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Structure Report

Crystallographic analysis of *Neisseria meningitidis* PorB extracellular loops potentially implicated in TLR2 recognition

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ABSTRACT

Among all *Neisseriae* species, *Neisseria meningitidis* and *Neisseria gonorrhoeae* are the only human pathogens, causative agents of bacterial meningitis and gonorrhoea, respectively. PorB, a pan-*Neisseriae* trimeric porin that mediates diffusive transport of essential molecules across the bacterial outer membrane, is also known to activate host innate immunity via Toll-like receptor 2 (TLR2)-mediated signaling. The molecular mechanism of PorB binding to TLR2 is not known, but it has been hypothesized that electrostatic interactions contribute to ligand/receptor binding. Strain-specific sequence variability in the surface-exposed loops of PorB which are potentially implicated in TLR2 binding, may explain the difference in TLR2-mediated cell activation *in vitro* by PorB homologs from the commensal *Neisseriae lactamica* and the pathogen *N. meningitidis*. Here, we report a comparative structural analysis of PorB from *N. meningitidis* serogroup B strain 8765 (63% sequence homology with PorB from *N. meningitidis* serogroup W135) and a mutant in which amino acid substitutions in the extracellular loop 7 lead to significantly reduced TLR2-dependent activity *in vitro*. We observe that this mutation both alters the loop conformation and causes dramatic changes of electrostatic surface charge, both of which may affect TLR2 recognition and signaling.

1. Neisserial PorB structure and functions

Neisseria meningitidis is a Gram-negative opportunistic human pathogen carried by approximately 10–15% of the adult population (Cartwright et al., 1987; Yazdankhah and Caugant, 2004). Upon colonization of the nasopharyngeal epithelium, host cell invasion and meningococci dissemination via the bloodstream may occur, leading to meningitis and septicaemia. Among thirteen existing *N. meningitidis* serogroups, the majority of meningococcal disease worldwide is caused by A, B, C, X, Y and W135 types, categorized according to their capsular polysaccharides. Despite recent advances in meningococcal vaccine developments, *N. meningitidis* serogroup B strains still pose a threat worldwide. Nasopharyngeal colonization by *Neisseria lactamica* is also frequent, particularly in infants and young children, but systemic infections are very rare and this organism is considered a commensal (Gold et al., 1978). Within the *Neisseria* family, *Neisseria gonorrhoeae* is also a human pathogen and the causative agent of gonorrhoea (Virji, 2009).

Porins, major Gram-negative bacteria outer membrane proteins, mediate diffusive transport of essential solutes across the membrane (Nikaido, 2003). *N. meningitidis* expresses two porins, PorA and PorB (either class 2 or class 3), while *N. lactamica* only expresses PorB. PorB has a 16-stranded β-barrel structure with eight surface-exposed loops (L1–L8) and corresponding shorter periplasmic turns. While the β-barrel regions share a high level of sequence homology among the different strains, amino acid sequence variability characterizes the surface-exposed loops (Bennett et al., 2008; Derrick et al., 1999; van der Ley et al., 1991). The variable loop regions (VR) 1–4, located in L1, L5, L6 and L7, are used for the classification of *N. meningitidis* clonal complexes. *N. meningitidis* PorB (strain W135) and *N. gonorrhoeae* $PorB_{IA,}$ (strain N242) have been recently crystallized (Tanabe et al., 2010; Zeth et al., 2013), confirming the predicted PorB topology model.

Besides having conventional porin functions, PorB influences host cell invasion, intracellular bacteria survival, immune evasion

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and even onset of disease. For example, PorB-induced host cell actin nucleation and reorganization during host cell infection possibly influences bacterial invasiveness (Wen et al., 2000), and PorB translocation into the host cell mitochondrial membrane modulates host cell apoptosis (Binnicker et al., 2004; Follows et al., 2009; Jiang et al., 2011; Kozjak-Pavlovic et al., 2009; Massari et al., 2000, 2010).

