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Vaccine Candidate P6 of Nontypable *Haemophilus influenzae* is Not a Transmembrane Protein Based on Protein Structural Analysis

Lea Vacca Michel, PhD¹, Breanna Kalmeta¹, Mark McCreary², Joy Snyder¹, Paul Craig, PhD¹, and Michael E. Pichichero, MD³

¹Department of Chemistry, Rochester Institute of Technology, Rochester, New York

²Department of Biology, Rochester Institute of Technology, Rochester, New York

³Rochester General Hospital Research Institute, Rochester, New York

Abstract

P6 has been a vaccine candidate for nontypable *Haemophilus influenzae* (NTHi) based on its location on the outer membrane and immunogenicity. Because P6 is attached to the inner peptidoglycan layer of NTHi, and is putatively surface exposed, it must be a transmembrane protein. We examined the P6 structure using computational modeling, a P6 modified by site-directed mutagenesis to study monoclonal antibody attachment to P6, and nuclear magnetic resonance spectroscopy. We found that P6 cannot be a transmembrane protein, and therefore may not be surface exposed. We conclude that there may be another protein on the surface of NTHi that has epitopes similar if not identical to P6.

Keywords

nontypable *Haemophilus influenzae*; P6 protein; outer membrane protein

1. Introduction

For over twenty years, the outer membrane protein (OMP) P6 has been a leading vaccine candidate against nontypable *Haemophilus influenzae* (NTHi) [1–3]. NTHi is an important cause of acute otitis media (AOM), sinusitis, acute exacerbations of chronic bronchitis and pneumonia (in developing countries). The incidence of ear infections alone in the United States (75% of children experience at least one episode of AOM by their 3rd birthday) points to the urgent need for a vaccine against NTHi [4]. However, one of the major barriers to NTHi vaccine development has been identification of antibody targets that are conserved among all or virtually all strains of the bacteria. P6 has been shown to be conserved among all of the tested strains of NTHi [1,21].

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Correspondence: Michael E. Pichichero, MD Rochester General Hospital Research Institute 1425 Portland Avenue Rochester, NY 14621 Phone: 585-922-5959 and Fax: 585-922-4289 michael.pichichero@rochestergeneral.org.

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Although studies have demonstrated that monoclonal antibodies interact with P6 on the surface of the bacterial cell [5,6] and that P6 is the target of bactericidal antibodies [7–10], our analysis of a recent protein structure of P6 [11] suggests that P6 may not be a surface exposed OMP. In addition, studies on Pal, the homologue to P6 in *Escherichia coli* (*E. coli*), mark Pal as a non-surface exposed protein which interacts with the peptidoglycan layer and various other proteins inside of the cell [12–14]. These observations led us to pursue new experimental evidence to corroborate or refute the physical location and orientation of P6. Our findings, presented here, strongly suggest that it is unlikely for P6 to exist as a transmembrane protein in the outer membrane of NTHi.

2. Materials and Methods

2.1. Computational modeling of P6 as a transmembrane protein

Currently, there are several computational tools available on the Expert Protein Analysis System (ExPASy, Swiss Institute of bioinformatics) server [15] for predicting the topology of transmembrane proteins. The topology prediction programs required an input of the P6 primary sequence, minus the first 19 amino acid leader sequence which gets cleaved from the protein and is replaced by the lipid moiety. The programs yielded an output of the predicted transmembrane regions and/or the likelihood that certain regions within the protein are contained within the membrane.

2.2 Protein expression and purification

The NTHi P6 gene in the pET28-a (Kanamycin resistant) vector was a generous gift from Dr. John Orban (University of Maryland Biotechnology Institute). The P6 protein is non-lipidated (the 19 residue N-terminal signal sequence was removed) and contains a 6-Histidine tag at its N-terminus. The protein was expressed and purified as described in the literature with some modifications [11]. Briefly, the protein was expressed in *E. coli* BL21 (DE3) cells in LB for the ELISA experiments and ^{15}N -labeled minimal media with ^{15}N , 99% $[\text{NH}_4\text{Cl}]$ (Cambridge Isotope Laboratories, Inc.) as the sole nitrogen source for the nuclear magnetic resonance (NMR) experiments, and induced with 1 mM IPTG. The cells were harvested by centrifugation at 5000g for 15 minutes and the pellets were frozen overnight. The thawed cells were lysed via sonication and centrifuged at 20,000g for 25 minutes. The supernatant was then purified via TALON resin beads (Clontech) according to the manufacturer's instructions. The protein was eluted in imidazole buffer and exchanged into 50 mM NaPi, 50 mM NaCl pH 7.0 with a PD-10 column desalting column (GE Healthcare). The estimated protein concentration was determined using a BCA assay (Pierce) and using an extinction coefficient (280 nm) of $14,350 \text{ cm}^{-1}\text{M}^{-1}$.

