Translocator Proteins in the Two-partner Secretion Family Have Multiple Domains*

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Neeraj K. Surana1‡, Amy Z. Buscher2‡, Gail G. Hardy3‡, Susan Grass3‡, Thomas Kehl-Fie1‡, and Joseph W. St. Geme, III1‡§

From the †The Edward Mallinckrodt Department of Pediatrics and the Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110 and the §Departments of Pediatrics and Molecular Genetics and Microbiology, Duke University Medical Center, Durham, North Carolina 27710

The two-partner secretion pathway in Gram-negative bacteria consists of a TpsA exoprotein and a cognate TpsB outer membrane translocator protein. Previous work has demonstrated that the TpsB protein forms a β-barrel structure with pore forming activity and facilitates translocation of the TpsA protein across the outer membrane. In this study, we characterized the functional domains of the Haemophilus influenzae HMW1B protein, a TpsB protein that interacts with the H. influenzae HMW1 adhesin. Using c-Myc epitope tag insertions and cysteine substitution mutagenesis, we discovered that HMW1B contains an N-terminal surface-localized domain, an internal periplasmic domain, and a C-terminal membrane anchor. Functional and biochemical analysis of the c-Myc epitope tag insertions and a series of HMW1B deletion constructs demonstrated that the periplasmic domain is required for secretion of HMW1 and that the C-terminal membrane anchor (HMW1B-(234–545)) is capable of oligomerization and pore formation. Similar to our observations with HMW1B, examination of a (234–545) is capable of oligomerization and pore formation. Sim-

and are known or presumed virulence factors in all cases, generally functioning as adhesins or hemolysins (5).

Most studies of the TPS pathway have focused on characterizing TpsA proteins, leaving important questions regarding the mechanism of activity of the TpsB proteins. According to the prevailing model for the TPS pathway, both the TpsA protein and the TpsB protein are delivered to the periplasm by the Sec apparatus. Subsequently, the TpsB protein inserts into the outer membrane and is believed to form a β-barrel with a central pore. The TpsA protein then interacts with the TpsB protein via a conserved N-terminal "secretion domain." Following this interaction, the TpsA protein is translocated across the outer membrane, presumably through the TpsB pore. Interestingly, the TpsA secretion domain and the TpsB protein are not functionally interchangeable between unrelated TPS systems, suggesting that the interaction between TpsA and TpsB has a high level of specificity (6).

In the current model, the TpsB protein has at least two distinct functions. First, the TpsB protein must recognize and interact with the secretion domain of the TpsA periplasmic intermediate. Second, the TpsB protein must form a pore to allow for secretion of the TpsA protein. Regarding these functions, previous work with FhaC, ShlB, and HMW1B has suggested that the entire TpsB sequence contributes to β-barrel formation with a total of 19–22 transmembrane β-strands and 9–11 periplasmic loops and that TpsB proteins form pores with a diameter of 1–3 nm (3, 7–10).

The H. influenzae hmw1 locus consists of three genes, designated hmw1A, hmw1B, and hmw1C (11). The hmw1A gene encodes the HMW1 adhesin, the hmw1B gene encodes the HMW1B outer-membrane translocator, and the hmw1C gene encodes a protein with possible glycosyltransferase activity (3, 10–12). HMW1 is synthesized as pre-pro-protein and undergoes two cleavage events prior to surface localization. First, amino acids 1–68 direct the pre-pro-protein to the Sec apparatus and are cleaved. Second, amino acids 69–441 (containing the TPS secretion domain) direct the pro-protein to HMW1B and are cleaved (3, 10, 13, 14). HMW1B forms a tetrameric channel and facilitates translocation of mature HMW1 across the outer membrane (10). Mature HMW1 remains noncovalently associated with the bacterial surface, with small amounts released into the culture supernatant (3, 13).

In this study, we characterized the functional domains of the H. influenzae HMW1B TpsB protein. We found that HMW1B has an extracellular N terminus, a large internal periplasmic domain, and a C-terminal membrane anchor with 10 predicted β-strands. Further analysis revealed that the periplasmic domain is required for recognition of HMW1 and that the C-terminal membrane anchor forms a tetrameric β-barrel with pore-forming ability important for translocation of HMW1. In additional work, we found that the C terminus of the β. pertussis FhaC TpsB protein (residues 232–584) was sufficient for pore

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2 Present address: Dept. of Biology, Washington University, St. Louis, MO 63110.

3 Present address: Dept. of Biology, Indiana University, Bloomington, IN 47401.

4 To whom correspondence should be addressed: Dept. of Pediatrics, Duke University Medical Center, Children’s Health Center, Rm. T901, DUMC 3352, Durham, NC 27710. Tel.: 919-681-4080; Fax: 919-6812714; E-mail: j.stgeme@duke.edu.

