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The holin of bacteriophage lambda forms rings with large diameter

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Summary

Holins control the length of the infection cycle of tailed phages (the Caudovirales) by oligomerizing to form lethal holes in the cytoplasmic membrane at a time dictated by their primary structure. Nothing is currently known about the physical basis of their oligomerization or the structure of the oligomers formed by any known holin. Here we use electron microscopy and single-particle analysis to characterize structures formed by the bacteriophage λ holin (S105) *in vitro*. In non-ionic or mild zwitterionic detergents, purified S105, but not the lysis-defective variant S105_{A52V}, forms rings of at least two size classes, the most common having inner and outer diameters of 8.5 and 23 nm respectively, and containing approximately 72 S105 monomers. The height of these rings, 4 nm, closely matches the thickness of the lipid bilayer. The central channel is of unprecedented size for channels formed by integral membrane proteins, consistent with the non-specific nature of holin-mediated membrane permeabilization. S105 present in detergent-solubilized rings and in inverted membrane vesicles showed similar sensitivities to proteolysis and cysteine-specific modification, suggesting that the rings are representative of the lethal holes formed by S105 to terminate the infection cycle and initiate lysis.

Introduction

The transcriptional programmes that stage the infection cycle of tailed phages (the Caudovirales) have been characterized in great detail and are among the best-understood regulatory phenomena in biology (Friedman and Court, 2006; Little, 2006). However, it is

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Supplementary material

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not widely appreciated that the duration of the infection cycle is determined at a post-translational level by a class of small membrane proteins, the holins. Throughout the late gene expression period, holins accumulate in the membrane without a detectable effect on host physiology. Suddenly, at an allele-specific time, the holins trigger to form non-specific lesions or holes in the membrane, sufficiently large to allow the release of ~500 kDa protein complexes (Wang *et al.*, 2003). This allows a phage-encoded endolysin to attack the cell wall, and lysis, with concomitant liberation of the viral progeny, which ensues within seconds. Nothing is known about either the structure of these unique membrane lesions or about the timing mechanism intrinsic to the primary structure of each holin. Even conservative missense changes in a holin can result in dramatic shortening or lengthening of the infection cycle (Raab *et al.*, 1988; Johnson-Boaz *et al.*, 1994; Gründling *et al.*, 2000a). It is thought that the ubiquity of holin-mediated lysis systems results from the ability of phages to rapidly evolve to shorter or longer infection cycles, to adjust to changes in host quality or density (Wang *et al.*, 1996; Wang, 2006; Zheng *et al.*, 2008).

Holins constitute a very diverse functional group, with more than 50 unrelated gene families and at least three different membrane topologies. The best studied holin is that of phage λ (Wang *et al.*, 2000) whose holin and endolysin genes, *S* and *R*, respectively, are transcribed from the pR' late promoter, beginning about 8 min after infection (Zagotta and Wilson, 1990; Chang *et al.*, 1995). *R* is a small 18 kDa muralytic transglycosylase (Bienkowska-Szewczyk *et al.*, 1981). The *S* gene encodes two proteins, S105 and S107 (Wang *et al.*, 2000) (Fig. 1). Although S105 and S107 only differ by the presence of a MetLys-dipeptide extension at the N terminus of the latter, the two proteins have opposite functions (Bläsi *et al.*, 1990). The shorter product, S105, is the holin for phage λ while the longer product, S107, acts as a holin antagonist. The S105 protein has been shown to have three transmembrane domains (TMDs) (Gründling *et al.*, 2000b), adopting an N-out, C-in topology with respect to the cytoplasmic membrane (Graschopf and Bläsi, 1999).

The nature of the hole formed by S105 remains uncertain. Lesions formed by S105 have been shown to be large enough to allow the passage of a fully folded, tetrameric R- β -gal fusion protein to the periplasm (Wang *et al.*, 2003). Such a hole would have to be larger than 12 nm in diameter and constitute the largest known membrane pore, by far, formed by an α -helical inner membrane protein. Earlier studies had also shown that membrane-associated S105 could be cross-linked into dimers, trimers and tetramers using a bifunctional reagent, dithiobis-succinimidylpropionate (DSP) (Gründling *et al.*, 2000a). Importantly, many non-functional, missense mutants of S105, e.g. S105_{A52V}, could only be cross-linked into dimers; the higher oligomers were not formed. This suggests that the ability of S105 to oligomerize in the membrane is central to its function. To account for the observation that missense changes along any face of all three TMDs of S105 can have dramatic, but unpredictable, effects on lysis timing, Wang *et al.* (2003) proposed that holins form large, two-dimensional aggregates in the membrane, and that at the time of triggering, the packed holin TMDs re-organize to form large protein-bounded lesions in the membrane. In the present study, we characterize multimeric complexes formed by purified S105 in detergent solution. Single-particle analysis of these complexes provides the first three-dimensional structure for a holin assembly that is consistent with its function.

