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Functional Significance of Factor H Binding to Neisseria meningitidis

Muriel C. Schneider,* Rachel M. Exley,* Hannah Chan,† Ian Feavers,† Yu-Hoi Kang,‡ Robert B. Sim,‡ and Christoph M. Tang2*‡

Neisseria meningitidis is an important cause of septicemia and meningitis. To cause disease, the bacterium must successfully survive in the bloodstream where it has to avoid being killed by host innate immune mechanisms, particularly the complement system. A number of pathogenic microbes bind factor H (fH), the negative regulator of the alternative pathway of complement activation, to promote their survival in vivo. In this study, we show that N. meningitidis binds fH to its surface. Binding to serogroups A, B, and C N. meningitidis strains was detected by FACS and Far Western blot analysis, and occurred in the absence of other serum factors such as C3b. Unlike Neisseria gonorrhoeae, binding of fH to N. meningitidis was independent of sialic acid on the bacterium, either as a component of its LPS or its capsule. Characterization of the major fH binding partner demonstrated that it is a 33-kDa protein; examination of insertion mutants showed that porins A and B, outer membrane porins expressed by N. meningitidis, do not contribute significantly to fH binding. We examined the physiological consequences of fH binding to the bacterial surface. We found that fH retains its activity as a cofactor of factor I when bound to the bacterium and contributes to the ability of N. meningitidis to avoid complement-mediated killing in the presence of human serum. Therefore, the recruitment of fH provides another mechanism by which this important human pathogen evades host innate immunity. The Journal of Immunology, 2006, 176: 7566–7575.

The complement system plays a pivotal role in innate immune defense against pathogenic microbes. Initiation of the complement cascade through any of three pathways, classical (CP),1 lectin, or alternative (AP), leads to cleavage of C3 to C3b by C3 convertases. Deposition of C3b on the surface of an invading pathogen results in its elimination through phagocytosis or lysis following assembly of the membrane attack complex (MAC) (1, 2). Activation of complement within the host must be precisely controlled as inappropriate and excessive activity can damage host cells, especially within the intravascular compartment (3). Several factors are responsible for down-regulating complement activation in vivo. Soluble regulators are present in the systemic circulation and at mucosal surfaces, while other molecules that reduce complement activation are expressed on cells to prevent local deposition of complement factors. Factor H (fH) is a 150-kDa soluble protein that is present in the secretions in the nasopharynx and is the main regulator of the AP (4). The AP both amplifies complement activation initiated by the CP and/or LP and is continually activated by the spontaneous, low-level conversion of C3 to C3b; C3b interacts with factor Bb to generate C3bBb, the C3 convertase of the AP (5). fH inhibits the AP in three ways by accelerating the dissociation of C3bBb, acting as a cofactor for factor I (fI)-mediated inactivation of C3b, and competing with factor B (1).