5 The abbreviations used are: TPS, two-partner secretion; AMS, 4-acetamido-4’-maleimidyldistibene-2’-disulfonic acid; OGM, Oregon Green 488 maleimide; DDM, n-dodecyl β-maltoside; HAT, His affinity tag.
Domain Architecture of TpsB Proteins

forming activity, raising the possibility that all TpsB proteins have a modular structure with discrete domains involved in TpsA protein recognition and pore formation.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions—Escherichia coli DH5α (Invitrogen) and E. coli BL21(DE3) (15) are laboratory strains that have been described previously. E. coli BL21(DE3)omp8 is a porin-deficient strain that lacks outer membrane proteins OmpF, OmpC, OmpA, and LamB (16) and was a generous gift from V. Braun (University of Tubingen, Tubingen, Germany). B. pertussis strain Tohama I was a generous gift from W. Goldman (Washington University, St. Louis) (17).

Plasmids that were used in this study and have been described previously include pHMW1-15, which contains the entire hmwlI locus cloned into pT7-7 (18), pHAT::Hia1–49, which contains the coding sequence for the Hia signal peptide upstream of the HAT epitope in pHAT10 (10), pHAT::P-49/OmpA21–191, which contains the coding sequence for the Hia signal peptide upstream of the HAT epitope in pHAT10 (10), pHAT::HMW1B, which contains the coding sequence for the Hia signal sequence, the HAT epitope, and the indicated HMW1B residues. To construct these plasmids, pHMW1-15 was used as a template to amplify the coding sequence for the relevant HMW1B residues, engineering a Sall site at the 5′ end and a BamHI site at the 3′ end. The resulting products were digested with Sall and BamHI and ligated into Sall-BamHI-digested pHAT::Hia1–49.

The plasmids pHAT::HMW1B27–545, pHAT::HMW1B107–545, pHAT::HMW1B121–545, and pHAT::HMW1B234–545 encode the Hia signal sequence, the HAT epitope, and the indicated HMW1B residues. To construct these plasmids, pHMW1-15 was used as a template to amplify the coding sequence for the relevant HMW1B residues, engineering a Sall site at the 5′ end and a BamHI site at the 3′ end. The resulting products were digested with Sall and BamHI and ligated into Sall-BamHI-digested pHAT::Hia1–49.

To construct these plasmids, chromosomal DNA from B. pertussis strain Tohama I was used as template to amplify the coding sequence for the relevant FhaC residues, engineering a Sall site at the 5′ end and a BamHI site at the 3′ end. The resulting products were digested with Sall and BamHI and ligated into Sall-BamHI-digested pHAT::Hia1–49. All constructs were examined by nucleotide sequencing to ensure that PCR products were free of unintended mutations.

Cell Fractionation and Protein Analysis—Whole-cell sonicates were prepared by resuspending bacterial pellets in 10 mM HEPES (pH 7.4) and sonicating to clarify. Outer membrane proteins were recovered on the basis of Sarkosyl insolubility as described by Carlone et al. (23).

For dot immunoblot analysis of outer membranes, aliquots of outer membrane protein preparations were applied to a nitrocellulose filter using a dot-blot manifold apparatus. Samples were incubated for 30 min and then pulled through the filter by vacuum suction. Protein was detected by a standard Western blot analysis.

To assess extracellular secretion of HMW1-(69–441), cells were resuspended from a plate to an A600 of 0.6–0.9 and then diluted to an A600 of 0.3. After incubation at room temperature for 45 min, bacteria were harvested by centrifugation. The cell pellet was resuspended in 10 mM HEPES (pH 7.4) and sonicated to clarify. Culture supernatants were precipitated by adding trichloroacetic acid to a final concentration of 10% (v/v), incubating for 10 min at 4 °C, and then centrifuging at 15,600 × g at 4 °C for 10 min. The trichloroacetic acid-precipitated proteins were resuspended in 0.2 M Tris (pH 9.0) and resolved by SDS-PAGE.

Western blots were performed with a mouse monoclonal antiserum raised against the c-Myc epitope (monoclonal antibody 9E10; Roche Applied Science), a guinea pig polyclonal antiserum raised against HMW1 residues 1–591, a guinea pig polyclonal antiserum raised against the mature form of HMW1, or a rabbit polyclonal antiserum raised against HMW1B. Appropriate secondary antibodies conjugated to horseradish peroxidase (Sigma) were used, and detection of antibody reactivity was accomplished by incubation of the membrane in a chemiluminescent substrate solution (Pierce) and exposure to film.

