Acquisition of Heme Iron by *Neisseria meningitidis* Does Not Involve Meningococcal Transferrin-Binding Proteins

NATHALIE MARTEL AND B. CRAIG LEE*

Department of Microbiology and Infectious Diseases, The University of Calgary,
Calgary, Alberta, Canada T2N 4N1

Received 25 August 1993/Returned for modification 29 September 1993/Accepted 10 November 1993

**Summary**

In the meningococcus, hemin-binding proteins (HmBPs) are critical determinants of virulence (3). These proteins are known to be expressed at a high level in meningococcal meningitis (21) and are involved in bacterial iron acquisition (5). HmBPs are also known to play a role in iron-dependent host cell invasion (3, 23). The specific interaction of heme and hemoglobin with these HmBPs has been proposed to constitute the initial step in heme uptake in the meningococcus (15), analogous to the receptor-mediated uptake mechanism of transferrin iron acquisition in eukaryotic cells (11, 23). Properties such as their surface-exposed location (15) and their size homogeneity (15) among clinically significant meningococcal serogroups favor such a function, but definitive genetic evidence to support this premise is lacking.

Despite differences in their binding specificities (15, 23), similarities in size between HmBP1 and TBP1, a protein whose size of approximately 100,000 Mₐ (11, 17) is also highly conserved among the meningococci, suggest that these two proteins may be functionally homologous. The construction of defined isogenic meningococcal TBP mutants (11) provided the tools both to preliminarily address this question and to supply circumstantial evidence supporting the role of the HmBPs in heme iron uptake in *N. meningitidis*.

The meningococcal strains used in this study were kindly provided by A. B. Schryvers (University of Calgary, Calgary, Alberta, Canada). *N. meningitidis* B16B6 is a serogroup B, serotype 2A strain. Isogenic derivatives of B16B6 deficient in the expression of TBP1 and/or TBP2 were constructed by shuttle mutagenesis or cassette mutagenesis (11) and have been described previously (11). The mutants used were N16TIE (a *tbpA* mutant deficient in the expression of TBP1), N16T2K (a *tbpA* mutant deficient in the expression of TBP2), and N16T2EK (a *tbpA* *tbpB* double mutant that expresses no TBPs). All isolates were maintained at −70°C in skim milk and glycerol and, prior to use, were grown at 35°C in an atmosphere of 5% CO₂-chocolate agar supplemented with 1% (vol/vol) CVA enrichment (Difco).

Human hemoglobin, bovine hemin, iron-saturated human transferrin, hemin-agarose (27), and EDDA (ethylenediamine-di-o-hydroxyphenylacetic acid) were purchased from Sigma. Horseradish peroxidase-conjugated human transferrin was obtained from Jackson Immunoresearch Laboratories (Avondale, Pa.). Biotinylated Mr 70,000 poxysuccinimide ester was from Calbiochem (San Diego, Calif.). Streptavidin-agarose was obtained from Bethesda Research Laboratories (Bethesda, Md.). The rabbit polyclonal antiserum raised against the *N. meningitidis* B16B6 TBPs was a gift courtesy of A. B. Schryvers (11, 17) and has been shown to be a pan-reactive reagent, recognizing epitopes present on TBP1 and TBP2 from other meningococcal serogroups (21a).

Iron-saturated human transferrin was biotinylated by the method of Schryvers and Morris (23).

Crude total membranes were prepared as described previously (16) from cells grown in brain heart infusion (BHI) broth rendered iron deficient by addition of the iron chelator EDDA to a final concentration of 100 µM. Total membranes were fractionated into inner and outer membrane components by sucrose isopycnic ultracentrifugation (4, 6). The protein content was determined by the method of Lowry et al. (18) with bovine serum albumin as the standard.

To assess the ability of hemin and hemoglobin to serve as the sole exogenous source of iron for the parental strain *N. meningitidis* B16B6 and the three isogenic mutants, a series of growth experiments were performed. After 12 h of growth on chocolate agar plates at 35°C in an atmosphere of 5% CO₂, organisms were inoculated into BHI broth made iron deficient with the addition of 100 µM EDDA to a starting optical density at 600 nm (OD₆₀₀ of 0.03, as measured with a Pye Unicam PU8800 spectrophotometer. This culture was shaken at 35°C in a 5% CO₂ atmosphere until mid-logarithmic-phase growth was achieved. Aliquots were removed to inoculate 6 ml of warm iron-deficient BHI broth containing 100 µM EDDA.
to which had been added heme and hemoglobin at molar equivalent amounts of heme to an initial OD600 of 0.05. Growth at 35°C in 5% CO2 was monitored hourly. All experiments were performed in triplicate.

