnight at 4°C. The dialyzed medium was supplemented with 20 mM imidazole and loaded onto a HisTrap HP (GE Healthcare) column overnight at room temperature and eluted with a linear imidazole gradient (20 to 500 mM imidazole) in 25 mM tris (pH 7.5) and 500 mM NaCl. The eluted proteins were pooled together and deglycosylated with Endo F1 overnight at 4°C, concentrated, and applied to a size-exclusion chromatography column (Superdex 75 16/600), and a peak corresponding to the homogeneous oligomers was concentrated to about 12 mg/ml in 10 mM Hepes (pH 7.5) and 150 mM NaCl. The proteins were flash-frozen in liquid nitrogen and stored in −80°C until further use.

Ectodomain of mPFN2 was purified from the medium using anti-1D4 antibody–coupled agarose resin. Six liters of mammalian expression medium with secreted mPFN2 was concentrated to 500 ml using a QuikStand device (Watson, Marlow) with a 10-kDa cutoff membrane, filtered, and incubated with anti-1D4 monoclonal antibody–coated resin preequilibrated in 25 mM tris (pH 7.5) and 500 mM NaCl buffer overnight at 4°C. The agaroase resin with mPFN2 bound was then washed with 25 mM tris (pH 7.5) and 500 mM NaCl buffer before being eluted with 2 ml of 1D4 peptide at a concentration of 0.5 mg/ml. The sample was concentrated using a 30-kDa centrifugal concentrator (Millipore) to about 1.2 mg/ml and flash-frozen in liquid nitrogen for storage at −80°C. The mutants of the ectodomain were purified in the same way.

Cryo-EM sample preparation and data collection

The mPFN2 ectodomain was incubated with buffer containing 50 mM citric acid buffer (pH 5.5) and 150 mM NaCl at 0.5 mg/ml at 37°C overnight to form pre-pore oligomers, which were further purified through size-exclusion chromatography (Superose 6 10/300). Fractions corresponding to the homogeneous oligomers were pooled together and concentrated to ~0.5 mg/ml. Tween 20 was added to the protein solution at a final concentration of 0.01% and incubated for 30 min before cryo-EM grids were prepared. The mPFN2 pore sample was prepared by lowering the pH of the pre-pore samples to pH 3.6 to 4.0 in the presence of 0.056% Cymal-6 (final concentration) at room temperature for 30 min. The pH was estimated by large-scale mixing of the pre-pore assembly buffer [50 mM citric acid (pH 5.5) and 150 mM NaCl] with 1 to 1.5× volume of 170 mM citric acid (pH 3.3) and 150 mM NaCl.

For cryo-EM, 3 μl of mPFN2 pre-pore (~0.3 mg/ml) was applied to a glow-discharged lacy carbon grid with ultrathin carbon support film (Agar, UK), blotted for 3.5 s in 100% humidity at 22°C, and flash-frozen in liquid ethane with an FEI Vitrobot Mark IV. The mPFN2 pore (~0.12 mg/ml) was frozen in the same way. The mPFN2 pre-pore grids were imaged using an FEI Polara electron microscope operating at 300 kV with semi-automated image acquisition via SerialEM. Micrographs were acquired with a Gatan K2 Summit direct electron detection camera operated in the counting mode at a calibrated magnification of ×48,076 and defocus values ranging from −1.0 to −2.5 μm. The mPFN2 pore data were collected using a Thermo Scientific Glacios operating at 200 kV with a Falcon III camera in linear mode. The details of data collection parameters are summarized in table S1.

Cryo-EM image processing

For cryo-EM data of mPFN2 pre-pore, beam-induced motion correction was performed using MotionCor2 (34) to generate dose-weighted micrographs from all frames. The contrast transfer function was estimated using Gcft (35). XMIPP (36) was used for automatic particle picking, and the particles were further manually inspected. In total, 246,755 particles were extracted and initially binned 2x. After several rounds of reference-free 2D classification in RELION (37), good class averages representing the C16 symmetry were selected and further processed. About 5000 particles were used to generate an ab initio model in cryoSPARC (38), and the resulting initial model was used for 3D classification in RELION with C16 symmetry imposed. Particles from two classes (41,693 particles) with similar density maps were selected and reextracted at full size for further 3D refinement, resulting in a map with overall resolution of ~3.5 Å. The overall B factor was automatically determined using the RELION PostProcess tool, with an estimated B-factor applied of −144 Å².