Results

Oligomeric state of purified S105

Previous studies have indicated that oligomerization of S105 is necessary for it to disrupt the membrane (Gründling *et al.*, 2000a). To determine whether the presumptive S105 oligomers were preserved by detergent extraction, the S105 protein purified from strains overexpressing an allele of *S* encoding an internal hexahistidine tag was analysed by chromatography on Superose 6 (fractionation range, 5–5000 kDa). When S105 was extracted and purified using the zwitterionic detergent Empigen BB (EBB), the majority of the protein eluted in a single peak (Fig. 2) near the included volume for the column. When this sample was chromatographed on S75 resin (fractionation range, 3–100 kDa), the protein eluted as a single, symmetrical peak midway between v_0 and v_i for the column (not shown). As SDS-PAGE under non-reducing conditions indicated that a significant fraction of the S105 protein in these samples existed as disulphide-linked dimers (not shown), peaks eluting from the Superose 6 and S75 columns correspond to EBB-solubilized S105 dimers rather than monomers. Apparently, EBB disrupts the higher-order oligomers that S105 is known to form in the membrane (Gründling *et al.*, 2000a).

We then replaced EBB with the non-ionic detergent n-dodecyl- β -D-maltopyranoside (DDM) for the extraction and purification of S105, which was obtained in nearly identical quantity and purity as with EBB. When S105 purified in DDM was subjected to gel-permeation chromatography, three peaks were obtained (Fig. 2). The first peak corresponded to a > 5 MDa species that eluted in the void volume of the column. The second peak contained > 670 kDa S105 complexes and the last peak probably corresponded to the dimers observed in the EBB extracts. Strikingly, when the lysis-defective mutant, S105_{A52V}, was purified in DDM and analysed similarly, all of the protein eluted as the lower molecular mass species (Fig. 2). Finally, chemical cross-linking experiments confirmed that S105 formed higher oligomers in DDM but not in EBB. Protein purified in either detergent was treated with DSP and analysed by SDS-PAGE under non-reducing conditions. Only S105 in DDM was cross-linked into species larger than the dimer (Fig. 2, inset).

The finding that S105 adopted markedly different quaternary structures in EBB as compared with DDM prompted further investigation into the gross structure of S105 in the two detergents. S105 in either EBB or DDM gave almost identical overlapping circular dichroism spectra showing an α -helical content of 48% or approximately 55 residues (Fig. S1). The lysis-defective protein, S105_{A52V}, purified in EBB behaved similarly. This amount of α -helix is sufficient to account for the three TMDs predicted by the susceptibility of S105 in membranes to chemical modification (Gründling *et al.*, 2000b). A construct lacking the first TMD (S105_{TM1}) was found to have 38% α -helical content, corresponding to a 19 residue reduction, consistent with the loss of TMD1.

S105 oligomers in DDM exist as rings

Images of negatively stained samples revealed that DDM-solubilized S105 formed large, well-dispersed ring-shaped structures (Fig. 3A and B). These rings were able to stack onto each other to form larger assemblies. Fractions from the void volume of the Superose 6

column consisted mostly of large stacks (Fig. 3A) while the second peak contained shorter stacks with fewer than six rings (Fig. 3B). The majority of shorter stacks were composed of only two rings (ring dimers). Only a small number of single rings were ever observed. When the protein eluting in the second peak was re-chromatographed, the majority of it now eluted as dimeric S105 (not shown). Moreover, rings were observed at low frequency when the dimer fraction was examined by electron microscopy (EM) (not shown). Thus, the rings present in the DDM-solubilized samples are dynamic structures that are being continuously assembled and disassembled. Several other commonly used detergents also supported ring formation by purified S105. Electron microscopic analysis of S105 purified in n-octyl- β -D-glucopyranoside (OG) also revealed rings and stacks. However, these samples were not as homogeneous as the DDM-purified protein and showed evidence of small aggregates and precipitation (not shown). The formation of rings was not restricted to non-ionic detergents. S105 purified in the mild, zwitterionic detergent lauryldimethylamine-oxide (LDAO) also formed rings and stacks, although the length of the stacks was somewhat greater than what was observed with DDM (Fig. 3C).