The TBPs were isolated from iron-limited total membranes by a batch affinity method as described previously (23). Eluted proteins were applied on a 10% polyacrylamide–sodium dodecyl sulfate (SDS) gel for analysis by SDS-polyacrylamide gel electrophoresis (PAGE).

HmbPs were isolated by affinity purification with heminagarose as the affinity resin in a batch method as described previously (13, 14). Eluted proteins were separated by SDS-PAGE.

For Western blots (immunoblots), 1-µg samples of iron-deficient outer membranes, affinity-purified HmbPs, and affinity-purified TBPs derived from the parental strain N. meningitidis B16B6 and a group X meningococcal strain were separated by SDS-PAGE, and the proteins were subsequently electroblotted at 10 V constant voltage for 12 h at 4°C onto polyvinylidene difluoride (PVDF) membranes (Immunobilon-P, 0.45 µm; Millipore Canada) in 25 mM Tris–192 mM glycine–20% (vol/vol) methanol, pH 8.3, by the method of Towbin et al. (26) with a Bio-Rad MiniTransblot apparatus. For the immunodots, 1-µl aliquots (1 mg/ml) of the above proteins were immobilized on nitrocellulose-cellulose-acetate paper (Immobilon-NC, 0.45 µm, HAHY paper; Millipore Canada). After blocking with 0.5% (wt/vol) skim milk in 50 mM Tris-HCl (pH 7.5)–1 M NaCl (TBS/M) for 1 h at 35°C, the membranes were reacted with a 1:500 dilution of the polyclonal anti-TBP1/TBP2 antibody for 1 h at 35°C. The immunoblots were then rinsed three times for 5 min each with TBS/M. A 1:1,000 dilution of the second antibody (goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate [Sigma]) was allowed to bind for 1 h at 35°C, and the blot was washed three times for 5 min each with TBS/M. The blots were developed with a chloronaphthol-hydrogen peroxide substrate mixture (HRP reagent; Bio-Rad) for 10 min. The papers were washed with water to stop the reaction.

Affinity-purified proteins were analyzed by SDS-PAGE with the discontinuous buffer system of Laemmli (12). The resolving gel was 10% acrylamide and the stacking gel was 5% acrylamide with 8% bisacrylamide. Gels were electrophoresed at 150 V constant voltage for 1 h on a Bio-Rad minigel apparatus. Gels were stained with silver (19). Molecular weights were determined by using known proteins as standards.

Isolation of HmbPs. Two HmbPs of 97,000 and 50,000 molecular weight were isolated by hemin affinity chromatography from the parental strain B16B6 and from N16T12EK, the double isogenic mutant that expresses no TBPs (Fig. 1). Identical HmbPs were affinity purified from the isogenic strains deficient in the expression of either TBP1 (N16T1E) or TBP2 (N16T2K) (data not shown). No significant quantitative or qualitative differences in the HmbPs were seen in the isogenic mutants deficient in one or both TBPs compared with the parental strain.

Growth curves. When added as equivalent molar amounts of heme, both bovine hemin and human hemoglobin were capable of supplying the necessary iron to support the growth of iron-limited cultures of the TBP1 and TBP2 isogenic mutants (data not shown). No differences in the growth-promoting effects of bovine hemin and human hemoglobin were discerned in comparisons of the three mutants or the isogenic mutants and the parental isolate. Similar cell densities were achieved when bovine hemin and human hemoglobin served as the sole exogenous iron source. These results indicate that the isogenic TBP1 and TBP2 mutants retained the same capacity as the parental isolate to acquire iron from heme and hemoglobin.

Comparison of TBPs and HmbPs from N. meningitidis group X. Because the sizes of the meningococcal HmbP1 and TBP1 appear to be strongly conserved (15, 22), the affinity-isolated HmbPs from a group X meningococcal strain were compared with its homologous TBPs (Fig. 2). When total membranes derived from iron-restricted cultures of a group X meningococcal isolate were subjected to transferrin affinity chromatography, two TBPs with molecular weights of 100,000 (TBP1) and 85,000 (TBP2) were isolated (22). In contrast, when the same membrane preparations were bound to heminagarose, two proteins of different molecular weights were eluted from the affinity resin, of 97,000 (HmbP1) and 50,000 (HmbP2) molecular weight. The sizes of these HmbPs are clearly different from those of the TBPs (Fig. 2).