A variety of microbes avoid complement-mediated killing by recruiting fH to their surfaces including parasitic and viral pathogens such as Echinococcus granulosus, Onchocerca volvulus, and HIV (6–8). fH is also used by a number of bacteria to promote their survival in the host in the face of an immune response. fH binds to OspE, an outer membrane lipoprotein expressed by the spirochete Borrelia burgdorferi, the causative agent of Lyme disease (9, 10). Furthermore the M protein of Group A Streptococcus and YadA of Yersinia enterocolitica both recruit fH leading to reduced opsonophagocytosis but not complement-mediated lysis (11, 12). Neisseria gonorrhoeae, an important cause of sexually transmitted disease, also recruits fH to its cell surface. Isolates of N. gonorrhoeae can be classified as serum sensitive or serum resistant (SR) on the basis of their resistance against complement-mediated killing (13). fH appears to bind to two distinct targets on N. gonorrhoeae (14). The bacterium incorporates exogenous 5′-cytidine monophospho-N-acetyl neuraminic acid (CMP-NANA) into its LPS as sialic acid (15, 16), which can recruit fH to the cell surface (17). However, even in the absence of CMP-NANA some gonococcal strains are SR. This is mediated by binding of fH to a subtype of Por1, the most abundant outer membrane protein. N. gonorrhoeae can express one of two alleles of porin (Por) I, Por1A or Por1B. Strains expressing Por1A are SR through binding fH, whereas serum-sensitive strains express Por1B instead. Examination of strains expressing hybrids of Por1A and Por1B indicates that fH binds to surface exposed loop 5 of Por1A (18). An effective complement system is critical for resistance against infections caused by the closely related pathogen Neisseria meningitidis. This is evident from observations of individuals lacking components of the MAC who have between 1,000- and 10,000-fold increased lifetime risk of developing disseminated meningococcal infection compared with the general population (19).
role of the AP in resistance against meningococcal infections is highlighted by the increased susceptibility to neisserial infections in patients lacking properdin, which stabilizes the AP C3 convertase (19). Furthermore, individuals with polymorphisms in the promoter of the gene encoding fH have lower serum fH levels and are at increased risk of N. meningitidis infection (20). N. meningitidis has several mechanisms to avoid lysis by the complement system, including siaylation of its LPS and expression of a polysaccharide capsule (21). Most infections in industrialized countries are caused by serogroup B and C strains (26), which both express a polysaccharide capsule. Most infections in industrialized countries are caused by serogroup B and C strains (26), which both express a polysaccharide capsule. Most infections in industrialized countries are caused by serogroup B and C strains (26), which both express a polysaccharide capsule.

Although the binding of fH to N. gonorrhoeae has been extensively characterized, the role of fH in the biology of meningococcal infection has not been investigated. Here, we characterize the basis of fH binding to the meningococcus and define the consequences of this interaction, particularly in relation to resistance against complement-mediated lysis, the most critical aspect of innate immunity against this important human pathogen.

**Materials and Methods**

**Bacterial strains and growth conditions**

N. meningitidis was grown on brain heart infusion medium supplemented with 5% Levanthal’s base at 37°C in the presence of 5% CO2. Antibiotics were added as required at the following concentrations: erythromycin, 2 μg/ml; tetracycline, 2.5 μg/ml; kanamycin (kan), 75 μg/ml; CS8 H44/76 (multilocus sequence typing, ST-32, electrophoretic type, ET-5 (http://pubmlst.org)) and 528 (ST-18) are serogroup B isolates of N. meningitidis, Z2491 (ST-4, subgroup IV) and F6124 (ST-5, subgroup III) are serogroup A strains, and the serogroup C isolates studied were FM20 (ET-37) and 90/18311 (ST-11, ET-37) (28). The Streptococcus pneumoniae isolate, D39, and the corresponding mutant lacking PspC were provided by Dr. L. Briles (University College, London, U.K.) and were grown on blood agar plates.

The construction of MC58ΔsisD, MC58Δlst, and MC58ΔsisA has been described previously (29, 30). siaC encodes the enzyme polysialyltransferase that is required for the polymerization of sialic acid during the biosynthesis of serogroup B capsule (31), while list is the LPS-specific sialyltransferase (32). The siaC gene is necessary for sialic acid biosynthesis (33); mutants lacking siaC are unable to express a capsule or siaylate their LPS without exogenous CMP-NANA. The MC58ΔporA and MC58ΔporB mutants were derived by insertional inactivation of the genes with a cassette encoding kan resistance. Primers (porA forward, 5'-ATGCGAAAAACACTTGCCCTC-3'; porA reverse, 5'-AATGAGGAGGAGCAGCTCAAAAC-3'; porB forward, 5'-GGGAAAGCTTCTCCTCGGTG-3'; porB reverse primer, 5'-GGGAAAGCTTCTCCTCGGTG-3') were designed based on the genome sequence of N. meningitidis strain MC58 to amplify porA and porB from N. meningitidis. The PCR products were ligated into pCR2.1 TOPO (Invitrogen Life Technologies), and the constructs were verified by restriction endonuclease digestion. A kan-Δpsl cassette was amplified using primers with KpnI recognition site at the 5' ends, and the product was inserted into the porA gene at a unique KpnI site in the open reading frame. Another pair of primers with BsoDI sites at the 5' ends was used to amplify the kan resistance cassette from Tn5, which was ligated into a unique BsoDI site in porB. The porAkanΔpsl and porBkan constructs were amplified by PCR, and the products were used to transform N. meningitidis by standard methods. Recombinants were selected on medium containing kan and, following replacement was confirmed by PCR and Southern blot analysis in back-crossed strains (data not shown).