One of the best-characterized PorB functions *in vitro* and *in vivo* is induction of host cell signaling via Toll-like receptor 2 (TLR2) and TLR1 (Massari et al., 2002, 2006; Toussi et al., 2012), resulting in its immune adjuvant properties (Burke et al., 2007; Chiavolini et al., 2008; Liu et al., 2008; Mackinnon et al., 1999; Wetzler et al., 1996). TLRs recognize pathogen-associated molecular patterns (PAMPs) and their structural features have been extensively studied (Botos et al., 2011). PAMP recognition by the extracellular domain of TLR2 and TLR1 induces dimerization and rearrangement of their cytoplasmic domains, recruitment of the adaptor protein MyD88 and downstream activation of NF-κB, MAPKs and AP-1 signaling pathways (Medzhitov et al., 1998).

Although the TLR2-dependent activity of PorB is well-characterized, studies on the molecular details of PorB/TLR2 interaction are scarce. A possible model proposes electrostatic interaction of negatively charged residues on the TLR2 ectodomain and positively charged residues in PorB surface-exposed regions (Tanabe et al., 2010). PorB directly binds to TLR2 *in vitro* and to cell surface-bound TLR2 (Massari et al., 2006; Liu et al., 2010; Toussi et al., 2012). However, PorB from different *Neisseriae* strains have different apparent affinity for TLR2 and induce variable levels of cell activation, possibly via different intracellular signaling pathways. Strain-specific *porB* gene sequence variability within these regions may influence the modality of interaction with TLR2 and subsequent cell activation, as shown by studies using *N. meningitidis*/*N. lactamica* hybrid PorB loop mutants (Liu et al., 2010; Massari et al., 2006; Toussi et al., 2012).

As a step towards the characterization of PorB/TLR2 interaction, we report the crystal structure and molecular differences between PorB from *N. meningitidis* serogroup B strain 8765 ($PorB^{WT}$, which shares 63% sequence identity with PorB from serogroup W135 ($PorB^{W135}$) (Tanabe and Iverson, 2009) and the $PorB^{DDE255-262AKR}$ mutant ($PorB^{DDE/AKR}$), in which mutation of three residues in loop 7 for corresponding residues from the sequence of *N. lactamica* PorB ($PorB^{Nl}$) causes a significant reduction in TLR2-dependent host cell responses *in vitro* (Toussi et al., 2012). Comparison of $PorB^{WT}$ and $PorB^{DDE/AKR}$ crystal structures shows an altered L7 loop conformation, modification in charge distribution and electrostatic surface differences that likely influence PorB/TLR2 interaction and subsequent cell activation levels. Understanding the mechanism of PorB recognition by TLR2 at a molecular level will provide invaluable information for studies of its functions *in vitro* and *in vivo*.

2. Recombinant PorB expression and purification

The plasmid pNVK15, encoding PorB from *N. meningitidis* serogroup B strain 8765 (B:15:P1,3) and generation of PorB loop mutants have been previously described (Qi et al., 1994; Toussi et al., 2012). All PorB molecules were expressed as inclusion bodies in *Escherichia coli* BL21(*DE3*) and purified as also previously described (Tanabe and Iverson, 2009), with minor modifications. Briefly, prior to a single-step size exclusion chromatography (HiPrep Sephacryl S-100 16/60), samples were incubated for 4 days at 4 °C in 20 mM Tris–HCl pH 7.5, 200 mM NaCl and 0.1% lauryldimethylamine N-oxide (LDAO) (Buffer A) to enhance refolding and dialyzed against Buffer A (30:1 ratio) using a 10 kDa molecular weight cut-off dialysis tubing.