Site-directed Fluorescence Labeling—Site-directed fluorescence labeling of cysteine residue substitutions was carried out as described previously (24). Briefly, E. coli BL21(DE3) derivatives harboring pHAT10:HMW1B cysteine substitutions were resuspended from a plate to A600 ~0.8, back diluted 1:25, and grown to A600 ~1.0. Cells were harvested and resuspended in Buffer A (100 mM potassium sulfate, 50 mM potassium phosphate (pH 8.0)) and divided into 2 equal aliquots. Freshly prepared 4-acetamido-4′-maleimidylstilbene-2-2′-disulfonic acid (AMS; Molecular Probes) was added to 1 aliquot to a final concentration of 100 μM. After incubation at 25 °C for 7 min, both aliquots were washed three times and resuspended in Buffer A. Oregon Green 488 maleimide (OGM, Molecular Probes) was added to both aliquots to a final concentration of 40 μM, and samples were incubated at 25 °C in the dark for 15
min. Reactions were quenched by addition of 6 mM β-mercaptoethanol, and samples were washed three times. Samples were resuspended in 10 mM HEPES (pH 7.4) supplemented with Complete Mini Protease Inhibitor mixture tablets (Roche Applied Science), and outer membrane proteins were prepared on the basis of Sarkosyl insolubility (23). Outer membrane proteins were solubilized with 1% Eluent (Calbiochem), 20 mM HEPES (pH 8.0), 150 mM NaCl by rocking overnight at 4 °C. Insoluble material was pelleted by centrifugation at 12,000 × g for 30 min at 4 °C. The soluble outer membrane fraction was incubated at 25 °C for 30 min with Talon affinity resin (Clontech) pre-equilibrated with 1% Eluent, 20 mM HEPES (pH 8.0), 150 mM NaCl. The Talon resin was washed three times, resuspended in Laemmli buffer, and boiled for 5 min. Proteins were resolved via SDS-PAGE. The fluorescence profile was obtained by scanning gels with a Typhoon 9410 Variable Mode Imager (Amersham Biosciences) utilizing the Blue Fam Laser Blue 2 (excitation wavelength of 488 nm). Fluorescent values for each band were obtained using ImageQuant software (Amersham Biosciences). The same gels were then stained with Coomassie Blue and scanned using an Alpha Innotech MultiImager (San Leandro, CA), performing densitometry using the AlphaEase system software (San Leandro, CA). The fluorescence value for each HMW1B protein was normalized to its protein level as determined by Coomassie Blue staining. Subsequently, the fluorescence profile (normalized OGM signal without AMS pretreatment) was obtained by scanning gels with a Typhoon 9410 Variable Mode Imager (Amersham Biosciences) utilizing the Blue Fam Laser Blue 2 (excitation wavelength of 488 nm). Fluorescent values for each band were obtained using ImageQuant software (Amersham Biosciences). The same gels were then stained with Coomassie Blue and scanned using an Alpha Innotech MultiImager (San Leandro, CA), performing densitometry using the AlphaEase system software (San Leandro, CA). The fluorescence value for each HMW1B protein was normalized to its protein level as determined by Coomassie Blue staining. Subsequently, the fluorescence ratio (normalized OGM signal without AMS pretreatment divided by the normalized OGM signal with AMS pretreatment) was calculated for each HMW1B derivative to obtain the fold reduction in OGM labeling due to pretreatment to AMS. All HMW1B mutants were analyzed a minimum of three times. Data were analyzed for statistical significance using the z-test. Criteria for periplasmic residues included a fluorescence ratio ≤6 and a z-test value ≤0.05. Criteria for surface-localized residues included a fluorescence ratio ≥10 and a z-test value ≤0.05.

Flow Cytometry—Flow cytometry was performed as described previously (12), using polyclonal antiserum raised against mature HMW1, a mouse monoclonal antiserum raised against the c-Myc epitope (monoclonal antibody 9E10; Roche Applied Science), or a mouse antiserum against the HAT epitope.

Adherence Assays—Adherence assays were performed with Chang epithelial cells (Wong-Kilbourne derivative, clone 1-5c-4, human conjunctiva; ATCC CCL20.2) as described previously (18).

Protein Purification—HMW1B-(234–545) was purified using the conditions for purifying wild type HMW1B, as described previously (10). Briefly, outer membrane proteins from overnight cultures of BL21(DE3)omp8::pHAT::HMW1B234–545 were recovered on the basis of Sarkosyl insolubility (23). Outer membrane proteins were solubilized with 1% Eluent (Calbiochem), 20 mM HEPES (pH 8.0), 150 mM NaCl by rocking for 1 h at 25 °C. The insoluble outer membrane fraction was pelleted by centrifugation at 40,000 × g for 45 min at 4 °C. The soluble outer membrane fraction was loaded onto a column containing Talon beads (Clontech) and equilibrated with 0.1% n-dodecyl β-D-maltoside (DDM), 20 mM HEPES (pH 8.0), 150 mM NaCl. HMW1B-(234–545) was purified using a linear gradient of imidazole. The fractions containing HMW1B-(234–545) were concentrated using an Amicon Ultra 10 concentrator (Millipore) and further purified by size-exclusion chromatography using a HiPrep 16/60 Sephacryl S-200 HR column (Amersham Biosciences) that had been equilibrated previously with 0.1% DDM, 20 mM HEPES (pH 8.0), 150 mM NaCl. Fractions containing HMW1B-(234–545) were concentrated using an Amicon Ultra 10 concentrator (Millipore). The N-terminal β-barrel domain of OmpA was purified in a similar manner, starting with a culture of BL21(DE3) harboring pHAT::P-49/OmpA21–191.

