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Original article

Immunogenicity and structural characterisation of an in vitro folded meningococcal siderophore receptor (FrpB, FetA)

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Abstract

The iron-limitation-inducible protein FrpB of *Neisseria meningitidis* is an outer-membrane-localized siderophore receptor. Because of its abundance and its capacity to elicit bactericidal antibodies, it is considered a vaccine candidate. Bactericidal antibodies against FrpB are, however, type-specific. Hence, an FrpB-based vaccine should comprise several FrpB variants to be capable of providing broad protection. To facilitate the development of a meningococcal subunit vaccine, we have established a procedure to obtain large quantities of the protein in a native-like conformation. The protein was expressed without its signal sequence in *Escherichia coli*, where it accumulated in inclusion bodies. After in vitro folding, the protein was biochemically, biophysically and biologically characterised. Our results show that in vitro folded FrpB assembles into oligomers, presumably dimers, and that it induces high levels of bactericidal antibodies in laboratory animals.

Keywords: In vitro folding; *Neisseria meningitidis*; Outer membrane protein; Vaccine

1. Introduction

Neisseria meningitidis (meningococcus) is a Gram-negative bacterium that is capable of causing life-threatening septicaemia and meningitis. Effective vaccines based on the capsular polysaccharides of the majority of pathogenic serogroups have been developed [1]. However, a vaccine against serogroup B meningococci, which is responsible for most disease cases in North America and Europe, is not available. Development of a polysaccharide-based vaccine against serogroup B meningococci is a cumbersome task, since the type B capsule resembles human self-antigens [2]. Outer membrane vesicle (OMV) vaccines have been successfully used to combat serogroup B epidemics, but these vaccines offer little cross-protection although recent data suggest that adjustment of the procedure used to produce OMVs may solve this problem [3].

An attractive alternative for OMV vaccines would be a subunit vaccine consisting of outer membrane proteins (OMPs). Due to its abundance and its capacity to induce bactericidal antibodies, the general diffusion porin PorA is the most

Abbreviations: CFU, colony-forming units; EDDHA, ethylenediamine di-*o*-hydroxyphenylacetic acid; FrpB, Ferric-repressed protein B; FetA, Ferric enterobactin transport protein A; GMT, geometric mean titre; HBSS, Hank's balanced salt solution; HPI, hexagonally packed intermediate; IPTG, isopropyl-β-D-thiogalactopyranoside; MH, Mueller Hinton; OD, optical density; OMP, outer membrane protein; OMV, outer membrane vesicle; PMSF, phenylmethylsulfonyl fluoride; RT, room temperature; SB-12, n-dodecyl-*N*,*N*-dimethyl-1-ammonio-3-propanesulfonate; STEM, scanning transmission electron microscopy; TEM, transmission electron microscopy; TSB, tryptic soy broth.

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extensively studied OMP with regard to its vaccine potential. PorA induces high levels of bactericidal antibodies, which is believed to be the best correlate for protection against serogroup-B meningococci [4]. Unfortunately, however, the PorA protein shows extensive antigenic variation among strains, which complicates the development of a broad-spectrum vaccine. In the search for antigenically more conserved OMPs, proteins involved in iron acquisition are under investigation. The meningococcus is remarkably efficient in scavenging iron from its host by using a wide variety of OMP receptors, the expression of which is regulated by the availability of iron. Together, these receptors enable the meningococcus to use various human iron-loaded proteins, such as lactoferrin, transferrin and haemoglobin [5]. Furthermore, although the meningococcus does not appear to produce its own siderophores, it has the ability to use siderophores secreted by other bacteria [6]. The major iron-limitation-induced OMP of *N. meningitidis* is a ~70-kDa protein designated FrpB (Ferric-repressed protein B) [7]. The FrpB protein of *Neisseria gonorrhoeae* was shown to be involved in the transport of enterobactin, a siderophore produced by *Escherichia coli*, and it was, therefore, renamed FetA (Ferric enterobactin transport protein A). Enterobactin transport by FrpB is specific and dependent on the energy-coupling protein TonB [8]. However, as the affinity of FrpB for enterobactin is relatively low, the possibility exists that another, enterobactin-related siderophore is the actual ligand of this protein in vivo.