Architecture of the S105 ring

To further define the structure of the S105 rings, negatively stained preparations were subjected to single-particle analysis. Reference-free classification of end-on projections yielded class averages with no noticeable subunit features. Visual inspection of the classes and eigenimages indicated a slight heterogeneity in the diameter of the rings (not shown). This heterogeneity is not surprising as it is likely that the number of subunits that make up a ring may vary. Such variability in subunit number has previously been reported for the cholesterol-dependent cytolysins that also form multimeric rings of large diameter (Tilley *et al.*, 2005). The data sets were therefore analysed using an approach similar to the one performed on heterogeneous pneumolysin rings (White *et al.*, 2004; Tilley *et al.*, 2005). Reference-free classification was carried out using only the first five eigenvectors in order to allow the data to be separated on the basis of low-resolution features, such as particle diameter. Inspection of 15 class averages indicated two clusters based on the outer diameter: 24.5 nm (8 classes, 65% of particles) and 26–28 nm (7 classes, 35% of particles). When C16, C18 and C20 symmetries were imposed on class averages from the 24.5 nm cluster, a reinforcement of structural features was only obtained for C18 (Fig. 3D). The same procedure was followed for classification of a subset of the larger particles (27 nm outer diameter). These classes were found to comply with a C20 rotational symmetry (Fig. 3E). From these observations, it can be concluded that S105 forms rings with some variability in subunit number, ranging from 18 to 20 protomers. Given the dimensions of the ring and the molecular mass of S105 (12.3 kDa), each protomer must be an oligomer of S105.

Examination of the S105 assemblies by cryo-EM (Fig. 4A) and imposition of C18 symmetry allowed us to calculate a 3D structure of a ring dimer at 2.6 nm resolution (Fig. 4B). The reconstruction indicates that each ring has an outer diameter of 23 nm and an inner diameter of 9 nm for the upper ring and 8 nm for the lower ring. The difference in the inner diameters of the lower and upper rings may arise from conformational changes required for the interaction or possibly from a difference in the number of protomers in each ring. A cross-section through the ring dimer reveals that each ring has a flat lower surface and a tapered

upper surface. This gives rise to a slight radial asymmetry, with each ring measuring 4 nm in height towards the periphery and tapering towards the central hole. The cross-section does not have a twofold symmetry axis, indicating a head-to-tail arrangement of the upper versus the lower ring rather than head-to-head arrangement.

Accessibility of S105 in detergent-solubilized rings and membrane-associated oligomers to proteolysis and chemical modification

To determine whether the structure of the S105 rings was related to S105 oligomeric complexes formed in membranes, we compared their sensitivities to proteolysis and chemical modification. For the proteolysis experiments, inverted inner membrane vesicles (IMVs) produced by the passage of cells expressing S105 from a plasmid were used as the source of the membrane-associated S105. In these preparations, it is expected that S105 had been triggered to form lesions, allowing the movement of macromolecules across the IMV membrane. IMVs and DDM-purified S105 were treated with either proteinase K or trypsin and the digestion products were analysed by SDS-PAGE and immunoblotting using antibodies against the N and C termini of S105. The C terminus of S105 was sensitive to trypsin in both IMVs and in DDM rings (Fig. 5A), giving rise to a ~8.5 kDa product detected by antibodies raised against an N-terminal peptide. The size of this product is consistent with cleavage at either Lys92 or Arg93 in the cytoplasmically exposed C-terminal segment of S105. Both the N- and C-termini of membrane-associated S105 were sensitive to proteinase K, which is not unexpected as the IMVs should contain S105 lesions allowing the protease to enter the lumen of the vesicles. In DDM rings, only the C terminus was digested by proteinase K. The protection of the N terminus in DDM might be due to stack formation that could shield the N terminus from the protease. This possibility is corroborated by the fact that S105_{A52V} in DDM, which does not form rings, is susceptible to cleavage at the N terminus (not shown). If this interpretation is correct, the region between TMD3 and the highly charged C terminus of S105 is relatively exposed in the stacked rings present in DDM, as it is sensitive to both trypsin and proteinase K.