Heat Modifiability Assay—Samples were incubated in sample buffer for 5 min at either 25 or 100 °C prior to loading on a 15% polyacrylamide gel. Electrophoresis was performed at 4 °C using pre-cooled running buffer (0.1% SDS, 25 mM Tris base, 192 mM glycine).

Circular Dichroism—CD spectra were obtained on a Jasco 600 CD spectrophotometer using a quartz cell with a 0.1-cm path length. Purified HMW1B-(234–545) was used at a concentration of 0.15 mg/ml. Five scans were performed at room temperature, and the contribution of buffer alone was subtracted. Secondary structure content was estimated using the CDSSTR algorithm on the DICHROWEB website (25, 26). Mean residue ellipticities were calculated considering a mean residue weight of 111.55 Da.

Liposome Swelling Assay—Liposome swelling assays were performed as described previously (10, 27).

Antibiotic Susceptibility Assay—Antibiotic susceptibility of E. coli strains was determined using the standard disk diffusion technique (28).

RESULTS

HMW1B Has Multiple Domains—In previous work, secondary structure analysis of HMW1B suggested a model with 22 transmembrane β-strands (3). To validate this model, we elected to map the topology of HMW1B experimentally. As a first step, we inserted the c-Myc epitope (EQKLISEEDL) in predicted periplasmic and extracellular loops, ultimately creating 21 different constructs with insertions in 18 of the predicted 22 loops (see Fig. 1). These constructs were then introduced into E. coli DH5α, allowing assessment of outer membrane fractions for the effect of the insertions on HMW1B stability and examination of whole bacteria for surface localization of the c-Myc epitope. Outer membrane fractions were analyzed by Western blot analysis using a polyclonal antiserum raised against HMW1B and by non-denaturing dot immunoblot assays using either an antiserum against HMW1B or an antibody against the c-Myc epitope. In addition, whole bacteria were examined by flow cytometry using an antibody against the c-Myc epitope. Criteria for concluding that the c-Myc epitope was surface-localized included detection of the tagged HMW1B protein in the outer membrane at wild type levels, detection of the c-Myc epitope by dot immunoblot analysis of outer membranes, and detection of the c-Myc epitope by flow cytometry. If the recombinant protein was present in the outer membrane at wild type levels and the c-Myc epitope was detectable by dot immunoblot analysis of outer membranes but was not detectable by flow cytometry, we concluded that the c-Myc epitope was on the periplasmic side of the outer membrane. If the recombinant protein was present in the outer membrane at wild type levels but was not detectable with an antibody against the c-Myc epitope by dot immunoblot analysis of outer membranes, we concluded that the location of the c-Myc epitope was ambiguous. As summarized in Fig. 1 and supplementary Table I, 17 of the 21 tagged proteins were expressed. Among these 17 proteins, the c-Myc epitope was localized on the bacterial surface in 6, in the periplasm in 6, and in an ambiguous location in 5. To
complement these results, we generated a recombinant protein with the HAT epitope at the immediate N terminus of mature HMW1B (HAT-HMW1B-(27–545)) and then expressed this protein in E. coli DH5α/H9251.

Based on flow cytometry, the HAT epitope in DH5α/H9251/pHAT-HMW1B-(27–545) was detectable on the bacterial surface (data not shown). Although tagging with the c-Myc epitope and the HAT epitope provided helpful information regarding the topology of HMW1B, this approach was not sufficient to generate a full topology map. As an additional strategy to map the HMW1B surface and periplasmic loops, we employed site-directed fluorescence labeling, a technique that uses small molecule thiol-reactive fluorescent (OGM) and nonfluorescent (AMS) probes differing in their ability to cross the E. coli outer membrane. This method relies on the fact that OGM is readily permeable across the outer membrane, labeling surface and periplasmic thiol groups equally well. In contrast, AMS crosses the outer membrane very slowly. Therefore, when used to pre-treat cells, AMS blocks OGM labeling of surface thiol groups. Given that mature HMW1B contains no cysteine residues, we substituted cysteine residues at 27 positions in HMW1B, corresponding to 21 of the 22 predicted loops.

Twenty five of the 27 mutant proteins were produced at wild type levels, and the remaining two mutant proteins were not detectable (suggesting degradation) (supplemental Table II). Four of the cysteine substitution residues did not label with OGM without solubilization of the protein from the outer membrane and therefore provided no information regarding HMW1B topology (supplemental Table II). Based on measurement of fluorescence ratios and calculations of statistical significance, eight substitution mutations were clearly localized to the periplasm, and five substitution mutations were clearly localized to the surface (Fig. 1 and supplemental Table II). The A29C, T95C, S109C, S177C, G243C, S256C, S478C, and T503C mutations yielded intermediate fluorescence ratios with large standard deviations, precluding definitive localization.