FrpB is considered a promising vaccine candidate to protect against meningococcal disease. Although FrpB, like PorA, shows significant antigenic variation among strains [9], its ability to induce relatively high levels of bactericidal antibodies justifies a further analysis of this protein [10,11]. Here, we describe the production of large quantities of FrpB, which were used for structural and immunological analysis. It was suggested recently that a vaccine comprising five FrpB (FetA) variants and six variants of PorA should afford protection against a large panel of hyperinvasive meningococcal isolates [12]. Together with our previously developed method to obtain large amounts of correctly folded PorA [13,14], the current work enables the development of such a multivalent meningococcal subunit vaccine.

2. Materials and methods

2.1. Expression and isolation of FrpB

The FrpB protein used throughout this study was derived from *N. meningitidis* strain H44/76. The variable region of this particular FrpB was designated F3-3 [9]. The part of *frpB* encoding the mature protein (i.e., FrpB without its signal sequence) was PCR amplified from genomic DNA with the primer pair 5′-gctacatatggcagaaaataatcggaaggtc-3′ and 5′-gctaggatccttagaacttgtagttcacgcc-3′ containing *Nde*I and *Bam*HI restriction sites, respectively (underlined). The resulting product was cloned into pCRII-TOPO according to the instructions of the manufacturer (Invitrogen). From there, the *frpB* fragment was obtained by digestion with *Nde*I and *Bam*HI and cloned downstream of the inducible T7 promoter of plasmid pET11a (Novagen), which was digested with the same restriction enzymes. This resulted in the plasmid pET11a-*frpB*. *E. coli* strain BL21(DE3) [15], which contains the gene for T7 RNA polymerase under an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter, was transformed with the plasmid. An overnight culture of the resulting strain was diluted 1:50 into fresh Luria–Bertani (LB) medium supplemented with 100 μg/ml ampicillin. When an optical density of 0.6 at 550 nm (OD_{550}) was reached, protein expression was induced by the addition of 1 mM IPTG (Sigma). After 2.5 h of growth, the bacteria were harvested by centrifugation and washed with 0.9% (w/v) NaCl. Cell pellets were stored at −20 °C. FrpB was present in inclusion bodies, which were isolated as described [16].

2.2. In vitro folding of FrpB

Inclusion bodies were solubilized in 20 mM Tris–HCl, 100 mM glycine (pH 8.0) containing either 6 M guanidinium hydrochloride or 8 M urea. After incubation on a turning wheel for 1.5 h, residual membrane fragments were removed by centrifugation for 1 h at 356,000 × *g*. The protein concentration in the supernatant was determined with the Pierce protein assay (Pierce, Rockford, IL, USA) using BSA (Sigma) as a standard. In vitro folding of FrpB was initiated by 20-fold dilution of fresh solubilized inclusion bodies (50 mg/ml) in 27 mM ethanolamine (pH 10.8) containing 0.5% (w/v) n-dodecyl-*N*,*N*-dimethyl-1-ammonio-3-propanesulfonate (SB-12) (Fluka, Buchs, Switzerland). Prior to use, the detergent was dissolved in methanol/chloroform (1:1) and purified by passage over an Al_2O_3 column [16]. All in vitro folding experiments with FrpB were initiated at room temperature (RT), and the protein was stored at 4 °C until analysis. When appropriate, buffers were exchanged using a PD-10 desalting column (Amersham). After exchange, some aggregates were observed, which were removed by filtration through a filter with a pore size of 0.22 μm (Millipore).

2.3. SDS–PAGE and Western blotting

Proteins were separated by standard sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). To detect folded forms of FrpB, an SDS–PAGE system that we refer to as semi-native SDS–PAGE was used [17]. In this technique, to prevent denaturation of the folded proteins the slab gels are devoid of SDS, while the amount of SDS present in the electrode buffer and loading buffer depend on the SDS sensitivity of the protein of interest and is empirically determined for each protein. For FrpB, the electrode and loading buffers contained 0.01% and 0.075% (w/v) SDS (end concentration), respectively. The samples were kept at RT before loading. To denature the protein, a loading buffer with 2% (w/v) SDS (end concentration) was used, and these samples were boiled for 2 min before loading. The samples were loaded on polyacrylamide gels composed of a stacking gel with 3% (w/v) acrylamide and a 7% (w/v) resolving gel.