The global resolution was estimated by the gold-standard Fourier shell correlation (FSC) = 0.143 criterion, with a soft mask applied around the protein density. The local resolution was estimated using RELION and presented in Chimera (39). The dataset of mPFN2 pores was processed with similar procedures. In brief, automated particle picking was performed using Gautomatch (www.mrc-lmb.cam. ac.uk/kzhang/Gautomatch). A total of 44,046 particles contained in good 2D classes of all side and top views of C16 symmetry pores were selected after three rounds of 2D classification in RELION. An ab initio model generated with these particles in cryoSPARC was used for 3D classification in RELION without applying symmetry. The best class (24,936 particles) was further refined with C16 symmetry in RELION, resulting in a map with local resolution ranging from 4.1 to 9.2 Å. All subsequent post-processing steps were performed in the same way as described above for the pre-pore dataset.

Model building into the cryo-EM reconstruction, refinement, and structure visualization

Phyre2 (40) was used to generate a homology model of the MACPF domain of mPFN2, using the perforin-1 crystal structure as the template [Protein Data Bank (PDB) entry code: 3NSJ]. The MACPF homology model was first fitted into the density map and manually rebuilt. The P2 domain crystal structure was manually fitted into the map, with major adjustment in the extended β hairpin. The remaining
The derivative dataset was collected at a wavelength of 1.04 Å (see the crystals were soaked with concentrated gold cyanide for 1 hour. At Diamond Light Source (Didcot, UK). For experimental phasing, the alignment of residue Asn269 to its attached glycan density, followed by construction of two TMH1 and TMH2 using Coot (41). A further round of iMODFIT (43) to then refine the MACPF-EGF domain structure into the density, whereby normal modes analysis is used to facilitate flexible fitting. This led to a straightening of the MACPF domain central β sheet in line with the deployment of the TMH1 and TMH2 regions. We then fitted the P2 domain, making use of all 11 structures to hand (10 crystals and 1 cryo-EM from the pre-pore). We found that the best fit was for chain B of the P1 crystal form and so used that in rigid body fitting into its density within the pore map. This process placed the C terminus of the EGF and the N terminus of the P2 domain close enough in space that the connectivity of the model could be restored manually in Coot (41). A further round of iMODFIT refinement was followed by the manual construction of two β hairpins for TMH1 and TMH2 using Coot (41) constrained by the alignment of residue Asn269 to it attached glycans density, followed by construction of a full 16-mer pore and refinement using phenix. real_space_refine (42) in isolation and incorporated in the Namdinator online server (44, 45), until convergence was achieved. Namdinator runs made use of implicit solvent in the latter cycles of refinement.

Structural phylogeny construction
Superimposition of homologous protein structures was performed using SHP (46) as previously reported (47). The phylogenetic tree was calculated using a pairwise evolutionary distance matrix determined from the superimposed domains. The tree representation was generated using the programs FITCH and DRAWTREE as part of the PHYLIP package (48).

Protein crystallization, x-ray diffraction data collection, and structure determination
Crystallization screening was carried out by sitting-drop vapor-diffusion methods in CrystalQuick 96-well plates by mixing 100 nl of protein solution with 100 nl of reservoir and equilibrating against 95 µl of reservoir at 20°C. Deoxyxylated P2 domain was concentrated to 10 to 15 mg/ml and crystallized in 0.1 M MES (pH 6.0) and 10% PEG-6000 (polyethylene glycol, molecular weight 6000) with 50 nl of additives (0.02 M l-cysteine stock). The crystals appeared after 2 weeks and grew to full size within 1 month. The crystals were harvested with 25% glycerol as the cryoprotectant in liquid nitrogen. The crystal diffraction data were collected on I24 and I03 beamlines at Diamond Light Source (Didcot, UK). For experimental phasing, the crystals were soaked with concentrated gold cyanide for 1 hour. The derivative dataset was collected at a wavelength of 1.04 Å (see table S2 for details of data collection).

A gold-derivative single anomalous dispersion (Au-SAD) analysis was first performed in HKL2map (49) to identify the heavy atom positions. The resulting heavy-atom coordinates were subsequently used together with a native dataset to carry out single isomorphous replacement anomalous scattering analysis in phenix.autosol (50). The resulting electron density was used in phenix.autobuild (51) to generate a partial model. The subsequent model building and refinement were carried out in Coot (41) and phenix.refine (42). The final refined model contains residues from Phe376 to Ile579, with one missing loop between Val538 and Pro549. The other crystal form was phased by molecular replacement using the refined P2 domain model with β-hairpin truncation. The electron density representing the β hairpin was clearly visible after rigid-body refinement. The subsequent model building and refinement were carried out in Coot and phenix.refine (41, 42).