Results from analysis of c-Myc epitope insertions, cysteine substitution mutants, and the original computer-generated map of HMW1B were combined to generate an overall topology for HMW1B. As depicted in Fig. 1, this topology includes a surface-localized N terminus, a large internal periplasmic domain, and a C terminus composed of 10 β-strands. There are two regions (between residues 276 and 325 and between residues 483 and 526) where the nature of the predicted β-strands is unclear (i.e. transmembrane or nontransmembrane). Based on the premise that a pore-forming β-barrel must have at least 10 β-strands, in both of these regions we depicted the predicted β-strands as transmembrane. There are also two regions (between residues 219 and 276 and between residues 442 and 483) where either of two predicted β-strands could be depicted as transmembrane, maintaining the orientation of other experimentally derived regions. In these cases, we arbitrarily assigned one of the β-strands as transmembrane.

The N Terminus of HMW1B Is Required for Secretion of HMW1—To assess whether the c-Myc epitope tags or cysteine substitution mutations interfered with the function of HMW1B, we analyzed all mutant
HMW1B proteins in an *E. coli* derivative expressing HMW1 and HMW1C. Using immunoblot analysis of whole-cell sonicates, flow cytometry, and adherence assays, we examined the processing, secretion, and function of HMW1. All of the cysteine substitution mutants that were present in the outer membrane had wild type HMW1B function in these assays (supplemental Table II). However, five c-Myc epitope-tagged HMW1B constructs were associated with defects in the surface localization of HMW1 and can be divided into two distinct groups. The first group includes HMW1B derivatives with c-Myc epitope tags following residues 141, 149, and 157 and was associated with a lack of processing of the pro form of HMW1, a lack of HMW1 on the bacterial surface, a lack of adherence to Chang epithelial cells, and a lack of HMW1 in the culture supernatant, reflecting a lack of secretion of HMW1 (Table I). The second group includes HMW1B derivatives with c-Myc epitope tags following residues 276 and 325 and was associated with normal processing of HMW1 but a lack of HMW1 on the bacterial surface and a lack of adherence to Chang epithelial cells. Interestingly, increased levels of HMW1 were detected in the supernatants of the strains expressing these HMW1B constructs, indicating a defect in anchoring to the bacterial surface. These data suggest that the presence of the c-Myc epitope at certain locations in HMW1B interferes with processing, secretion, and/or anchoring of HMW1.

To explore further what region of HMW1B is critical for secretion of HMW1, we constructed a series of HMW1B deletion mutants. In previous work, we demonstrated that HMW1-(1–441) is necessary and sufficient for translocation through HMW1B, resulting in free release of HMW1-(69–441) (10). (HMW1-(1–68) represents the HMW1 signal peptide and is cleaved in the periplasm.) In this study, we co-expressed HMW1-(69–441) (10). (HMW1-(1–68) represents the HMW1 signal peptide and is cleaved in the periplasm.) In this study, we co-expressed HMW1-(69–441) with a plasmid encoding HMW1 and HMW1C. Using immunoblot analysis of whole-cell sonicates, flow cytometry, and adherence assays, we examined the processing, secretion, and function of HMW1. All of the cysteine substitution mutants that were present in the outer membrane had wild type HMW1B function in these assays (supplemental Table II). However, five c-Myc epitope-tagged HMW1B constructs were associated with defects in the surface localization of HMW1 and can be divided into two distinct groups. The first group includes HMW1B derivatives with c-Myc epitope tags following residues 141, 149, and 157 and was associated with a lack of processing of the pro form of HMW1, a lack of HMW1 on the bacterial surface, a lack of adherence to Chang epithelial cells, and a lack of HMW1 in the culture supernatant, reflecting a lack of secretion of HMW1 (Table I). The second group includes HMW1B derivatives with c-Myc epitope tags following residues 276 and 325 and was associated with normal processing of HMW1 but a lack of HMW1 on the bacterial surface and a lack of adherence to Chang epithelial cells. Interestingly, increased levels of HMW1 were detected in the supernatants of the strains expressing these HMW1B constructs, indicating a defect in anchoring to the bacterial surface. These data suggest that the presence of the c-Myc epitope at certain locations in HMW1B interferes with processing, secretion, and/or anchoring of HMW1.

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### Table 1: c-Myc-tagged HMW1B with defects in HMW1 processing and secretion

| Residue preceding c-Myc epitope | Immunoblot analysis of HMW1 | Fluorescence-activated cell sorter of surface-localized HMW1 | Adherence to Chang cells | HMW1 in supernatant |
|-------------------------------|---------------------------|----------------------------------------------------------|-------------------------|---------------------|
| Wild type                     | Wild type                 | +a                                                      | +                       | +                   |
| 141                           | Pro only                  | –c                                                      | –                       | –                   |
| 149                           | Pro only                  | –                                                       | –                       | –                   |
| 157                           | Pro only                  | –                                                       | –                       | –                   |
| 276                           | Wild type                 | –                                                       | –                       | –                   |
| 325                           | Wild type                 | –                                                       | –                       | –                   |

a Western analysis of whole-cell sonicates with an antiserum directed against HMW1. Wild type indicates presence of both pro-HMW1 (corresponding to residues 69–1536) and mature HMW1 (corresponding to residues 442–1536). Pro only refers to the detection of pro-HMW1 only.

b + indicates wild type levels.

c – indicates lack of detection.
d + + indicates greater than wild type levels.