In order to determine if the C-terminal region of S105 is required for ring formation, S105 digestion products from both proteases were negatively stained and imaged by EM. EM of these products indicated that rings and stacks of rings were still formed by S105 despite the lack of the estimated 24 C-terminal residues (not shown). These results indicate that the C-terminal domain of S105 is not required for ring formation, consistent with previous *in vivo* studies that showed that S105 truncated at the C terminus does not abolish hole formation (Bläsi *et al.*, 1999).

Cysteine-modification studies were also used to assess the exposure of the N terminus, TMD2, and the C terminus of S105 rings to the aqueous environment. As a result of the propensity of the cysteines in S105 to become oxidized forming disulphide-linked dimers, the three forms of S105 used in these experiments were extracted in buffer containing 2,2'-dithiodipyridine. After purification, the thiopyridyl-protecting groups were removed by treatment with tris(2-carboxyethyl)phosphine. Rings composed of wild-type S105 (which has a Cys at position 51 in TMD2), S105_{H7C,C51S} and S105_{C51S,A99C} were subjected to modification by PEG-maleimide in the presence and absence of SDS. As can be seen in Fig.

5B, the amino-terminus of S105_{H7C,C51S} is easily modified by PEG-maleimide. By contrast, Cys51 is less accessible in the rings and undergoes a significant degree of modification only in SDS. These results are consistent with previous chemical modification studies showing that in IMVs, a Cys at position 7 was readily modified by 4-acetamido 4' [(iodoacetyl)amino]stilbene-2,2'-disulfonic acid (IASD), while Cys51 was unreactive (Gründling *et al.*, 2000b). Finally, a cysteine at position 99 (S105_{C51S,A99C}) in the C-terminal region reacts with the maleimide reagent in either condition. In our previous study with IMVs, cysteines at the C terminus remained unreactive unless boiled in SDS. This unreactivity to IASD was thought to derive from interactions with negatively charged phospholipids in the inner leaflet of the bilayer which are absent from the purified protein.

Discussion

Holins control the duration of the infection cycle of most tailed phages and can be regarded as perhaps the simplest and most fundamental of biological timing systems. A key characteristic of holin function is that the protein is able to accumulate in the membrane without compromising its electrochemical integrity (Gründling *et al.*, 2001), thus preserving the maximum biosynthetic capacity of the host until the instant of the programmed lysis event. Then, at the time of triggering programmed into the primary structure, holins form very large, non-specific holes. Despite the ubiquity and diversity of holins in double-stranded DNA phages, including well-defined holin-dependent lysis systems in paradigm phages like λ and T4, little has been known about the oligomeric structure of holins before or after triggering. In the current study, we have examined the ability of the purified λ holin, S105, to exist as defined oligomeric structures in detergent solution.

Several zwitterionic and non-ionic detergents permitted the formation of S105 oligomers. EM examination of these oligomers revealed that they were ring-shaped structures that could further polymerize into head-to-tail filaments. Single-particle analysis of end-on projections revealed a slight heterogeneity in ring diameter with rings consisting of 18–20 protomers. A cryo-EM 3D reconstruction of a ring dimer from 60% of the particle population (C18 symmetry) allowed further structural details of the rings to be evaluated. The rendering threshold for the 3D reconstruction was chosen based on the good correlation of the original class averages with the re-projections (Fig. 4A, inset) and the 18-fold symmetry estimated from the negative stain data for the particles in this size group. These criteria led to a model in which each protomer consists of four S105 monomers giving rise to an 860 kDa complex with 72 S105 monomers per ring. This number is consistent with calculations assuming a diameter of 10 Å for an α -helix and an average shortest Ca–Ca inter-helix distance of 5.5 Å (as calculated from 16 known membrane protein crystal structures) (Gimpelev *et al.*, 2004). The calculated cross-sectional surface area of the ring can accommodate approximately 62 molecules of S105 assuming that all helices are perpendicular to the membrane plane.

The form of S105 that is likely to be found in the inner membrane is that of a single ring (Fig. 4C). The stacking of rings observed *in vitro* is most likely due to the charge distribution of S105 (net negative charge on the periplasmic side versus a net positive charge on the cytoplasmic side) (Fig. 1). This polarity enables the stacking of rings with closely

matched diameters into filament-like structures and is also corroborated by the head-to-tail arrangement seen in the 3D reconstruction.