2.3. Site-directed mutagenesis

The P6 D59N mutant was prepared using the QuikChange II site-directed mutagenesis kit (Stratagene/Agilent Technologies) according to the manufacturer's instructions. P6 D59N is a P6 variant where the aspartic acid at position 59 was substituted with an asparagine. The non-lipidated P6 gene in pET28-a was used as a template for the mutagenesis, and the following forward and backward mutant primers were purchased from Integrated DNA Technologies. Forward: 5'-gttacaataccgtttatttcggtttgataaataacattactggtgaatacg-3' Backward: 5'-cgtattcaccagtaatgttatattatcaaaaccgaaataaacggtattgtaac-3' The P6 D59N protein was expressed in *E. coli* and purified as described above for wild-type P6.

2.4. ELISA

50 ng of purified recombinant P6 or P6 D59N protein were added to each well of an Apogent medium binding plate (Nunc), incubated at room temperature for approximately 3

hours and then refrigerated overnight. The plate was washed 3 times with PBS with 0.1% TWEEN-20. The plate was blocked with PBS/3% skim milk/ 0.1% TWEEN-20 (200 μ l/well) for 1 hour at 37°C. After the plate was washed 3 times, 100 μ l of the unpurified 7F3 and 4G4 monoclonal antibodies (kindly provided by Dr. Timothy Murphy, University at Buffalo) were added to each well at different dilutions (10 fold, 20 fold, 40 fold and 80 fold in PBS/3% skim milk/0.1% TWEEN-20) and allowed to incubate for 1 hour at room temperature. The plate was washed again; the goat anti-mouse IgG with HRP (1:10,000 dilution in PBS/3% skim milk/ 0.1% TWEEN-20, 100 μ l/well) was added to the wells and allowed to incubate at room temperature for 1 hour. After washing, 100 μ l of TMB substrate (KPL) was placed in each well and allowed to develop for 30 minutes at room temperature, and the reaction was stopped by adding 100 μ l of 1M phosphoric acid to each well. The plates were read using an automated ELISA reader at 450nm.

2. 5. NMR Spectroscopy

The NMR data were collected on a Varian INOVA 500 MHz spectrometer (operating at 499.839 MHz for ^1H) at 299 K. A ^1H - ^{15}N HSQC spectrum (8 scans, 1024 \times 128 points) was collected for both the purified recombinant wild-type P6 and P6 D59N (~1 mM protein concentration, pH 7.0). The NMR spectra were processed using NMRpipe [16] and visualized (the two spectra were overlaid) using Sparky [17]. The residue chemical shift assignments for wild-type P6 have been published in the literature [11].

3. Results

3.1. Computational modeling of P6 as a transmembrane protein

Seven protein topology programs were used for prediction analyses of the location of P6 in NTHi. None of the programs predicted membrane spanning regions contained within P6 (Table 1).

3.2. Immunologic assays to detect P6

ELISA was performed on purified recombinant wild-type P6 and P6 D59N. P6 D59N did not show any binding to the 7F3 monoclonal antibody. The 4G4 monoclonal antibody did bind to P6 D59N at approximately half the binding seen to wild-type P6 (Fig. 1).

3.3. Nuclear magnetic resonance

NMR spectroscopy was utilized to confirm that the D59N mutation did not alter the protein structure from that of wild-type P6. The ^1H - ^{15}N HSQC “fingerprint” spectra for both proteins, confirmed that the structure of P6 D59N is highly similar to that of wild-type P6, with only minor chemical shift differences between the two spectra (data not shown).

3.4. Protein structure analysis

When the P6 protein structure is oriented such that its longest measurement is vertical, the protein is about ~7 nm in length. The OM of NTHi has been measured to be 7.5–10 nm thick [18]. Residue 59 is located in the center of the protein (Fig. 2). Monoclonal antibodies 7F3 and 4G4 interact with P6 residue 59. Therefore, P6 structure analysis shows it is impossible for P6 to interact with the peptidoglycan layer in the interior of the bacterial cell and span the OM to become surface-exposed and have residue 59 interact with monoclonal antibodies on the cell surface.