### Figure 2: Role of the HMW1B N terminus in secretion of HMW1

Immunoblot analysis of whole-cell sonicates (lanes labeled with an S) and trichloroacetic acid-precipitated culture supernatants (lanes labeled with a T) generated from *E. coli* DH5α expressing HMW1B-(1–441) in the presence of the indicated region of HMW1B. Immunoblotting was performed with a polyclonal antiserum against HMW1-(1–591).

### Figure 3: HMW1B-(234–545) β-barrel conformation

A, Coomassie Blue-stained gel of purified HMW1B-(234–545). B, heat modifiability of purified HMW1B-(234–545). Samples were incubated at either 100 or 25 °C for 5 min, resolved on an SDS-polyacrylamide gel, and stained with Coomassie Blue. C, circular dichroism spectrum of HMW1B-(234–545). Purified HMW1B-(234–545) in 0.1% DDM, 20 mM HEPES (pH 8.0), 150 mM NaCl was analyzed at room temperature, and the average of five buffer-corrected scans is shown.
The molecular weight of a solute that results in 10% of mass of 38 kDa. HMW1B-(234–545) proteoliposome for arabinose was pore forming activity (data not shown). The specific activity of serum albumin were used as negative controls and demonstrated no with the apparent topology of HMW1B-(234–545), which contains 10 β-strands corresponding to 29% of the HMW1B-(234–545) sequence.

To determine whether HMW1B-(234–545) forms a multimer similar to wild type HMW1B (10), we examined purified HMW1B-(234–545) by size-exclusion chromatography. As shown in Fig. 4, HMW1B-(234–545) eluted with a retention volume consistent with a molecular mass of ~155 kDa, indicating that HMW1B-(234–545) forms an oligomer. Of note, the presence of dodecyl maltoside contributes to the hydrodynamic radius of the protein complex and makes it difficult to know exactly how many subunits are in the oligomer. As an additional method to define the quaternary state of HMW1B-(234–545), we analyzed the protein by blue native PAGE, which allows membrane proteins to be electrophoresed in the absence of detergents or denaturants by using Coomassie Blue G to maintain protein solubility (30). Similar to wild type HMW1B, HMW1B-(234–545) migrated at molecular masses consistent with a tetramer, a dimer, and a monomer (data not shown). Taken together, these data provide strong evidence that HMW1B-(234–545) forms an oligomer, most likely a tetramer.

In previous work, we demonstrated that wild type HMW1B has pore forming activity with a pore size of ~2.7 nm (10). To investigate whether the pore forming activity of HMW1B is contained within the C-terminal region of the protein, we examined HMW1B-(234–545) using the liposome swelling assay, an established method for studying the pore-forming properties of proteins (8, 10, 31–34). As shown in Fig. 5A, porin activity was directly proportional to the amount of HMW1B-(234–545) added to the liposomes. OmpA-(21–191) (purified from the bacterial growth fraction was determined to be 296 Da, close to the published value for OmpF (280 Da) (27). The best fit regression line for liposomes reconstituted with the outer membrane proteins (triangles) in the presence of solutes of various molecular weights. Swelling rates are presented as the percentage of the swelling rate obtained with l-arabinose as the solute. Solutes included l-arabinose (150 Da), d-galactose (180 Da), N-acetyl-D-glucosamine (221 Da), sucrose (342 Da), and l-raffinose (504 Da). The plot represents the average ± S.E. of three experiments. C, antibiotic susceptibility assays with the indicated antibiotics. Bars represent the mean zone of inhibition of bacterial growth ± S.D. Asterisks indicate a statistically significant difference between the bars joined by the brackets.

FIGURE 5. Pore activity of HMW1B-(234–545). A, swelling rates from liposome swelling assays obtained with HMW1B-(234–545) proteoliposomes using l-arabinose as the solute. A representative experiment is shown. Each data point represents the means ± S.E. of at least three readings. B, swelling rates from liposome swelling assays of proteoliposomes reconstituted with 2–30 μg of HMW1B-(234–545) (squares) or 0.5 μg of E. coli BL21(DE3) outer membrane proteins (triangles) in the presence of solutes of various molecular weights. Swelling rates are presented as the percentage of the swelling rate obtained with l-arabinose as the solute. Solutes included l-arabinose (150 Da), d-galactose (180 Da), N-acetyl-D-glucosamine (221 Da), sucrose (342 Da), and l-raffinose (504 Da). The plot represents the average ± S.E. of three experiments. C, antibiotic susceptibility assays with the indicated antibiotics. Bars represent the mean zone of inhibition of bacterial growth ± S.D. Asterisks indicate a statistically significant difference between the bars joined by the brackets.