A comparison of the negative stain and cryo-EM data revealed a difference in the inner and outer ring diameters: the outer diameter decreased from 24.5 (negative stain) to 23 nm (cryo) and the inner diameter decreased from 11 (negative stain) to 9 nm (cryo). This type of size discrepancy has been previously reported for detergent-solubilized membrane protein complexes and is thought to be due to the stain exclusion by detergent surrounding the protein, which overemphasizes the size of the complex in projection (Grigorieff, 1998; Nield *et al.*, 2003). The increase in the inner diameter can be explained by an accumulation of stain in the gap between the lower and upper rings. As the ring–ring interface and the surface of the hole are thought to be hydrophilic, stain may also bind to these parts of the structure, thus further exaggerating the size of the hole.

Although the rings observed in detergent have not been directly observed in native or artificial membranes, several points of structural, biochemical and genetic evidence argue that the assemblies observed *in vitro* are representative of a state that is present in the bilayer at a specific time during holin function. Foremost, it was found that the non-lytic variant S105_{A52V}, which is blocked as a dimer *in vivo* (Gründling *et al.*, 2000a), never oligomerized in any of the detergent conditions tested. This finding supports the hypothesis that the intermolecular contacts formed by S105 in detergent, but not by S105_{A52V}, are related to, if not identical with, those formed in the bacterial inner membrane. The thickness constraints imposed by the lipid bilayer determine the length and tilt angle of membrane-spanning helices (Dumas *et al.*, 1999; Venturoli *et al.*, 2005). The 4 nm height of each ring agrees well with the estimated thickness of the lipid bilayer (Dubochet *et al.*, 1983; Lewis and Engelman, 1983). Thus the rings comply with structural constraints that would be present in the membrane.

The stage of lysis at which such rings would participate is unclear. The average inner diameter of the rings (8.5 nm) is large enough to allow the λ endolysin, R, which has the dimensions 40 Å × 32 Å × 32 Å (Evrard *et al.*, 1998), to transverse the inner membrane. However, this is not consistent with the ability of S105 holes to allow release of the R– β -gal fusion protein (Wang *et al.*, 2003), which would measure at least 12 nm in the smallest dimension. These results may be reconciled by the finding of different size classes of S105 rings in DDM. The most populated size classes may represent the lowest free energy form of DDM-solubilized S105. In the context of the bilayer, conditions would allow a higher fraction of S105 to be incorporated into rings of sufficient size to allow the passage of R– β -gal to the periplasm. Finally, it has been previously estimated that during phage infection, approximately 1000–3000 S molecules accumulate in the membrane (Chang *et al.*, 1995). This would correspond to 15–45 rings if all the S105 molecules are recruited into such assemblies. The coalescence of several of these rings into a single lesion could account for the ability of larger molecules, such as the R– β -gal fusion to escape.

Clearly, the next step in understanding holin structure and function requires that S105 be observed in native membranes or reconstituted into artificial vesicles. The toxicity of the holin to the host, the catastrophic effect on the membrane and the precise timing of lysis

onset pose technical obstacles for direct visualization of S105 in isolated IMVs or in chemically fixed ultra-thin sections of holin-expressing cells. However, cryo-electron tomography allows millisecond, chemical-free preservation of whole cells to be observed at better than 5 nm resolution. These studies as well as reconstitution of purified S105 into liposomes using conventional detergent-based techniques or by detergent-free, chaperone-assisted reconstitution (Deaton *et al.*, 2004) (Dewey, J.S., Struck, D.K and Young, R., unpubl. data) are underway to provide us with a better understanding of the holin interaction with the lipid bilayer. Finally, the stability and monodispersity of the rings formed in DDM suggest that favourable conditions for the crystallization of monomeric and oligomeric forms of S105 may be found.

Experimental procedures

Antibodies

Antibodies against the C-terminal sequence of S105 have been described (Chang *et al.*, 1995). Antibodies against the peptide formyl MPEKHDLLAC corresponding to the N-terminal sequence of S105 were prepared and affinity-purified by Bethyl Laboratories (Conroe, Texas).

S overexpression

All alleles of *S* used in this work are isogenic to *S105_{τ94}*, which encodes S105 with the sequence G₂H₆G₂ embedded between amino acid positions 94–95 (Smith *et al.*, 1998). Overexpression of *S* alleles was carried out as previously described (Deaton *et al.*, 2004) except that the vector pET30b was used instead of pET11a and the host used for induction was *Escherichia coli* C43(DE3) (Miroux and Walker, 1996).