Western blot and immunodot analysis. Polyclonal antiserum raised against TBP1 and TBP2 of N. meningitidis B16B6 recognized the affinity-purified 100-kDa TBP1 and the 85-kDa TBP2 in Western blots (Fig. 3B) of the group X meningococcal strain (22). Bands of identical size were detected in the Western blot of iron-deficient (Fig. 3B) but not of iron-rich (data not shown) outer membrane proteins. A weakly immu-
noretic band of 90 kDa was seen in both the iron-limited outer membrane and the affinity-purified TBP preparations and may represent either a protein with common epitopes or a protein contaminating the immunizing mixture of affinity-purified TBPs to which a minor population of antibodies was raised. Significantly, no bands were seen when the electrophoretically transferred affinity-purified HmBPs were probed with the anti-TBP1/TBP2 antiserum (Fig. 3B). The immunodot results mirrored those seen in the Western immunoblots (Fig. 3A). The lack of a signal emanating from the electroblotted sample buffer indicated that the bands detected from both the affinity-isolated TBPs and the iron-restricted outer membrane preparation were due to specific interaction of the antiserum with these proteins and did not represent nonspecific attachment of the antiserum or second antibody to the Immobilon-P paper. The concordance of both the Western blot and immunodot assays coupled with the absence of reacting bands corresponding to the molecular weights of the HmBPs on the outer membrane protein immunoblot indicated that the HmBPs are immunologically distinct from the TBPs. The ability of the anti-TBP1/TBP2 antiserum to recognize TBP1 and TBP2 in a whole-cell immunoblot assay (21A), in which the proteins are presumably in their native conformation, also suggested that the lack of reactivity against the HmBPs was unlikely to be the result of loss of conformational epitopes during affinity purification or electrophoretic transfer. Identical results were seen when the corresponding preparations from N. meningitidis B16B6, which expresses a 100-kDa TBP1 and a 68-kDa TBP2 (11), were probed with the anti-TBP1/TBP2 antiserum (data not shown).

This study demonstrates that the TBPs and HmBPs in N. meningitidis are distinct proteins. First, a functional difference exists, as reflected most cogently by the demonstration that the double isogenic TBP mutant N16T12EK, which does not express either TBP1 or TBP2, had the same capacity as its parental strain to use heme and human hemoglobin as iron sources. This functional distinction is consistent with, and is reinforced by, the differences in binding specificities of the TBPs and HmBPs noted in previous studies (15, 23). In competitive binding assays, neither hemin nor human hemoglobin could abrogate binding of human transferrin to the meningococcal TBPs (23), and in reciprocal experiments, iron-loaded human transferrin was incapable of blocking binding to the HmBPs (15).

Second, the TBPs and HmBPs display an immunological distinctiveness, as exemplified by the failure of the anti-TBP1/TBP2 antiserum to recognize the HmBPs in immunodot and Western immunoblot assays. However, two alternative explanations may account for this observation. Despite the size conservation of the HmBPs among clinical meningococcal isolates (15), the HmBPs may exhibit antigenic heterogeneity, a property that may obscure similarities in the reactivity of the TBPs and HmBPs to the polyclonal antiserum. In view of the inability of the anti-TBP1/TBP2 antiserum to react with the HmBPs isolated from two meningococcal strains belonging to different serogroups, this possibility appears unlikely. In addition, the polyclonal reagent may preferentially recognize immunodominant epitopes, compromising its ability to detect minor antigenic domains common to both binding proteins. These results indicate that heme iron acquisition in N. meningitidis does not involve the meningococcal transferrin receptor. Although the precise mechanism of heme iron uptake in the meningococcus is unknown, the apparent differentiation of the TBPs enhances the candidacy of the HmBPs as participants in this process. Such a role is strengthened by the observations that both the use of heme iron and the expression of the HmBPs in the TBP mutants were unimpaired compared with these activities in their parent strain. Although circumstantial, this evidence in aggregate is consistent with the proposed function of the HmBPs as receptors in the meningococcal heme iron uptake pathway (15). The recent genetic demonstration that heme iron uptake in Yersinia enterocolitica (25) and Vibrio cholerae (8) requires the presence of an outer membrane heme receptor supports this contention.

The HmBPs augment the expanding portfolio of meningococcal surface receptors that specifically bind host iron-containing proteins (23, 24). Exemplifying the high degree of adaptability that the bacterium displays for its obligate host, such an arsenal of iron-binding proteins would handsomely equip the meningococcus with the versatility to acquire the iron sequestered in the variety of human host proteins. Definitive proof of the role and delineation of the physiological significance of the meningococcal HmBPs will await genetic analyses.

We thank A. B. Schryvers for his generous provision of the bacterial strains and of the polyclonal antiserum.

This work was supported by the Alberta Heritage Foundation for Medical Research.

REFERENCES


6. Hancock, R. E. W., and H. Nikaido. 1978. Outer membranes of gram-negative bacteria. XIX. Isolation from Pseudomonas aerugi-

CRC Press Inc., Boca Raton, Fla.


15. Lee, B. C. Isolation and characterization of the hemin-binding proteins from *Neisseria meningitidis*. Submitted for publication.