To determine the pore size of HMW1B-(234–545), swelling rates were obtained for a variety of sugars differing in molecular weight and were expressed relative to swelling rates obtained for arabinose (Fig. 5B). As a positive control, liposomes were reconstituted with outer membrane proteins isolated from E. coli BL21(DE3) and examined in a similar manner. The molecular weight of a solute that results in 10% of the swelling activity of arabinose (M,0.1 Ara) can be used to determine the inner diameter of pore-forming proteins (27). The M,0.1 Ara for the liposomes reconstituted with the E. coli BL21(DE3) outer membrane fraction was determined to be 296 Da, close to the published value for OmpF (280 Da) (27). The best fit regression line for liposomes reconstituted with HMW1B-(234–545) revealed a M,0.1 Ara value of 482 Da. Taking into account the known M,0.1 Ara value for OmpF and the known pore diameter of OmpF (1.1 nm) (36), the calculated pore diameter for HMW1B-(234–545) was ~1.9 nm.

It is interesting that liposomal swelling assays demonstrated a pore size for HMW1B-(234–545) that is smaller than the pore size for wild type HMW1B. To further investigate this difference, we examined HMW1B-(234–545) and wild type HMW1B for pore activity in vivo by using an antibiotic susceptibility assay. In these assays, the zone of growth inhibition around a disk impregnated with antibiotic provides a measure of pore activity. In performing this experiment, we compared E. coli DH5α/HMW1B, DH5α/HMW1B-(234–545), and DH5α with vector alone. As shown in Fig. 5C, chloramphenicol (M, 323) inhibited growth of all three strains, suggesting that this antibiotic is able to freely
diffuse through the outer membrane, most likely through the porins. In contrast, rifampicin (M, 823) and vancomycin (M, 1485) selectively inhibited growth of organisms expressing HMW1B or HMW1B-(234–545), demonstrating that these proteins have pore activity in vivo (Fig. 5C). Of note, vancomycin (M, 1485) inhibited growth of organisms expressing HMW1B and organisms expressing HMW1B-(234–545) to different degrees (Fig. 5C). Thus, the antibiotic susceptibility assay revealed that HMW1B-(234–545) has pore activity and suggested that this pore activity is different from that of wild type HMW1B, paralleling observations with the liposome swelling assay.

The C Terminus of FhaC Harbors Pore Forming Activity—In light of our finding that HMW1B has a modular structure with a pore-forming domain at the C terminus, we wondered whether other TpsB translocator proteins have a similar modular architecture. To test this hypothesis, we generated constructs encoding B. pertussis FhaC-(31–585) (wild type, mature FhaC) and FhaC-(232–585). These proteins were expressed in E. coli DH5α and were examined for pore forming activity via in vivo antibiotic susceptibility assays. As shown in Fig. 6, rifampicin selectively inhibited the growth of organisms expressing either FhaC-(31–585) or FhaC-(232–585), demonstrating that FhaC-(31–585) and FhaC-(232–585) have pore forming activity in vivo. Vancomycin also inhibited the growth of organisms expressing FhaC-(31–585) or FhaC-(232–585) but had a greater effect on organisms expressing FhaC-(232–585). Therefore, similar to observations with HMW1B-(234–545), FhaC-(232–585) has pore forming activity, and this activity appears to differ from that observed for wild type FhaC.

DISCUSSION

In earlier work, we demonstrated that HMW1B forms a tetrameric pore that interacts directly with the HMW1 adhesin, the cognate exo-protein (10). In this study, we have used c-Myc epitope tag insertions and cysteine mutagenesis to map the topology of HMW1B experimentally. These studies demonstrated that HMW1B has an extracellular N terminus, a large internal periplasmic domain, and a C-terminal β-barrel with 10 β-strands. Functional characterization demonstrated that the internal periplasmic domain of HMW1B is required for secretion of HMW1 and that the C-terminal region of HMW1B forms a multimer with pore-forming ability. Further analysis revealed that the C terminus of the B. pertussis FhaC protein is sufficient for pore formation, raising the possibility that all TpsB proteins have a modular structure.

Our topology analysis of HMW1B made use of two independent experimental approaches that were ultimately correlated with computer-generated secondary structure predictions. Importantly, our findings with the c-Myc epitope insertions and cysteine substitutions were generally in accord with one another. Of the 18 instances where we had a c-Myc epitope insertion within a few residues of a cysteine substitution, we observed only one discrepancy in terms of implications for localization. In the region of discrepancy, the c-Myc epitope tag following residue 526 was detectable on the bacterial surface by antibody reactivity, whereas S532C was localized in the periplasm based on fluorescence labeling data. In considering this discrepancy, we concluded that both amino acids were located in the periplasm.

Our finding that HMW1B has a tripartite organization differs from published information on the topology of ShlB and FhaC, which have been reported to contain 19–20 transmembrane β-strands and no large periplasmic domain (7, 9). However, analysis of the topology of ShlB and FhaC has relied principally on sequence alignments and computer predictions and has been predicated on the underlying assumption that the majority of the protein sequence contributes to β-barrel formation. In contrast, in this study we defined the topology of HMW1B based primarily on our experimental results and secondarily on algorithms to predict transmembrane β-strands (38–40).