S105 purification

Purification of S105 in EBB was carried out as previously described (Deaton *et al.*, 2004). Purification in DDM, β-OG and LDAO was performed as follows. Cell pellets were re-suspended in lysis buffer (10 mM Tris pH 7.9, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF) and lysed by passage through a French press. Lysates were cleared of whole cells by centrifugation for 15 min at 10 000 *g* and at 4°C in a Sorvall SS-34 rotor. IMVs and outer membranes were harvested by ultracentrifugation for 90 min (100 000 *g*, 4°C) in a Ti50.2 (Beckman) rotor. Total membrane pellets were solubilized in 10 mM Tris pH 7.9, 150 mM NaCl, 1 mM PMSF, 10 mM MgCl₂, 1% EBB (v/v) or 1% (w/v) DDM or 2% (w/v) β-OG or 20 mM LDAO and extracted overnight with gentle shaking at 4°C. Insoluble material was removed by ultracentrifugation for 1 h at 100 000 *g* and 4°C. Solubilized material was applied to 250 μl of TALON resin and S105 was eluted in buffer containing 10 mM Tris pH 7.0, 150 mM NaCl, 500 mM imidazole, 1% EBB (v/v) or 0.1% (w/v) DDM or 1% (v/v) β-OG or 4 mM LDAO.

Gel permeation chromatography

Gel permeation chromatography was carried out using Superose-6 or S75 columns on an AKTA FPLC (Pharmacia). Calibration standards were purchased from Bio-Rad and used according to the manufacturer's directions.

DSP cross-linking of S105 purified in EBB and DDM

Protein samples were purified as above, except that Tris was substituted with Hepes in all buffers. Ten microlitres of purified protein (130 μ M) was diluted into 90 μ l of Hepes buffer [50 mM Hepes pH 7.2, 150 mM NaCl, 1% (v/v) EBB or 0.1% (w/v) DDM]. Twenty microlitres of 6 mM DSP dissolved in anhydrous DMSO (or 20 μ l of DMSO for un-cross-linked samples) was added to the diluted protein, and the samples were incubated with gentle shaking at 25°C for 30 min. Reactions were terminated by addition of 0.1 M glycine. Cross-linking products were analysed by SDS-PAGE on a 10% Tris-Tricine gel and Western-blotted with antibodies directed against the N terminus of S105.

Circular dichroism spectroscopy

S105 alleles were purified in PBS (20 mM Na₂HPO₄ pH 7.0, 150 mM NaCl) with either 1% (w/v) EBB or 0.1% (w/v) DDM. CD measurements were carried out in an Aviv 62DS CD spectrometer using a 0.1 cm pathlength cuvette with wavelength scans from 250 to 200 nm.

Negative stain and cryo-EM

Negatively stained specimens were prepared according to Valentine *et al.* (1968) by floating carbon-coated mica onto sample droplets followed by staining with aqueous uranyl acetate (2% w/v). Electron micrographs were recorded in a JEOL 1200 EX TEM at a calibrated magnification of 24 380 $\times$. Micrographs were digitized using the Leafscan 45 microdensitometer to a final pixel size of 4.1 $\text{\AA}$ pixel⁻¹. For cryo specimens, S105 in DDM (0.5 mg ml⁻¹) was applied to C-Flat holey carbon grids that had been freshly glow-discharged. Vitrification was carried out in an FEI Vitrobot at 100% humidity with 3 s blotting. Specimens were observed in a JEOL 2200 FS TEM using a Gatan 626 cryo-specimen holder. Electron micrographs were acquired using a Tietz TVIPS F224HD CCD camera under low-dose conditions (< 10 e⁻ $\text{\AA}^{-2}$). The final pixel size was 4.2 $\text{\AA}$ pixel⁻¹.