4. Discussion

P6 has been reported to comprise 1–5% (by weight) of the total protein in the outer membrane of NTHi [5]. Several experimental approaches have been utilized to demonstrate the surface exposure of P6. Monoclonal and polyclonal antibodies to P6 have been shown to stain the entire organism by immunofluorescence and immunoelectron microscopy [1,5,6,19]. NTHi strain 3524 was radio-labeled with ^{125}I , and then after disruption of the bacterial outer membrane, the OMPs were dispersed on an agarose gel. The presence of a band at ~16 kDa that was extrinsically labeled suggested that P6 was surface exposed [1]. Adsorbed antiserum was shown to immunoprecipitate P6 [19], P6 was shown to be accessible to bactericidal antibodies [7–10,20] and the anti-P6 antibody was shown to be eluted from the surface of intact NTHi [5]. It is also generally accepted that NTHi P6 interacts with the peptidoglycan layer in the periplasmic space of the bacterial cell [11,21].

There have been several key immunological experiments involving monoclonal antibodies to P6 that have suggested that P6 is surface exposed [5–10]. Apicella *et al.* performed immunoelectron microscope experiments that utilized the P6 monoclonal antibodies 3B9 and 4G4 [5,6]. The presence of gold-labeled secondary antibodies on NTHi cells (visualized via an electron microscope) suggested that both monoclonal antibodies bound to P6 on the surface of the cells [5,6]. A third monoclonal antibody, 7F3, was proposed to bind to the same or a closely related epitope on P6 as the 4G4 antibody [6]. Pre-incubation of NTHi with monoclonal antibody 7F3 inhibited human serum bactericidal killing, suggesting that P6 was indeed surface exposed and the target of bactericidal activity [20]. While the specific amino acids that make up the epitope for 7F3 and 4G4 have not been fully identified, aspartic acid at position 59 has been implicated in antibody binding to P6 [21]. The results of our experiments demonstrate that D59 does indeed participate in the binding of P6 to both 7F3 and 4G4 monoclonal antibodies. The OM typically measures between 7.5 and 10 nm thick. At its longest measurement, P6 is only ~7 nm long, making it unlikely that P6 would be able to interact with monoclonal antibodies on the cell surface via residue 59 (which is located in the middle of the protein) and span the OM such that it can also interact with the peptidoglycan layer (Fig. 2).

Transmembrane proteins typically demonstrate a pattern of hydrophobic/hydrophilic amino acids which can be used to predict the probable “membrane spanning” regions. In the case of NTHi P6, seven out of seven transmembrane prediction programs (available on the ExPASy server) did not recognize any membrane spanning regions within the P6 amino acid sequence (Table 1). These results, along with our protein structure analysis of P6 and the NTHi OM, suggest that P6 cannot possibly span the OM in order to interact with monoclonal antibodies (at residue 59) on the cell surface *and* with the peptidoglycan layer in the interior of the bacterial cell.

It is important to mention that the experiments presented in this work were performed on recombinant non-lipidated P6 protein in which the leader sequence containing the first 19 amino acids was removed. During *in vivo* processing of P6, the N-terminal leader sequence is removed and replaced by a lipid moiety. The ELISA and NMR experiments were attempted on the lipidated version of P6; however, purified lipidated P6 unfolds and/or aggregates such that NMR spectroscopy cannot be performed on the protein (data not shown). The lipid moiety of a bacterial lipoprotein anchors it to the membrane by insertion into the inner or outer leaflet of the lipid bilayer. Since P6 has to interact with antibodies and peptidoglycan through its amino acids (and not through its lipid moiety), using the non-lipidated version of P6 does not prevent us from making conclusions about the length of native P6 or the location of its epitopes.

The structural similarity between NTHi P6 and *E. coli* Pal is notable. The NMR structure of NTHi P6 was determined in 2006 [11]. It showed a high degree of similarity between NTHi P6 and *E. coli* Pal [12]. The N-terminally truncated 108-residue Pal crystal structure can be overlaid onto the NTHi P6 NMR structure (Fig. 3). The overall folds of the proteins are highly similar, suggesting the potential for similar binding partners and/or similar functions *in vivo*. Although the functions of Pal in *E. coli* are not definitive, Pal is known to be anchored to the inner leaflet (*i.e.*, the inner lipid layer) of the OM of *E. coli* via its lipid moiety and is proposed to interact with the peptidoglycan layer as well as several Tol proteins and outer membrane protein A (OmpA) [12–13,22–23]. Importantly, there is no controversy that *E. coli* Pal is attached to the OM via its lipid moiety, but is otherwise defined as non-membrane spanning protein. In other words, with the exception of the N-terminal lipid, Pal does not span either layer of the OM and faces in towards the intermembrane (*i.e.*, periplasmic) space of *E. coli*. Although there is no definitive evidence demonstrating a similar orientation for P6 in the NTHi OM, the remarkable similarity between the structures of NTHi P6 and *E. coli* Pal points to the possibility of similar membrane orientations.

