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Original article

The *Moraxella* IgD-binding protein MID/Hag is an oligomeric autotransporter

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Abstract

The immunoglobulin D (IgD)-binding protein MID/Hag of the human respiratory pathogen *Moraxella catarrhalis* is an outer membrane protein of approximately 200 kDa belonging to the autotransporter family. MID also functions as an adhesin and hemagglutinin. In the present paper, the ultrastructure of MID was mapped. Using a series of *Escherichia coli* transformants, the last 210 aa of the C-terminal region were shown to translocate protein MID through the outer membrane suggesting that MID has a β-barrel structure comprising of 10 transmembrane β-sheets. Electron microscopy mapping with gold-labelled specific antibodies, and partial unravelling using guanidine hydrochloride showed that the rest of the MID protein forms an approximately 120 nm long, fibrillar structure in which the individual monomers fold back on themselves to expose a globular distal domain at their tips comprising both the IgD-binding (MID962–1200) and adhesive (MID764–913) regions. This positions their N-termini close to the C-terminal membrane spanning domains. Mass measurements by scanning transmission electron microscopy (STEM) verified that the MID molecule is an oligomer.

Keywords: Adhesin; MID; *Moraxella catarrhalis*

1. Introduction

After *Haemophilus influenzae* and *Streptococcus pneumoniae*, *Moraxella catarrhalis* is the third most common bacterial agent causing otitis media in children [1–3]. In adults and the elderly, *M. catarrhalis* causes lower respiratory tract infections, particularly in those patients with predisposing conditions such as chronic obstructive pulmonary disease (COPD).

The *M. catarrhalis* IgD-binding protein MID (also designated as hemagglutinin; Hag (for a review see Ref. [4])) has a molecular mass of approximately 200 kDa. However, native purified MID is an oligomeric complex as indicated by gel filtration [5] and ultracentrifugation [6]. MID exhibits unique IgD-binding properties and binds to both soluble and membrane-bound IgD on B cells. The smallest fragment with preserved IgD-binding capacity comprises 238 aa residues (MID962–1200) [6]. Ultracentrifugation experiments revealed that the native recombinant MID962–1200 fragment is a tetramer. Interestingly, tetrameric MID962–1200 binds IgD more than 20-fold more efficiently than the monomeric form, suggesting that a tetrameric structure of MID962–1200 is crucial for optimal IgD-binding [6]. MID is also a hemagglutinin and adhesin that binds to type II alveolar epithelial cells [7,8]. The adhesive part of MID is found within MID764–913 located upstream of the IgD-binding site. MID has sequence similarities with the *Yersinia enterocolitica* outer membrane protein (OMP) YadA and the *H. influenzae* adhesin (Hia), which

Abbreviations: MID, *Moraxella* IgD-binding protein; MPL, mass-per length; OMV, outer membrane vesicles; OMP, outer membrane protein; STEM, scanning transmission electron microscopy; TEM, transmission electron microscopy.

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belongs to the family of trimeric autotransporters [9–12]. This subgroup has also been referred to as the oligomeric coiled coil adhesin (oca) family.

The goal of this study was to map the ultrastructure of MID using electron microscopy and translocation analyses. We show that the MID oligomer has an approximately 120 nm long membrane-bound fibrillar structure with a globular domain at the distal end responsible for both the IgD-binding and the adhesive properties. Detailed sequence analysis of the C-terminal end suggests that MID belongs to the trimeric autotransporter family. Translocation analyses of C-terminal recombinant MID fragments of different lengths suggest that the MID translocator domain comprises 210 amino acids folding into a β-barrel structure comprising 10 transmembrane β-sheets. Furthermore, mass measurements by scanning transmission electron microscopy (STEM) showed that MID is a multimer.

2. Materials and methods

2.1. *Bacteria and* M. catarrhalis *outer membrane vesicle (OMV) preparation*

M. catarrhalis RH4 and the isogenic MID, UspA1, UspA2, double and triple mutants were constructed as described [6,7,13]. *M. catarrhalis* RH4 was cultured overnight and pelleted at 11,000 × g for 10 min at room temperature. Thereafter, the OMVs were collected from the supernatant by membrane filtration under magnetic steerage (Amicon, Beverly, MA) at 4 °C using a 100,000 Da Mw cut off ultrafiltration membrane (Millipore, Billerica, MA, USA).