**Preparation of fH-depleted human plasma**

Pooled citrated human plasma (30 ml; HD Supplies) was dialyzed at 4°C against 10 mM HEPES, 140 mM NaCl, 0.5 mM EDTA (pH 7.4) (running buffer), and the salt concentration was raised to 1 M to prevent Clq depletion. A 25-mL MRC OX23-Sepharose column (33) was equilibrated in running buffer, and the plasma run through at a rate of 10 ml/h. The unbound protein was collected in 1-ml fractions, and the protein concentration in each fraction was measured by reading the OD at 280 nm. Forty fractions (1 ml each) were pooled and dialyzed against the running buffer, and aliquots were frozen rapidly in liquid nitrogen then stored at −80°C. The plasma was diluted from 30 to 40 ml during this procedure. fH was then eluted from the column with 50 ml of 3 M MgCl2, adjusted to pH 6.8–7.0 by the addition of Tris base.

The extent of fH depletion from the plasma was >99.5% as assessed by ELISA as follows. Microtiter plates (MaxiSorp; Nunc) were coated with monoclonal anti-human fH MRC OX23 (50 μg/ml) and purified IgG in 0.1 M NaHCO3 (pH 9.6) 2.5 mg/ml (34). Plates were left for 1 h at 20°C, washed four times with PBS, and any remaining nonspecific binding sites were blocked with PBS-0.1% Tween20 (PBS-T) for 2 h at room temperature. Samples of plasma were assayed in doubling dilutions from 1/100 to 1/25,600 in PBS-T. Serially diluted samples (50 μl) were added to each well and incubated for 1 h at 20°C. fH of a known concentration (diluted 1/2 to 1/512, with a starting concentration of 10 μg/ml) was included as a standard and treated exactly as the plasma samples. The wells were washed four times in PBS-T, and 50 μl of rabbit anti-human fH (MRC Immunocytometry Unit) at a dilution of 1/32,000 in PBS-T was added to each well and incubated for 1 h at 20°C. The wells were washed again with PBS-T, then 100 μl of goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma-Aldrich) at 1/5000 dilution was added to each well. The plate was incubated as before, washed again in PBS-T, and developed with the substrate p-nitrophenyl phosphate (Sigma-Aldrich). The plate was read at 405 nm in a microtiter plate reader (Multiscan Ascent; Labsystems) after optimum color development had occurred.

**Flow cytometry**

FACS analysis was performed to quantify the binding of fH to N. meningitidis. Bacteria were grown overnight on solid medium and then harvested into PBS. The number of bacteria was estimated by measuring the OD at 260 nm of an aliquot of the bacterial suspension lysed in 200 mM NaOH/1% SDS (v/w). Bacteria (106 CFU) were fixed in 3% formaldehyde for 2 h at room temperature, washed three times in PBS, and stored at −80°C in PBS/15% glycerol. To estimate fH binding, 105 cells were resuspended in 10 μl of normal human serum (NHS) or alternative sources of fH for 30 min at 37°C. After two washes in PBS-T, binding was detected following incubation with a murine anti-human fH mAb (MRC OX23 or MRC OX24, 0.1 μg/ml, (34), or a polyclonal goat anti-human fH (1/200 dilution in PBS; Calbiochem) for 30 min at 37°C in 50 μl of PBS. An anti-human Talin IgG1 mAb (Sigma-Aldrich) and polyclonal goat anti-murine LAMP sera (Jackson ImmunoResearch Laboratories) were used as control primary Abs. The cells were washed twice in PBS-T, then resuspended in 50 μl of PBS with a polyclonal donkey anti-mouse IgG-FITC conjugate (1/200 dilution in PBS; Jackson ImmunoResearch Laboratories) or a polyclonal donkey anti-goat IgG FITC conjugate (1/200 dilution; Jackson ImmunoResearch Laboratories) and incubated for 30 min on ice. The cells were examined in a flow cytometer (Calibur FACSscan; BD Biosciences), and at least 4 × 104 events were recorded. fH binding to the cell surface was expressed as the percentage of fluorescent bacteria normalized using results from negative controls. Binding of fH to live bacteria was performed as follows: Bacteria (2 × 106 CFU) were incubated with 20 μl of purified IgG (870 μg/ml in 10 mM K2PO4, 100 mM KCl, 0.5 mM EDTA (pH 7.4)) or alternative source of fH (serum from a C7-deficient individual or heat-inactivated serum) for 30 min at 37°C. After two washes, the number of bacteria was estimated by measuring the OD at 260 nm, and the bacteria were fixed in 3% paraformaldehyde for 15 min, washed, and analyzed by FACS.