In conclusion, our results show that NTHi P6 is not a transmembrane protein since it is not of sufficient length to be surface-exposed and interact with the peptidoglycan layer on the inside of the bacterial cell [11,21]. In order for P6 to interact with the peptidoglycan layer and interact with monoclonal/bactericidal antibodies on the cell surface (as shown by immunological experiments), then P6 must either be inserted into the OM via two distinct “flipped” orientations or there is another OMP on the surface of NTHi that expresses an epitope similar to P6. Since the former has never been described for an OMP of any bacterial species, we are now pursuing studies to identify the OMP of NTHi that has structural epitopes similar to P6 but that is not P6.

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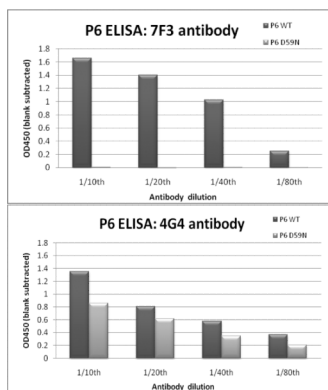


Fig. 1. ELISA data on purified recombinant P6 wild-type (WT) and P6 D59N proteins against 7F3 (left) and 4G4 (right) monoclonal antibodies.

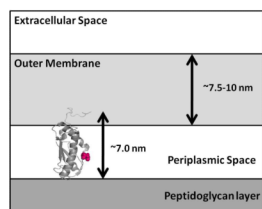


Fig. 2.

A cartoon representation of the backbone of NTHi P6 (PDB ID 2AIZ) is oriented vertically, with residue 59 highlighted in space-fill (left). Because the OM is measured between 7.5–10 nm thick, and P6 is proposed to interact with monoclonal antibodies on the cell surface via residue 59 (pink), it is unlikely that P6 is long enough to span the OM such that it can also interact with the peptidoglycan layer.

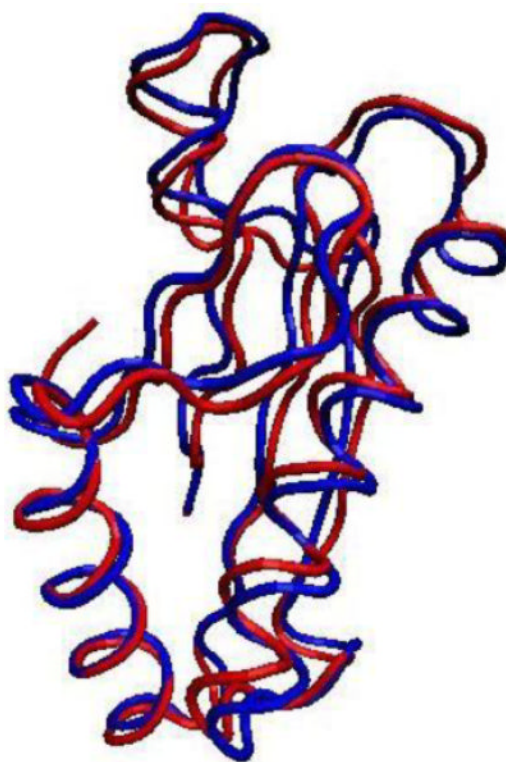


Fig. 3. Backbone representations of truncated P6 from NTHi (blue, PDB ID 2AIZ) and Pal from *E. coli* (red, PDB ID 1OAP) overlay with an RMSD of ~ 1.2 Å. The graphical representation was prepared using the Visual Molecular Dynamics program [31].

Table 1

Summary of outputs from the transmembrane prediction tools (19 amino acid leader sequence not included in analysis).

Topology Prediction Tool	Output
TMHMM Server v. 2.0 ²⁴	No predicted transmembrane
Dense Alignment Surface ²⁵	No predicted transmembrane
TMpred ²⁶	No predicted transmembrane
SOSUI ²⁷	No predicted transmembrane
HMMTOP ²⁸	No predicted transmembrane
PHDhtm ²⁸	No predicted transmembrane
Phobius ³⁰	No predicted transmembrane