2.2. *DNA constructs and recombinant proteins*

Eight fusion proteins were constructed using PCR and Overlap Extension PCR and MID764–2139 was amplified in a single step PCR (Fig. 3A). The final products were cloned into pET26b (Novagen, Darmstadt, Germany) and protein expression was monitored by flow cytometry, SDS-PAGE and Western blots as described [7]. All recombinant proteins used for antibody purification (Fig. 2) were recombinantly produced in *E. coli* [6,7]. The synthetic peptide consisting of the KTRAAS repeat (in MID775–822 and MID1023–1053) was also used [5,7,14].

2.3. *Antisera and immunopurification of antibodies*

Rabbits were immunized with purified recombinant MID proteins, synthetic peptide (KTRAAS), or recombinant UspA1 150–770 [7,13]. Resulting antisera were adsorbed on truncated MID fragments conjugated to Sepharose [13]. All antibodies were tested for specificity after purification using ELISA. Moreover, the antibodies detected MID in OMVs as revealed by Western blot. No cross reactivity was observed except for the UspA1 serum that had not been purified, which reacted with both recombinant UspA1 150–770 and A2 230–539 as verified by Western blotting [15].

2.4. *Transmission electron microscopy (TEM) and partial unravelling*

The location of different epitopes on MID was analysed by negative staining and electron microscopy as described [16]. Immunopurified antibodies were labelled with 5 nm colloidal thiocyanate gold [17]. OMV preparations were mixed with the antibodies and 5 μl aliquots were adsorbed onto carbon-coated grids for 1 min, washed, and stained on two drops of 0.75% uranyl formate. The grids were rendered hydrophilic by glow discharge at low pressure in air. A Jeol JEM 1230 electron microscope (JEOL, Tokyo, Japan) operated at 60 kV accelerating voltage was used. Images were recorded with a Gatan Multiscan 791 CCD camera (Gatan, Pleasanton, CA). To achieve partial unravelling bacteria and/or OMVs were mixed with guanidine hydrochloride to a final concentration of 0.5, 1, 2, 3, and 4 M, respectively, and incubated at room temperature for 10 min followed by negative staining as described above.

2.5. *Scanning transmission electron microscopy (STEM)*

The *Moraxella* OMV sample was diluted 10 times with buffer, and 7 μl aliquots were adsorbed for 60 s to glow-discharged thin carbon films that spanned a thick fenestrated carbon layer covering 200-mesh/inch, gold plated copper grids. The grids were then blotted, washed on six drops of quartz double-distilled water, and freeze-dried at $-80\,^{\circ}C$ and 5×10^{-8} Torr overnight in the microscope. Tobacco mosaic virus particles (TMV; kindly supplied by R. Diaz-Avalos of the Institute of Molecular Biophysics at Florida State University) served as mass standard. This sample was similarly adsorbed to separate microscopy grids, washed with 100 mM ammonium acetate prepared with quartz double-distilled water and air-dried.

A Vacuum Generators STEM HB-5 interfaced to a modular computer system (Tietz Video and Image Processing Systems, Gauting, Germany) was employed. Series of 512 pixel, dark-field images were recorded from the unstained samples at an accelerating voltage of 80 kV and a nominal magnification of 200,000. The recording dose ranged from 320 to 800 electrons/nm^2.

The principle of mass measurement by STEM is detailed in Ref. [18]. The digital images recorded were evaluated using the program INTMAN of the IMPSYS package [19]. Accordingly, the filament segments were not automatically tracked but defined by manually positioned vectors. The total electron scattering within an integration box matched to their length and width was then determined, the background scattering of the carbon support film subtracted and the average mass-per-length (MPL) was calculated [20]. Beam-induced mass-loss was estimated by linear regression analysis of the average MPL values measured at the various imaging doses. Finally, the data were scaled according to the mass measured for TMV, displayed in a histogram and described in a single Gauss curve. The number of MID monomers present was estimated: (1) by comparing the MPL expected for an alpha helical protein, (2) by comparing the MPL expected for an alpha helical protein but considering the possible effect of coiled coil formation and (3) by