**Far Western blot analysis**

Whole cell lysates of N. meningitidis were prepared by growing bacteria overnight on solid medium, harvesting around 106 CFU in 400 μl PBS, and centrifugation at 15,700 × g for 2 min. The pellet was resuspended in 100 μl of SDS-PAGE sample buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 100 μM 2-ME, 10% glycerol, 0.1% bromophenol blue). Outer membrane vesicles (OMVs) were prepared as described previously (35). Proteins were separated under reducing conditions with SDS-12% PAGE, then the proteins transferred to polyvinylidene difluoride membranes (Millipore). After nonspecific binding had been blocked with PBS containing 5% milk, the blots were incubated for 2 h with purified fH (final concentration of 3.36 μg/ml of

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in PBS-T (0.5% milk) or NHS as fH source (final dilution of 1/25 in PBS-T (0.5% milk), then incubated with MRC OX24 (86 ng/ml) in PBS-T (0.5% milk). Binding of MRC OX24 was detected using a donkey anti-mouse-IgG HRP-conjugated secondary Ab (Invitrogen Life Technologies).

**Cofactor activity assay of fH bound to N. meningitidis and serum sensitivity assays**

The cofactor activity of fH was assessed by analyzing fI-mediated conversion of C3b to iC3b. Briefly, fixed MC58 (10⁹ CFU) were preincubated with fH (870 μg/ml) or with buffer alone (10 mM K₂PO₄, 100 mM KCl, 0.5 mM EDTA (pH 7.4)) for 30 min at 37°C. After three washes, the bacteria were incubated with C3b (1 μg) and fI (0.4 μg) in 20 mM phosphate buffer (pH 6) in a final volume of 30 μl for 30 min at 37°C. fH was added directly to samples as a positive control for the assay, and reactions also were performed in the absence of fH as a negative control. The samples were separated under reducing conditions by SDS-PAGE, transferred to membranes, and subjected to Western blot analysis using a polyclonal rabbit anti-C3c Ab (1/1000 v/v; Dako) as the primary Ab, which was detected with a HRP-conjugated donkey anti-rabbit IgG (1/10,000 v/v; Dako).

For serum sensitivity assays, bacteria were grown overnight on solid medium, harvested into 400 μl of PBS, and centrifuged at 450 × g for 2 min to remove clumps. The supernatant was collected and spun at 15,700 × g for 2 min, the pellet was resuspended in 400 μl of PBS, and the number of bacteria was quantified as above. The bacteria (2 × 10⁹ CFU) were centrifuged, resuspended in 20 μl of purified fH (870 μg/ml), then incubated for 30 min at 37°C, or with the same buffer without fH as a negative control. After two washes in PBS-T, bacteria were quantified and added to DMEM with glutaMAX (Invitrogen Life Technologies) at a concentration of 2 × 10⁹ CFU/ml. The bacterial suspension was added to an equal volume of fH-depleted plasma already diluted 1/3 in DMEM (final dilution 1/6), then incubated at 37°C. Surviving bacteria were recovered by plating to brain heart infusion agar.