Single-particle analysis

Data sets were initially processed using the IMAGIC-5 software package (van Heel *et al.*, 1996) to produce reference-free classes using only the first five eigenvectors. For the cryo-EM data, 1836 ring dimers in end-on, side-on and intermediate projections were selected. After reference-free classification, 1079 particles from classes with a 23 nm outer diameter were extracted and processed separately. Further processing was performed using the EMAN single particle analysis package (Ludtke *et al.*, 1999) and a 3D reconstruction imposing C18 rotational symmetry was obtained. An initial 3D starting model was generated using the 'startcsym' command. The model was subsequently used for refinement through projection matching. The resolution of the map after eight iterations of refinement was 2.6 nm using the Fourier Shell Correlation 0.5 criterion (Frank, 1996). The final map was low pass-filtered to 2.6 nm and surface-rendered using UCSF Chimera (Pettersen *et al.*, 2004). The rendering threshold was adjusted to enclose protein densities of 1.72 MDa, assuming a protein density of 0.73 Da $\text{\AA}^{-3}$ (Zipper *et al.*, 1971).

Protease accessibility of S alleles

S105 IMVs and purified S105 samples were prepared as described above. For purified samples, 40 µg of protein in protease-DDM buffer [20 mM Tris pH 7.9, 150 mM NaCl, 5 mM CaCl₂ and 0.1% (w/v) DDM] was incubated with 2 µg of protease. For IMVs, 40 µg of total membrane protein in protease buffer (20 mM Tris pH 7.9, 150 mM NaCl, 5 mM CaCl₂) was incubated with 2 µg of protease. Digestion reactions were left for 30 min at 37°C and then terminated by addition of PMSF to a final concentration of 0.5 mM. Samples were run on 16.5% Tris-Tricine SDS-PAGE and Western-blotted using antibodies directed against either the N or C terminus of S105.

Cysteine modification

Protein samples for cysteine modification experiments were purified as described above but with a saturating concentration of 2,2'-dithiodipyridine included in the extraction buffer. The purified protein was reduced with 1 mM Tris (2-carboxyethyl) phosphine by a 5 min incubation at 25°C, then diluted 1:10 into buffer [500 mM Tris pH 7.0, 1 mM EDTA and 0.1% (w/v) DDM] for modification with PEG-maleimide (Creative PEGWorks; Winston-Salem, North Carolina). To 20 µl of protein (26 µg ml⁻¹), either 2 µl of 10% (w/v) SDS or water was added. Then, 3 µl of PEG-maleimide (600 µM) was added and the mixture left at room temperature for 30 min. Reactions were terminated by addition of 0.5 mM DTT. Samples were analysed by SDS-PAGE on a 16.5% Tris-Tricine gel and Western-blotted using antibodies directed against the N terminus of S105.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Protein topology and sequence diagram of the λ holin. Top: topology of S105 in the inner membrane, showing the charged residues in the termini and loops. Below: the sequence of S107 is shown. Horizontal arrow indicates Met3, the start of the S105 sequence; for simplicity, residue numbering is retained in the two sequences. The GGH₆GG sequence is shown inserted after residue Phe94 (Smith and Young, 1998). Arrows indicate the putative cleavage sites for trypsin (Lys92 or Arg93).

Fig. 2.

Gel permeation profiles of S105 in EBB (○—○), S105 in DDM (—) and of S105_{A52V} in DDM (----). The position of molecular mass standards is indicated by arrows, from left to right: 670, 158, 44 and 17 kDa. The oligomeric states of S105 in either EBB or DDM were also analysed by DSP cross-linking as shown in the inset.

Fig. 3. Electron microscopy of negatively-stained S105. Gel permeation peak fractions from S105 in DDM, corresponding to large stacks (A) and smaller ring assemblies (B). S105 purified in LDAO (C). Classification and rotational symmetry imposition for the 24.5 nm size group (D) and the 27 nm size group (E) (C1 = no symmetry imposed).

Fig. 4. Cryo-EM and single-particle analysis of S105 ring dimers

A. Electron micrograph indicating ring orientations. Side-on (squares), face-on (arrows) and intermediate projections (circles). Inset depicts characteristic class averages (top row) and corresponding re-projections (bottom row).

B. 3D reconstruction of an S105 ring dimer.

C. Putative single-ring form of the S105 multimer in the lipid bilayer.

Fig. 5. Protease accessibility and chemical modification of S105
A. S105 in IMVs or in 0.1% DDM was treated with trypsin (TR) or proteinase K (PK) and visualized by blotting with antibodies raised against the N (α -N) or C (α -C) terminus.
B. PEG-maleimide labeling of S105 in 0.1% DDM at positions 51, 7 and 99 in the presence/absence of SDS. Arrows indicate monomer (M), dimer (D) and pegylated products (P).