Negative-stain electron microscopy
Wild-type PFN2 ectodomain and the disulfide-linked mutants were incubated with liposomes at room temperature for 30 min. A 3-µl mixture was applied to a glow-discharged copper grid covered with carbon film (Electron Microscopy Sciences), washed twice with dH2O, stained with 0.75% uranyl formate or 2% uranyl acetate for 1 min, and blotted dry with filter paper. Negative-stained images were collected using FEI Tecnai T12 microscopes at a nominal magnification of ×67,000, with a calibrated pixel size of 1.7 Å. The particles were manually picked and extracted in EMAN2 (52), and the subsequent image analysis was carried out with Imagic (53).

Liposome sedimentation assay and sulforhodamine B liposome leakage assay
POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), POPS (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine), POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine), cardiopin, sphingomyelin (brain, Porcine), and E. coli total lipid extract were purchased from Avanti Polar Lipids Inc. LPSs from S. enterica (L1887) and E. coli (L2018) were purchased from Sigma. Liposomes with the compositions indicated were prepared following standard protocols. Briefly, a total of 1 mg of lipids dissolved in chloroform was mixed and dried in a clean Pyrex tube overnight by desiccation in a desiccator attached to a VARIO-SP diaphragm pump (Vacuubrand). The lipid film was subsequently hydrated by adding 0.5 ml of solubilization buffer [20 mM Hepes (pH 7.5) and 150 mM NaCl] followed by vigorous vortexing and 5 to 10 freeze-thaw cycles, and the resulting mixture was then extruded through 100-nm polycarbonate membrane (Whatman) 11 times using an extruder (Avanti Polar Lipids Inc.). To prepare liposomes with LPSs incorporated, LPS dissolved in buffer [20 mM Hepes (pH 7.5) and 150 mM NaCl] was added into the solubilization buffer before freeze-thaw cycles and extrusion. The liposomes were stored at 4°C and used within 2 days. To perform liposome sedimentation assays, 5 µg of mPFN2 protein (P2 domain; P2hairpin protein; ectodomain or ectodomainACTT) was incubated with 50 µl of liposome (2 mg/ml) for 1 hour at 37°C. The proteins incubated with buffer only [20 mM Hepes (pH 7.5) and 150 mM NaCl] served as negative controls. The protein buffer/liposome mixtures were then centrifuged at 67,000 rpm in an ultracentrifuge (Optima TL with TLA100.4 rotor) for 20 min at 10°C. The supernatant was collected to examine proteins unbound to the liposome. The pellets were resuspended in solubilization buffer with the same volume as the supernatant. Equal amounts of supernatant...
and pellicles were loaded for SDS–polyacrylamide gel electrophoresis analysis. Each liposome sedimentation assay was performed at least three times.

Sulforhodamine B liposome leakage assays were conducted as described (54). Briefly, the dried lipid mixtures as described above were rehydrated with 0.5 ml of different solubilization buffers [20 mM Hepes (pH 7.5)/50 mM citric (pH 5.5)/50 mM citric (pH 4.0)/50 mM citric (pH 3.0)] supplemented with 150 mM NaCl and 50 mM sulforhodamine B. After extrusion, the sulforhodamine B dye outside the liposome was removed by buffer exchange using a disposable PD 10 desalting column (GE Healthcare). The buffer exchange was performed twice to remove the residual sulforhodamine B outside the liposome. To perform the liposome leakage assay, the dye-encapsulated liposome was first diluted 50 times. The fluorescence of sulforhodamine B was measured with excitation wavelength of 585 nm and emission wavelength of 610 nm. The emission fluorescence before and after extrusion was continuously measured as F(tn) every 30 s for 60 min. The percentage of liposome leakage is defined as follows: Leakage (%) = F(tn) – F(t0) / F(t0) × 100. Each leakage assay was performed at least three times.

**Preparation and imaging of GUVs**

GUVs were prepared by the electroformation method (55), as described (56). Lipid mixtures [POPC:POPS:E. coli total lipid extract (Avanti Polar Lipids) = 80:10:10 (w/w)] were dissolved in chloroform to a final concentration of 5 mg/μl. Rhodamine-DHPE (Lissamine rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt) was added to fluoroescence label the membranes. Six microliters of the lipid mixture was spread on the conductive side of an indium tin oxide (ITO) slide and dried under vacuum. Electroformation was carried out in sucrose solution [300 mM sucrose and 1 mM MES (pH 5.7)] between two conductive ITO slides (Vesicle Prep Pro, Nanion Technologies, Germany) under the following conditions: initial increase of amplitude from 0 to 3 V with 5-Hz frequency for 30 min, followed by 3 hours of constant amplitude of 3 V and frequency of 5 Hz, and ending with decreasing frequency from 5 to 0 Hz at a constant amplitude of 3 V at 37°C. GUVs were sedimented by first adding glucose solution [300 mM glucose and 1 mM MES (pH 5.7)], next buffer on top [10 mM MES and 150 mM NaCl (pH 5.7)], and GUVs were left to sediment overnight. All solutions used for GUV preparation and experiments were isoosmolar (Osmotat 3000, Goenotec GmbH, Germany).