3. Crystallization, data collection, structure determination and molecular dynamics simulation

$PorB^{WT}$ crystals grew in 0.1 M MES pH 6.5–7.0, 50 mM CsCl and 29–35% (v/v) Jeffamine M-600 within 4 months and the L7 mutant $PorB^{DDE/AKR}$ crystals grew in 65 mM HEPES pH 7.5 and 1.1 M tri-sodium citrate in 2 weeks using previously described methods (Kattner et al., 2013). $PorB^{DDE/AKR}$ and $PorB^{WT}$ crystals were flash-cooled in liquid nitrogen in 1.5 M tri-sodium citrate with 20% Jeffamine M-600 or with 35% of Jeffamine M-600 as cryoprotectant, respectively. $PorB^{WT}$ diffraction data were collected at the Swiss Light Source (SLS) PX-II (X10SA) and for $PorB^{DDE/AKR}$ at the PX-III (X06DA) beamline at wavelengths of 1.00 Å. Datasets were processed using the HKL2000 suite (Otwinowski and Minor, 1997) and structures were solved by the molecular replacement (MR) method with Phaser (McCoy, 2007) using PDB ID: 3VZT as search model (Tanabe et al., 2010). Model building and refinement were performed using COOT (Emsley and Cowtan, 2004) and REFMAC5 (Murshudov et al., 1997) in CCP4 suite (Winn et al., 2011) and structure quality was analyzed by PROCHECK (Laskowski et al., 1993). Figures were generated with Pymol (DeLano, 2002) and sequence alignment performed by ClustalW (Thompson et al., 1994). The predicted structural models of selected PorB mutants were calculated by RosettaBackrub (Smith and Kortemme, 2008).

All-atom molecular dynamics simulations of PorB in bilayers were performed in GROMACS4.6 (Pronk et al., 2013). We used a combination of the Amber99sb-ildn force field (Lindorff-Larsen et al., 2010) for trimeric PorB, together with the virtual site model for hydrogen atoms (Feenstra et al., 1999), the Berger model for lipids (Cordomí et al., 2012) and the SPC/E water model (Berendsen et al., 1987), as employed previously for PorB (Kutzner et al., 2011). The trimers were inserted into fully hydrated and equilibrated POPC bilayers (Cordomí et al., 2012) by using the GROMACS utility g_membed (Wolf et al., 2010), equilibrated further for 25 ns under gradual release of position restraints on lipid head groups and the protein, and then freely simulated in the NpT-ensemble for 300 ns (integration time-step: 4 fs) at 1 bar and $T = 300$ K by using Berendsen pressure (Berendsen et al., 1984) and velocity-rescale temperature coupling (Bussi et al., 2007).

4. Structure of PorB from *N. meningitidis* strain 8765

Crystals of $PorB^{WT}$ were grown in Jeffamine M-600, similar to $PorB^{W135}$ (Tanabe and Iverson, 2009). Mutants of $PorB^{WT}$ in L4, L5 and L7 were recently described (Toussi et al., 2012). The L7 mutant, $PorB^{DDE/AKR}$, which induces significantly lower TLR2-dependent cell activation *in vitro* than $PorB^{WT}$, was instead crystallized using a salt-based precipitant, a condition that was not successful for $PorB^{WT}$. Crystals of $PorB^{WT}$ and $PorB^{DDE/AKR}$ diffracted to 3.3 and 2.4 Å, respectively (Table 1).

Initial MR modeling of the $PorB^{WT}$ and $PorB^{DDE/AKR}$ crystal structures based on the coordinates of $PorB^{W135}$ (PDB ID: 3VZT) presented some difficulties in refinement of the loop regions, since these molecules share only 63% of overall amino acid sequence identity and rms deviation values for the Cα atoms of 0.78 Å for (269 residues). The model rebuilding was facilitated by the structure of $PorB_{1A}$ from *N. gonorrhoeae* strain AN242 ($PorB^{Ng}$) (PDB ID: 4AUI) (Zeth et al., 2013). This porin has a 77% sequence identity to $PorB^{WT}$ and rms deviation values for the Cα atoms of 0.99 Å for (271 residues), allowing a significant improvement of loop tracing. Data collection and refinement statistics are summarized in Table 1. $PorB^{WT}$ has a final R_{work}/R_{free} value of 25.3%/29.3% for data refined to 3.3 Å and $PorB^{DDE/AKR}$ a final R_{work}/R_{free} value of 22.7%/25.5% for data refined to 2.4 Å.