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Recombinant truncated AniA of pathogenic *Neisseria* elicits a non-native immune response and functional blocking antibodies

Lucy K. Shewell [a], Shan C. Ku [b], Benjamin L. Schulz [b], Freda E.-C. Jen [a], Tsitsi D. Mubaiwa [a], Margaret R. Ketterer [c], Michael A. Apicella [c], Michael P. Jennings [a,*]

[a] Institute for Glycomics, Griffith University, Gold Coast, Queensland 4222, Australia
[b] School of Chemistry and Molecular Biosciences, University of Queensland, St. Lucia, Brisbane, Queensland 4072, Australia
[c] Department of Microbiology, University of Iowa, 51 Newton Rd., BSB 3-403, Iowa City, IA 52242, USA

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ABSTRACT

AniA of the pathogenic *Neisseria* is glycosylated in its C-terminal repeat region by the pilin glycosylation (*pgl*) pathway. AniA appears to be unique among bacterial nitrite reductases as it contains an N-terminal extension that includes a lipid modification site as well as a C-terminal extension that is glycosylated. Immunising with various glycoforms of the AniA protein demonstrated a strong humoral immune response to the basal monosaccharide. In addition, when animals were immunised with a truncated form of AniA, completely lacking the glycosylated C-terminal region, the antibody response was directed against AniA regardless of the glycosylation state of the protein. Immuno-SEM confirmed that AniA is expressed on the cell surface in *Neisseria gonorrhoeae*. Antisera generated against a truncated, non-glycosylated, recombinant form of the AniA protein are capable of blocking nitrite reductase function in a whole cell assay. We propose that recombinant modified AniA has potential as a vaccine antigen for *N. gonorrhoeae*.

1. Introduction

The pathogenic *Neisseria* species consist of *Neisseria meningitidis*, which causes bacterial meningitis and sepsis, and *Neisseria gonorrhoeae*, the causative agent of the sexually transmitted disease gonorrhoea. Currently there is no vaccine to prevent infection by *N. gonorrhoeae*, and there are now reports of *N. gonorrhoeae* strains that are resistant to the last remaining first-line treatment option for gonorrhea, the third-generation cephalosporin ceftriaxone [1], highlighting the pressing need for the development of gonoccocal vaccines.

In recent studies, we have identified an additional outer membrane glycoprotein in pathogenic *Neisseria*, the copper-containing nitrite reductase AniA, formerly known as Pan 1 [2,3], that is glycosylated in its C-terminal region by the *pgl* pathway [4]. Both *N. gonorrhoeae* and *N. meningitidis* contain the internal enzymatic steps of the denitrification pathway in which nitrite is reduced to nitric oxide (NO) by AniA [2], and nitric oxide is reduced to nitrous oxide (N_2O) by the nitric oxide reductase, NorB [5]. AniA is the major anaerobically induced outer membrane lipoprotein in *N. gonorrhoeae* [3,6] and is essential for the growth and survival of *N. gonorrhoeae* under oxygen-limiting conditions [2]. It has also been demonstrated that a strong immune response is generated against AniA in patients with various gonococcal infections [7]. *N. gonorrhoeae* has been shown to form biofilms *in vitro* and *in vivo* during cervical infection which may be associated with persistent gonococcal infection in asymptomatic women [8]. Expression of AniA is highly upregulated during biofilm growth of the gonococcus and anaerobic respiration mediated by AniA is essential to normal biofilm formation and is widespread in the substratum of gonococcal biofilms [9,10]. The cellular localisation of AniA is controversial with one model suggesting it is directed towards the periplasm [11], while a second model proposes that AniA is directed to the outside of the cell [12].

In this study we investigate the localisation of the AniA protein and examine the impact of glycosylation on the immune response to AniA as an adjunct to assessing its vaccine potential.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

All bacterial strains and conditions used in this study are listed in Supplementary Table 1 and associated text in Supplementary Experimental Procedures.

* Corresponding author. Fax: +61 7 5552 8098.
E-mail address: m.jennings@griffith.edu.au (M.P. Jennings).

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2.2. Construction and purification of glycoforms of FLAG-tagged AniA from N. meningitidis C311 strains

Strains expressing FLAG-tagged glycoforms of AniA were constructed as described in [4] with details provided in Supplementary Experimental Procedures.

2.3. Production of polyclonal antisera against glycoforms of AniA

Groups of 5 BALB/c mice were immunised on days 0, 21 and 28 with 5 μg of either FLAG-tagged AniA glycosylated with the DATDH monosaccharide, non-glycoslyated FLAG-tagged AniA or truncated FLAG-tagged AniA lacking the C-terminal glycosylation region. Following this immunisation schedule, terminal bleeds were collected and the serum from each mouse was harvested. These antisera were analysed by western blotting and ELISA as described in Supplementary Experimental Procedures.

