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Structure-based functional studies for the cellular recognition and cytolytic mechanism of pneumolysin from *Streptococcus pneumoniae*

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ABSTRACT

Cholesterol-dependent cytolysins (CDCs) contribute to various pathogenesis by Gram-positive bacterial pathogens. Among them, pneumolysin (PLY) produced by *Streptococcus pneumoniae* is a major contributor to pneumococcal infections. Despite numerous studies of the cytolytic mechanism of PLY, little structural information on its interactions with a specific receptor of the cell membrane is available. We report here the first crystal structures of PLY in an apo-form and in a ternary complex with two mannoses at 2.8 Å and 2.5 Å resolutions, respectively. Both structures contained one monomer in an asymmetric unit and were comprised of four discontinuous domains, similar to CDC structures reported previously. The ternary complex structure showed that loop 3 and the undecapeptide region in domain 4 might contribute to cellular recognition by binding to mannose, as a component of a specific cell-surface receptor. Moreover, mutational studies and docking simulations for four residues (Leu431, Trp433, Thr459, and Leu460) in domain 4 indicated that Leu431 and Trp433 in the undecapeptide might be involved in the binding of cholesterol, together with the Thr459–Leu460 pair in loop 1. Our results provide structure-based molecular insights into the interaction of PLY with the target cell membrane, including the binding of mannose and cholesterol.

1. Introduction

Cholesterol-dependent cytolysins (CDCs), which belong to the pore-forming toxin (PFT) family, have been identified in several Gram-positive bacterial genera including *Streptococcus*, *Clostridium* and *Listeria*, and are considered important virulence factors (Tweten, 2005). Among them, pneumolysin (PLY) produced by *Streptococcus pneumoniae* is a major contributor to pneumococcal infections, such as pneumonia, otitis media, sinusitis and meningitis (Hook et al., 1983; Kramer et al., 1987). While various attempts have been undertaken to understand the cytolytic mechanism of PLY, molecular-based studies of PLY have been interpreted only on the basis of the crystal structure of perfringolysin O (PFO) (Rossjohn et al., 1997) from *Clostridium perfringens* as a member of the CDC family, due to the lack of atomic structure information.

The molecular structure of members of the CDC family is highly conserved. CDCs are typically composed of four domains (D1–D4) and contain unique structural features consisting of three hydrophobic loops (L1–L3) and the undecapeptide region in D4, which is associated with receptor recognition on the target cell membrane (Farrand et al., 2010). Membrane cholesterol was initially thought to function as the sole CDC receptor and to interact with the undecapeptide of CDC directly (Rossjohn et al., 1997). However, Farrand et al. (2010) suggested that a simple motif consisting of a threonine–leucine pair in L1 is conserved in all CDCs and constitutes a common structural basis for recognition of cholesterol in the membrane. In addition, other recent studies have suggested that cholesterol recognition and binding of PLY requires the participation of at least two loops rather than a single loop structure (Taylor et al., 2013). These findings have resulted in exclusion of the undecapeptide from consideration in interactions with the cell membrane, including the binding of cholesterol; therefore, studies have focused on other possible roles of the undecapeptide in interactions with the target cell. Moreover, the structure–functional diversity of the undecapeptide remains elusive, since two distinct conformations—the furled-up and unfurled types—have been identified in the structures of CDCs. The discovery of intermedilysin (ILY), produced by *S. intermedius*, which uses human CD59 as a receptor (Giddings et al., 2004), was in stark contrast to the general understanding of CDCs, which were thought to use only cholesterol as their receptor. This finding suggested a new paradigm involving various receptors for membrane recognition by CDCs.

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Meanwhile, the studies of specific mechanisms for CDCs, including the recognition of and a stable association with the cell membrane, have focused on the non-lipid constituents (proteins and carbohydrates) as well as the lipid bilayer of the cell membrane (Howard and Buckley, 1982; Kato and Naiki, 1976; Saha and Banerjee, 1997). It has also been suggested that specific carbohydrate moieties may regulate not only the interaction of CDCs with the membrane bilayer but also their oligomerization. Our previous studies on the interaction of PLY with a carbohydrate moiety showed that the hemolytic activity of PLY was inhibited by mannose as a component of the cell membrane (Lim et al., 2013). These results suggested a new model of cellular recognition by PLY, which may interact with glycoproteins or glycolipids conjugated with mannose as a specific receptor.

To date, the crystal structures of several CDCs have been reported, but the available structural information on PLY remains sparse. Thus, more detailed investigations based on the molecular structure of PLY are required to improve understanding of its cytolytic mechanism. Here, we present the first crystal structures of PLY from *S. pneumoniae* in an apo-form and in a ternary complex with two mannoses at 2.8 Å and 2.5 Å resolutions, respectively. In addition, structural and mutational studies of PLY, together with the docking simulation, indicated that the undecapeptide in D4 might contribute to the binding of cholesterol as well as recognition of the cell membrane, in concert with the Thr–Leu pair and L3, respectively. Our results provide insightful structural information regarding the interaction of PLY with mannose as a recognition factor, and suggest that the functional properties of the undecapeptide in cholesterol binding should be reevaluated.