**FIGURE 1.** Flow cytometric quantification of fH binding to N. meningitidis MC58 using anti-fH mAbs or polyclonal anti-fH sera. Fixed bacteria (10⁸) were incubated with 10 μl of NHS (dark gray lines) or PBS (light gray lines), and fH binding detected with a polyclonal anti-human fH Ab (final dilution 1/1000) (A), or the mAbs, OX23 (B), or OX24 (C) or an isotype-matched (anti-Talin IgG1) mAb control (D) at 100 μg/ml final concentration. fH recruitment is observed by a shift to the right in the population of bacteria incubated with serum, compared with cells incubated with PBS.

**Results**

**fH binds to N. meningitidis**

We used three experimental conditions to detect fH binding to the serogroup B N. meningitidis isolate MC58 by FACS. First, NHS was used as the source of fH, and cells were incubated with PBS as a negative control. Second, MC58 was incubated with fH-depleted plasma supplemented with purified fH added at physiological concentrations (500 μg/ml; normal range, 100–600 μg/ml), and the results were compared with those obtained with bacteria incubated with depleted plasma without added fH. Finally, we incubated bacteria with purified fH alone. Fig. 1 shows fH binding to MC58 after incubation with NHS, evaluated by FACS analysis with a polyclonal anti-fH Ab (Fig. 1A) or with the mAbs MRC OX23 (Fig. 1B) or MRC OX24 (Fig. 1C). Similar levels of binding were detected regardless of the Ab used; no binding was detected using either an irrelevant polyclonal sera (data not shown) or an isotype matched mAb (Fig. 1D). Binding was observed using fH-depleted plasma only when supplemented with additional fH (Fig. 2A), and when fH was provided as a purified protein (Fig. 2B). Thus, fH binds to N. meningitidis independent of other components of the complement cascade such as C3b. To exclude the possibility that the binding, we detected was the result of an artifact from fixing bacteria, we examined whether live N. meningitidis was able to bind fH. Live bacteria recruited fH regardless of whether purified fH, heat-inactivated serum or serum from a C7-deficient individual (which lacks bacteriolytic activity) was used as the source of fH (Fig. 2C).
To further characterize the binding of fH to *N. meningitidis*, we analyzed the kinetics of binding of fH to the bacterial surface and whether binding was saturable. Binding occurred rapidly after incubation with purified fH (with maximal binding detected after 30 s (Fig. 3A) and was saturated at physiological concentrations (290 μg/ml; Fig. 3B).

To establish whether fH binds only to serogroup B meningococcal strains, we examined the capacity of serogroup A and C *N. meningitidis* isolates to recruit fH. FACS analysis demonstrated that Z2491 (serogroup A, ST4; Ref. 36) and FAM20 (serogroup C, ST2) bind fH at levels comparable to those observed with MC58 (Fig. 4, A–C). The findings were confirmed by examining further isolates of each serogroup (Fig. 4D).

The interaction between *N. meningitidis* and fH is independent of sialic acid on the bacterial surface

The LPS of *N. gonorrhoeae* can be sialylated in the presence of exogenous CMP-NANA if the strain expresses LPS of the appropriate immunotype (37) and the sialylated LPS modulates fH binding (17, 38). For serogroup B *N. meningitidis*, the situation is different as the bacterium synthesizes sialic acid, which is incorporated into the capsule as well as LPS. Therefore, we investigated the contribution of sialic acid to fH binding to MC58 as this strain expresses a polysialic acid capsule and LPS immunotype (L3, L7, L9) that can be sialylated (39). The extent of fH binding was examined in strains lacking a capsule (MC58/H9004 siaD) or unable to synthesize sialic acid (MC58/H9004 siaC). Following incubation in NHS, both MC58ΔsiaD and MC58ΔsiaC (Fig. 5A) exhibited similar levels of fH binding as the parental strain, MC58, regardless of which anti-fH mAb was used for detection. To exclude the possibility that fH binds to sialic acid on LPS, we assessed the binding to MC58Δlst, which lacks LPS sialylation (30). The lst mutant bound fH at wild-type levels (Fig. 5B), demonstrating that fH recruitment by the meningococcus is entirely independent of the presence of surface sialic acid. Binding of fH to live bacteria showed similar results (data not shown).
fH binds to a 33-kDa protein present in outer membrane vesicles