2.4. Expression and purification of His-tagged truncated AniA proteins

DNA sequences encoding AniA with various truncations of the N- and C-terminal regions (Antigens 1–8) were amplified from *N. gonorrhoeae* 1291 genomic DNA using the primers described in Supplementary Table 2. Primers were designed based on the *aniA* sequence from the *N. gonorrhoeae* 1291 genome sequence (Broad Institute NGAG_01981). Digested PCR products were cloned into pET-15b (Novagen) to create the expression plasmid constructs described in Supplementary Table 1. *Escherichia coli* BL21 (DE3) cells were transformed with the plasmid constructs and the His-tagged proteins were expressed and purified using TALON Metal Affinity Resin (Clontech) according to the manufacturer's instructions.

2.5. Production of polyclonal antisera against recombinant His-tagged AniA proteins

8 New Zealand white rabbits were immunised on days 0, 21, 42 and 63 with 100 μg of each of the purified His-tagged truncated AniA proteins (Antigens 1–8). Following this immunisation schedule, terminal bleeds were collected and the serum from each rabbit was harvested.

2.6. Isolation of outer membrane proteins

Outer membrane proteins were isolated essentially as described by [13].

2.7. Immuno-Scanning Electron Microscopy (SEM) analysis

N. gonorrhoeae 1291 cells used for immuno-SEM were grown anaerobically with 2 mM $NaNO_2$. Samples were incubated with pre-immune rabbit serum (1:1000 dilution) or anti-AniA polyclonal rabbit serum (raised against recombinant His-tagged AniA – Antigen 5) (1:1000 dilution) followed by incubation with anti-rabbit gold conjugated antibody. The images were collected by scanning EM on the Hitachi S4800.

2.8. Trypsin digestion of surface exposed proteins

Surface exposed proteins of *N. gonorrhoeae* 1291 were digested according to Rodriguez-Ortega et al. [14], with modifications as described in Supplementary Experimental Procedures.

2.9. Nitrite utilisation assays

Antisera raised against recombinant His-tagged AniA proteins (Antigens 1–8) were used in nitrite utilisation assays as described in Supplementary Experimental Procedures.

3. Results

3.1. Investigation of the immunogenicity of the AniA glycoforms in N. meningitidis C311

Typically bacterial nitrite reductases are periplasmic soluble enzymes [15]. The core region of the AniA nitrite reductase from *N. meningitidis* C311 is homologous to other characterised bacterial nitrite reductases, containing a number of conserved residues essential to the function of the enzyme, while the N- and C-termini of this protein appear to be distinct (Supplementary Fig. 1). The N-terminal region of AniA from *N. meningitidis* and *N. gonorrhoeae* contains a lipoprotein signal peptidase II-processing site (ALAAC) and regions of homology to two other *Neisseria* outer membrane lipoproteins, Lip/H.8 and Laz [3] (Supplementary Fig. 2A – underlined). This suggests that AniA from these *Neisseria* species, similar to Lip/H.8 and Laz, is an outer membrane protein anchored via this lipid modified N-terminus. AniA has been identified as a glycoprotein and the C-terminus encompasses the glycosylated serine residues which are present within the sequence AASAP [4] (Supplementary Fig. 2A).

To investigate the role of the glycosylation of AniA in the immune response to this protein, mice were immunised with AniA proteins purified from different *pgl* mutant strains and the reactivity of the resulting antisera was analysed against outer membrane proteins isolated from the wild-type strain, as well as selected *pgl* mutants (Fig. 1A). Mice were immunised with AniA purified from C311 *pglA*, which was glycosylated with a truncated glycan consisting of the 2,4-diacetimido-2,4,6-trideoxyhexose (DATDH) monosaccharide, with non-glycosylated AniA purified from C311 *pglL*, as this mutant is missing the *pglL* oligosaccharyltransferase, and with a truncated form of AniA completely lacking the C-terminal glycosylated region. Antisera raised against AniA glycosylated with the DATDH monosaccharide were the most reactive with AniA from the *pglA* mutant strain (Fig. 1B). Antisera generated against non-glycosylated AniA (Fig. 1C) and against the truncated form of AniA, lacking the C-terminal glycosylation region (Fig. 1D), were found to react equally with all glycoforms of AniA.

The reactivity of antisera from individual mice immunised with the different glycoforms of AniA was assessed in an ELISA against a recombinant, truncated form of AniA expressed in *E. coli*, lacking both the N-terminus and the glycosylated C-terminus (See Supplementary Fig. 2B – Antigen 5). Mice immunised with the truncated form of AniA were found to have the highest mean titre against the truncated AniA protein whereas those mice immunised with AniA glycosylated with the monosaccharide were found to have the lowest mean titre (Fig. 1F). The difference between the mean titres of these two groups was statistically significant (p value = 0.0433).

3.2. AniA is expressed on the cell surface of N. gonorrhoeae

It has been shown that AniA is required for the growth and survival of *N. gonorrhoeae* under oxygen-limiting conditions [2] and is essential for optimal biofilm formation in this species [9]. Furthermore, antibodies against AniA have been detected in patients with various gonococcal infections [7]. While 34% of *N. meningitidis* clinical isolates contain a frame-shift mutation within the AniA coding sequence resulting in loss of expression, 100% of *N. gonorrhoeae* strains examined were predicted to express full length AniA