Examination of the topology of HMW1B suggested that the C-terminal domain may be sufficient for β-barrel formation. Indeed, analysis of purified HMW1B-(234–545) demonstrated that this region forms an oligomeric, pore-forming β-barrel. Interestingly, the pore activity of HMW1B-(234–545) as assessed by liposome swelling assays and in vivo antibiotic susceptibility assays was different from the pore activity of full-length HMW1B. It is unclear why liposome swelling assays revealed a slightly smaller pore size for HMW1B-(234–545) than for wild type HMW1B. One possibility is that the biphase nature of the relative swelling data obtained with wild type HMW1B precluded accurate determination of pore size (10). Alternatively, the discrepancy between the linear relative swelling data obtained with HMW1B-(234–545) and the biphase relative swelling data obtained with wild type HMW1B may underscore an important difference in pore properties between these two proteins. In support of this second possibility is the finding of increased uptake of antibiotics such as vancomycin by HMW1B-(234–545)-expressing organisms compared with HMW1B-expressing organisms, potentially reflecting the formation of channels with different stabilities. To determine whether pore formation by a C-terminal domain is a general finding among TpsB proteins, we examined FhaC using in vivo antibiotic susceptibility assays. Similar to observations with HMW1B, comparison of wild type FhaC and FhaC-(234–545) revealed that the C-terminal portion of FhaC was sufficient for pore activity and conferred increased susceptibility to vancomycin. The hypothesis that the C-terminal domains of TpsB proteins form pores with increased stability compared with wild type TpsB proteins is consistent with earlier observations with ShlB, where ShlBΔ126–200 was demonstrated in black lipid bilayer assays to form more stable channels than did wild type ShlB, as defined by the lack of rapid opening and closing events (9).

The idea that the TpsB C-terminal domain expressed alone forms a channel that is more stable than the channel formed by the wild type protein raises the question as to how the TpsB N terminus affects pore activity. Of note, our findings indicate that the immediate N terminus of HMW1B is located extracellularly, similar to published findings with FhaC (7). One possibility is that the N terminus of TpsB proteins forms a structured domain within the pore itself, effectively forming a “cork” in the pore, similar to the situation with E. coli FhuA and E. coli FepA (7, 9, 41–43). Indeed, the observation that TpsB proteins have a pore diameter of 1–3 nm suggests the need for some mechanism to regulate the
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pore, in order to avoid leaching of periplasmic contents and indiscriminate uptake of compounds from the extracellular environment. Interestingly, FhaC has been noted to undergo a conformational change with co-expression of filamentous hemagglutinin (7), raising the possibility that pore activity of TpsB proteins is regulated by interaction with the cognate TpsA protein in the periplasm prior to translocation of the TpsA protein. Analysis of deletion and insertion mutations in HMW1B provided strong evidence that the periplasmic domain of HMW1B is required for processing and secretion of HMW1, likely underscoring an interaction with the secretion domain of HMW1. Specifically, examination of a series of in-frame deletion mutants revealed that the region between residues 107 and 120 is critical for secretion of HMW1. Furthermore, insertion of the c-Myc epitope after residues 141, 149, and 157 abrogated the ability of HMW1B to secrete HMW1. In this context, it is noteworthy that N-terminal deletions in FhaC abolish the ability to secrete filamentous hemagglutinin (7), raising the possibility that FhaC has a large periplasmic domain involved in substrate recognition as well. With this information in mind, we speculate that the sequence variability within the periplasmic domain of TpsB proteins and the secretion domain of TpsA proteins may provide the molecular basis for the observed specificity within TPS systems (5, 6).

Interestingly, two of the c-Myc epitope tag insertion mutants (insertions following residues 276 and 325) were capable of secreting HMW1, as evidenced by an abundant amount of HMW1 detected in the supernatant. However, the recombinant strains with these mutants had no HMW1 on the bacterial surface, indicating a defect in HMW1 anchoring to the bacterial surface. Of note, we have previously reported that HMW1 on the bacterial surface, possibly via interactions with the two HMW1 substrates, is required for surface-associated HMW1, indicating a defect in HMW1 anchor formation. In this context, it is noteworthy that N-terminal deletions in FhaC abolish the ability to secrete filamentous hemagglutinin (7), raising the possibility that FhaC has a large periplasmic domain involved in substrate recognition as well. With this information in mind, we speculate that the sequence variability within the periplasmic domain of TpsB proteins and the secretion domain of TpsA proteins may provide the molecular basis for the observed specificity within TPS systems (5, 6).

In summary, both topology mapping and functional studies reveal that the HMW1B protein has three distinct domains as follows: an extracellular N terminus, an internal periplasmic domain, and a C-terminal β-barrel. To our knowledge, this study provides the first experimental evidence that the periplasmic domain of a TpsB protein is critical for a productive interaction with its substrate and that the C terminus of TpsB proteins is sufficient for pore formation. Our results with HMW1B and FhaC highlight the possibility that all TpsB proteins have a modular structure.

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