To further characterize the basis of fH binding to *N. meningitidis*, Far Western blot analysis was performed. Whole bacterial cell lysates were separated by SDS-PAGE, transferred to membranes that were then incubated with purified fH. fH binding was assayed with MRC OX23 or MRC OX24, followed by an HRP-conjugated goat anti-mouse Ab for chemiluminescent detection. In lysates from *N. meningitidis* strains Z2491 and MC58, fH bound principally to a protein with an approximate molecular mass of 33 kDa (Fig. 5C); binding to a protein of the same molecular mass in serogroup C *N. meningitidis* was detected but was less marked despite equal loading of lysates (Fig. 5D); minor differences between strains were seen in the migration of the band reacting with fH. The specificity of the Far Western blot analysis was confirmed by incubating the membranes without fH or with an isotype-matched (anti-Talin IgG1) mAb instead of anti-fH mAbs (data not shown); the 33-kDa band was not detected under these circumstances. Furthermore, fH binding to *S. pneumoniae* in a PspC-dependent manner was detected with MRC OX23 (data not shown); PspC is the major fH receptor on the pneumococcus (40).

To exclude the possibility that fH was binding to the most abundant protein, whole cell lysates were examined by Coomassie brilliant blue staining. As shown in Fig. 5D, no predominant band of 33 kDa was evident. Binding also was detected by Far Western blot analysis of OMVs from *N. meningitidis* (Fig. 5C), and was abolished if the lysates were exposed to proteinase K, demonstrating that the 33-kDa band is proteinaceous in nature (data not shown).

In *N. gonorrhoeae*, fH binds to a region of external loop 5 of an outer-membrane porin, Por1A (18). *N. meningitidis* can express two outer-membrane porins, PorA and PorB. PorA is a 42-kDa protein (41) (www.tigr.org, NMB1429) with 51% amino acid identity to Por1A, whereas PorB has a molecular mass of 35.6 kDa (42, 43) (NMB2039) and shares 81% amino acid identity with Por1A (Fig. 6A). Therefore, we hypothesized that *N. meningitidis* PorA or PorB is necessary for recruitment of fH to the bacterial surface. Insertional mutants of *porA* and *porB* were constructed in MC58 and examined for fH binding by FACS and Far Western blot analysis. The absence of expression of the PorA and PorB proteins was confirmed by SDS-PAGE of OMV preparations from each mutant (data not shown). No significant difference was observed in the amount of fH binding detected by FACS to MC58, MC58*porA*, and MC58*porB* (Fig. 6B). Furthermore, the 33-kDa protein band was still detected by Far Western blot analysis in the insertion mutants (Fig. 6C). On prolonged exposure, additional minor bands of the predicted molecular mass of PorA and PorB were not detected in lysates from the corresponding mutants, suggesting that fH binds to PorA and PorB, but to a lesser extent than the 33-kDa protein.