GUVs were mixed with FDs of 4, 10, or 70 kDa (final concentration of 1 mg/ml) and PFN2 (final concentration of 0.1 mg/ml) and incubated for 60 or 120 min at room temperature. The buffer was used as a negative control instead of proteins. Images were recorded on a DM16000 CS inverted microscope with TCS SP5 laser scanning system (both Leica Microsystems, Germany) with a 63× oil immersion objective (numerical aperture, 1.25). FDs and rhodamine were excited with 488- and 543-nm lasers, and fluorescence emission was detected from 495 to 530 nm and from 580 to 630 nm, respectively. Permeabilization of GUVs was quantified in ImageJ software. For each experiment, between 50 and 300 vesicles were quantified in three independent experiments.

**Planar lipid bilayer experiments**

Planar lipid bilayers were prepared from a mixture POPC:POPS:E. coli total lipid extract = 70:10:20 (w/w) with a final concentration of lipids (5 mg/ml) dissolved in octane. For recording of electrical measurements, multielectrode cavity array chips (Ionera Technologies) were used on the integrated chip-based recording setup Orbit mini with EDR2 software (Nanion Technologies). Proteins were added to the cis side of the bilayer to a final concentration of ~32 ng/μl. MES (10 mM) and NaCl (500 mM) at pH 5.5 was used as a buffer. To promote pore insertion, a voltage from ±50 mV to 200 mV with a 20-kHz sampling rate was applied. For the analysis of current, the tracing software Clampfit (10.7.0.3) was used. The noise was quantified as described previously (4). In brief, the SD was determined on a short time interval (a few tens of milliseconds) of the individual current trace at which the pore was open, and noise was normalized by dividing SD with the average current of each trace at which the pore was open.

**Formation of supported lipid bilayer for HS-AFM**

Vesicles were prepared using 100% E. coli total phospholipid extract composed of PE:PG:CA:unknown = 57.5:15.1:9.8:17.6 (w/w; Avanti, USA). Lipids dissolved in chloroform were dried under argon flux, followed by 2-hour incubation in a vacuum desiccator. After that, lipids were fully rehydrated with buffer [10 mM Hepes-NaOH (pH 7.5) and 200 mM NaCl] for 5 min at room temperature, obtaining lipid solution (0.5 mg/ml). Last, the lipid suspension was bath-sonicated for 10 min to form vesicles. For mica-supported lipid bilayers, 3 μl of E. coli lipid solution (0.5 mg/ml) was deposited onto freshly cleaved mica (1.5-mm-diameter disc), incubated for 15 min, and rinsed thoroughly with buffer [10 mM Hepes-NaOH (pH 7.5), 200 mM NaCl, and 2 mM CaCl2].

**High-speed AFM**

The PFN2 assemblies on mica or on lipid bilayers were prepared by incubating PFN2 monomers (0.043 mg/ml) for 5 min, either directly on mica or on preformed supported lipid bilayers. Then, the samples were rinsed and directly imaged by HS-AFM. All images in this study were acquired using HS-AFM (SS-NEX, RIBM, Japan) operated in amplitude modulation mode using optimized scan and feedback parameters. Short (8 μm) cantilevers (NanoWorld, Switzerland) with nominal spring constant of 0.15 N/m, resonance frequency of ~0.6 MHz, and a quality factor of ~1.5 in buffer [20 mM Hepes-NaOH (pH 7.5), 200 mM NaCl, and 2 mM CaCl2] were used. To study the reversible lateral displacement of pre-pores on the membrane, the ratio of set point and free amplitude (Aset/Afree) was changed from ~0.95 to ~0.6 and back to ~0.95. Under such conditions, the average estimated applied force on the HS-AFM tip changes from ~30 to ~200 pN and back to ~30 pN (57). To study the pre-pore–to–pore transition, the fluid cell containing ~120 μl of buffer [20 mM Hepes-NaOH (pH 7.5), 200 mM NaCl, and 2 mM CaCl2] was supplemented with ~15 μl of 0.1 M HCl (through passive diffusion) to decrease the pH of the buffer solution to pH 3.6 to 4.0. The change in pH was estimated by large-scale mixing 150 μl of 0.1 M HCl into 1.20 ml of the imaging buffer.
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