2. Materials and methods

2.1. Preparation of wild type and mutant of PLYs

The expression and purification of wild type and mutant PLYs were performed as previously described (Lim et al., 2013). Briefly, the gene encoding PLY was amplified by PCR from *S. pneumoniae* genomic DNA, and the amplified DNA fragment was cloned into the pET-28a vector with a His_6 tag. Then, recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3). The expressed PLY was purified using immobilized metal ion affinity chromatography and size-exclusion chromatography. Site-directed mutagenesis was performed using the Quickchange mutagenesis method (Braman et al., 1996), wherein a pair of complementary designed primers (Supplementary Table 1) was used to amplify the entire plasmid by PCR with a high-fidelity non-strand-displacing *pfu* polymerase. Substitutions for the selected amino acids (L431A, W433A, T459S, T459G, L460I, and L460A) were introduced into the plasmid DNA, which was encoded with wild type PLY as a template for mutagenesis. After digesting the methylated plasmid with *Dpn*I, the mutated plasmid DNA was transformed into competent *E. coli* DH5α cells. All mutants were verified by DNA sequencing. Plasmids containing the desired mutations were transformed into *E. coli* BL21 (DE3) strain, and the PLY mutants were overexpressed and purified using the same protocol as for the wild-type protein. Following purification, the purified wild type and mutant PLYs were concentrated in buffer containing 20 mM Tris-HCl pH 7.9, 50 mM NaCl. The protein concentration was estimated using the Bradford assay, and the purity was confirmed by 15% SDS-PAGE to be >95%.

2.2. Crystallization and X-ray diffraction data collection

Prepared wild type PLY was crystallized by the hanging-drop vapor diffusion method at 22 °C. Each hanging drop was prepared by mixing 1 μl protein solution and 1 μl reservoir solution then equilibrated over a 500 μl reservoir solution. The apo-PLY crystals were obtained in reservoir solution containing 0.1 M Bicine-Tris pH 8.0–8.5, 30% (*w*/*v*) glycerol-polyethylene glycol 4000 (GOL_P4K), 0.1 M carboxylic acids mixture (Molecular Dimensions Inc.), and 0.7% 1-butanol. For co-crystallization with mannose, the protein solution was mixed with mannose in a 1:10 M ratio. Crystals of the ternary complex with two mannoses were obtained in a buffer consisting of 0.1 M Bicine-Tris, pH 8.0–8.5, 30% (*w*/*v*) ethylene glycol-polyethylene glycol 8000 (EDO_P8K), and 0.1 M cesium chloride.

For cryogenic experiments, the crystals of PLY in an apo-form and in a ternary complex were transferred into a cryoprotection solution containing 25% (*v*/*v*) ethylene glycol in a reservoir solution, and were flash-frozen in a stream of nitrogen gas. X-ray diffraction data of the PLY crystals in an apo-form and in a ternary complex with mannose were collected on beamline 5C and 7A at the Pohang Light Source (Pohang, South Korea) using an ADSC Quantum 315r and 270r CCD detector, respectively. The PLY crystals diffracted to resolutions of 2.8 Å for the apo-form and 2.5 Å for the ternary complex. All data sets were indexed, integrated and scaled using the *HKL-2000* software package (Otwinowski and Minor, 1997).

2.3. Structure refinement and comparison

The crystal structure was solved by the molecular-replacement (MR) method using the *MOLREP* program (Vagin and Teplyakov, 2010) in the *CCP4* package (Winn et al., 2011) with the PFO of *C. perfringens* (PDB ID: 1PFO) (Rossjohn et al., 1997) as the search model. The PLY crystals in an apo-form and in a ternary complex with mannose belonged to both the orthorhombic space group $P2_12_12_1$. Structural refinement was performed by the *PHENIX* program (Adams et al., 2002) with several cycles of torsion-angle-simulated annealing, energy minimization, and individual *B*-factor refinement. The model was completed by iterative cycles of model building with *COOT* (Emsley and Cowtan, 2004). Water molecules were automatically picked up by the *CNS* package (Brunger et al., 1998) and then confirmed based on peak height and distance criteria in the F_o-F_c and $2F_o-F_c$ maps. The final model of the apo-structure contained one monomer and the final R_{factor} and R_{free} values were 18.86% and 26.07%, respectively. The refined apo-form structure was then used as an initial model to solve the structure of ternary complex. Structural refinements of the ternary complex were performed using a simulated annealing refinement, followed by a rigid body refinement. Two mannose molecules were confirmed based on the F_o-F_c contoured at 2.5 σ and omitted $2F_o-F_c$ electron density maps. The final model of the ternary complex structure contained one monomer with two mannoses, and the final R_{factor} and R_{free} values were 21.25% and 27.09%, respectively. Structural validations of both structures were analyzed using the *PROCHECK* program (Laskowski et al., 1993), and no residue was detected in the disallowed region of the Ramachandran plot (Ramachandran et al., 1963). The data collection and final refinement statistics are given in Table 1.

Structure analysis was carried out using the following computer programs: *COOT* (Emsley and Cowtan, 2004) for superposition of molecules; *ESPript* server (Robert and Gouet, 2014) for preparation of the secondary structure-based alignment figure; *SSAP* Server (Orengo et al., 1997) for calculation of root-mean-square deviation (RMSD); *Adaptive Poisson Boltzmann Solver* (APBS) in *PMV* (Python Molecular Viewer) (Sanner, 1999) for calculation of molecular electrostatics and *PyMol* (Schrodinger, 2010) for the structure depiction. Other orthologs used in the structural comparison were *S. intermedius*, ILY (PDB ID: 1S3R) (Polekhina et al., 2005), *S. suis* suilysin, SLY (PDB ID: 3HVN) (Xu et al., 2010), *S. pyogenes* streptolysin