Role of fH in serum resistance of *N. meningitidis*

Because fH is recruited by *N. meningitidis*, we next examined the role of fH in protection against complement-mediated killing. First, we determined whether fH bound to the bacterium retains its activity as a cofactor for fI-mediated cleavage of C3b into inactive fragments including iC3b. Cleavage of C3b results in the loss of the 109-kDa band (corresponding to C3b), and the appearance of the 40-kDa component of iC3b and was dependent on the presence of fH (Fig. 7A). In the absence of fH, fI was able to cleave C3b, although this was clearly enhanced when fH was added directly to assays. Increased production of iC3b also was detected when *N. meningitidis* was preincubated with fH but not with buffer...
FIGURE 5. fH binds to N. meningitidis independently of sialic acid but is recognized by a protein with an approximate molecular mass of 33 kDa. A. Flow cytometric quantification of fH binding using OX23 and OX24 shows no significant difference between the wild-type strain and strains lacking a capsule (MC58ΔsiaD), or lacking both a capsule and LPS sialylation (MC58ΔsiaC), or (B) a strain lacking LPS sialylation only (MC58Δlsp). C. Far Western blot analysis showing fH binding to whole cell lysates of isolates of N. meningitidis (strains are indicated above each lane). Binding was detected to a major band with an apparent molecular mass of around 33 kDa in a range of isolates and in purified outer membrane vesicles. D. Coomassie brilliant blue staining of proteins separated by SDS-PAGE showing the profile of protein expressed by serogroups A, B, and C of N. meningitidis. No predominant band of 33 kDa is seen that could account for nonspecific binding of fH to the most abundant protein.

Discussion

The complement system is an important effector mechanism for both the innate and adaptive immune response against N. meningitidis (44). To avoid adaptive immune killing, the bacterium can vary its surface Ags and express nonimmunogenic, molecular mimics of host molecules as components of its LPS and capsule (45). Pathogenic microbes can use two main mechanisms to escape innate immune killing through the complement system. First, they can inhibit effector functions of complement activation. For example, bacterial capsules are thought to act by preventing the insertion of the MAC into the outer membrane (46, 47), while Streptococcus pyogenes secretes a protease that directly cleaves C5, the first component of the MAC (48, 49). Alternatively, pathogens can recruit negative regulators of the host complement system to their surface. N. gonorrhoeae binds both fH and C4bp, which down-regulate the AP and CP, respectively (50). Recent studies have shown that N. meningitidis PorA binds C4bp, leading to resistance against complement (51). In contrast, the role of fH in protection of meningococci against complement-mediated killing has not been reported even though it is known that this regulator of the AP is recruited by a number of pathogenic microbes to subvert the complement system (52).

We used two independent methods, FACS and Far Western blot analysis, to demonstrate that N. meningitidis recruits fH. To confirm the specificity of this interaction, binding was detected using any of three separate Abs, but not with irrelevant polyclonal sera or an isotype matched mAb. We confirmed the specificity of the Far Western blots by detecting fH binding to S. pneumoniae in a PspC-dependent fashion (40). Furthermore, we detected fH binding when it was provided either as serum or as a purified protein to live or fixed bacteria. Highest levels of binding were found when live bacteria were incubated in NHS, an environment that is relevant to understanding the pathogenesis of disseminated meningococcal infection. The finding that fH binding is saturable at plasma fH concentrations further supports the physiological significance of our findings.

We found that isolates of N. meningitidis bound fH regardless of their multilocus sequence type or serogroup. As the serogroup A capsule has a fundamentally different chemical composition to the polysialic acid capsule of serogroup B and C strains, it was unlikely that the capsule itself was responsible for the recruitment of
To the bacterium. In support of this, we found that fH recruitment was not affected by deletion of siaD, which is required for capsule biogenesis. However, siaD mutants can still synthesize sialic acid, and this could have mediated fH recruitment. We excluded this possibility by demonstrating that a siaC mutant, which is defective for the production of sialic acid, also recruits fH at levels comparable to that of the wild-type strain. Thus, the capsule is neither required for nor inhibits the recruitment of fH to N. meningitidis.

The binding of fH to the related pathogen, N. gonorrhoeae, has been extensively characterized (17, 18, 38). We found that, unlike the gonococcus, binding of fH by N. meningitidis was independent of the presence of LPS sialylation; the lst mutant of N. meningitidis bound fH at comparable levels to the wild-type bacterium. A recent report (38) indicates that sialic acid on N. gonorrhoeae LPS is not the actual target of fH binding but instead acts to enhance the interaction with Por1A. Therefore, our findings are entirely consistent with those described with N. gonorrhoeae, because the meningococcus does not express Por1A.

We investigated whether the meningococcal porins, PorA or PorB, are the target of fH binding given the precedent of Por1A mediating binding to N. gonorrhoeae (18). Because the level of fH binding was not reduced in the porA or porB mutant, neither of these outer membrane porins appears to act as the sole receptor for fH on N. meningitidis. However, it is still possible that PorA and PorB recruit fH independently to the bacterial surface but that their functions are redundant. Far Western blot analysis did indicate that fH does bind to N. meningitidis proteins independently of the presence of PorA and PorB proteins. Loss of porA or porB did not affect fH binding to a prominent band of ~33 kDa in lysates.

**FIGURE 6.** fH is recruited to the surface of porA and porB mutant strains. A. ClustalW alignment of the amino acid sequence of Por1A from N. gonorrhoeae and PorA and PorB from N. meningitidis. The region of Por1A involved in fH binding and the corresponding amino acid sequences in the meningococcal proteins are shown in bold. Identical amino acids are indicated with an asterisk. B. Similar levels of fH bound to strains lacking PorA or PorB as the wild-type strain, MC58. The proportion of bacteria binding fH (percentage gated) was normalized with readings obtained following incubation with PBS alone (error bars show the SD). C. Far Western blot analysis showing fH binding to N. meningitidis proteins independently of the presence of PorA and PorB proteins. Loss of porA or porB did not affect fH binding to a prominent band of ~33 kDa in lysates.
Far Western blot analysis indicated that a protein with an apparent molecular mass of 33 kDa binds to fH. Inspection of the sizes of bands detected in different isolates is consistent with there being allelic variants of the fH binding partner among circulating meningococcal strains (Fig. 5D). This is not surprising, because many structures expressed on the surface of *N. meningitidis*, especially those under immune selection, exhibit allelic variation (54). Interestingly, fH is recruited by immunogenic proteins in other bacteria such as M protein, PspC, and OspE of *S. pyogenes*, *S. pneumoniae*, and *B. burgdorferi*, respectively (9, 11, 40). *N. gonorrhoeae* Por1A also is the target of bactericidal Abs (14). Additionally, it has been shown recently that C4bp, another negative regulator of the complement system, binds to PorA (51), and is a major immunogenic protein on the surface of the meningococcus (55). Therefore, recruitment of negative regulators by Ags that elicit host immune responses might provide a mechanism to prevent deposition of complement components in a highly localized fashion; identification of the binding partner of fH on *N. meningitidis* might be informative about novel Ag(s) for inclusion in vaccines to prevent meningococcal disease.

To further understand the biological significance of fH recruitment by *N. meningitidis*, we examined the cofactor activity of fH bound to the bacterium. Although there have been several reports of cofactor activity when incubated with recombinant proteins (56–59), there has been only one other example in which the presence of fH on the surface of a bacterium has been demonstrated to retain its ability to enhance fI-mediated cleavage of C3b (60). Furthermore, fH serum sensitivity assays were performed. We examined the protective effect of fH bound to the bacterium against complement-mediated bacteriolysis. fH has a dual role in serum sensitivity assays, because depletion of fH in serum can lead to a reduction in effective C3 levels through unrestricted activation of the AP; individuals who lack fH become functionally C3 deficient and develop membranous glomerulonephritis and hemolytic uremic syndrome through inappropriate complement deposition (61). Despite this, in every assay with fH-depleted plasma, there was sufficient active complement to kill between 80 and 100% of wild-type bacteria. Although we preincubated bacteria with concentrations of fH that were above physiological levels (870 μg/ml, normal range 100–600 μg/ml), the concentration of bacteria (5 × 10⁹ CFU/ml) was much higher than found in disease in meningococcal bacteremia (10⁵–10⁶ CFU/ml) (62, 63). We found that preincubation of bacteria with fH protected *N. meningitidis* from complement killing, with >100% increase in bacterial survival if they were preincubated with fH. *N. meningitidis* avoids complement-mediated lysis by expression of a polysaccharide capsule and LPS (23). However, during rounds of bacterial replication in vivo in the systemic circulation or at the mucosal surface, binding of fH also will provide the bacterium with an advantage for survival and represents an additional mechanism by which *N. meningitidis* avoids complement-mediated killing.