Functional Comparison of the Binding of Factor H Short Consensus Repeat 6 (SCR 6) to Factor H Binding Protein from *Neisseria meningitidis* and the Binding of Factor H SCR 18 to 20 to *Neisseria gonorrhoeae* Porin^{∇†}

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Both *Neisseria meningitidis* and *Neisseria gonorrhoeae* recruit the alternative pathway complement inhibitory protein factor H (fH) to their surfaces to evade complement-dependent killing. Meningococci bind fH via fH binding protein (fHbp), a surface-exposed lipoprotein that is subdivided into three variant families based on one classification scheme. Chimeric proteins that comprise contiguous domains of fH fused to murine Fc were used to localize the binding site for all three fHbp variants on fH to short consensus repeat 6 (SCR 6). As expected, fH-like protein 1 (FHL-1), which contains fH SCR 6, also bound to fHbp-expressing meningococci. Using site-directed mutagenesis, we identified histidine 337 and histidine 371 in SCR 6 as important for binding to fHbp. These findings may provide the molecular basis for recent observations that demonstrated human-specific fH binding to meningococci. Differences in the interactions of fHbp variants with SCR 6 were evident. Gonococci bind fH via their porin (Por) molecules (PorB.1A or PorB.1B); sialylation of lipooligosaccharide enhances fH binding. Both sialylated PorB.1B- and (unsialylated) PorB.1A-bearing gonococci bind fH through SCR 18 to 20; PorB.1A can also bind SCR 6, but only weakly, as evidenced by a low level of binding of FHL-1 relative to that of fH. Using isogenic strains expressing either meningococcal fHbp or gonococcal PorB.1B, we discovered that strains expressing gonococcal PorB.1B in the presence of sialylated lipooligosaccharide bound more fH, more effectively limited C3 deposition, and were more serum resistant than their isogenic counterparts expressing fHbp. Differences in fH binding to these two related pathogens may be important for modulating their individual responses to host immune attack.

Factor H (fH) is a 155-kDa soluble plasma glycoprotein that negatively regulates the alternative pathway (AP) of complement by inhibiting the assembly of active C3 convertases, accelerating the decay of the AP C3 convertase (C3b,Bb), and acting as a cofactor in factor I-mediated cleavage of C3b to the hemolytically inactive molecule, iC3b (7, 36, 51, 52). fH is composed entirely of 20 short consensus repeat (SCR) domains (also called complement control protein domains, or CCPs); each SCR is ~60 amino acid residues in length and contains four invariant cysteines that coordinate folding of the domain (44). fH also binds to polyanions such as sialic acid, heparin, and sulfated glycosaminoglycans (31). Numerous human pathogens recruit fH to circumvent the deleterious effects of the AP of complement. Microbial fH binding proteins that contribute to pathogenicity have been described for several organisms, including group A (18) and group B streptococci, *Streptococcus pneumoniae* (20), *Yersinia enterocolitica* (1, 4),

human immunodeficiency virus, *Fusobacterium necrophorum* (14), and *Borrelia* spp. (2, 3).

The complement system is an important arm of the innate immune defense against pathogenic *Neisseria* spp. Individuals with deficiencies in components of the terminal complement pathway (C5 to C9) or the AP (such as properdin and factor D) are at an increased risk for disseminated neisserial infections (8, 9, 45). *Neisseria meningitidis* and *Neisseria gonorrhoeae* have evolved several mechanisms to combat complement-mediated killing. Each of these organisms recruits fH to its surface; however, the surface structures involved in binding to fH are different (27, 28, 34, 41–43, 46). Subtle differences in the ways these organisms bind fH may be important for survival in the unique niche occupied by each organism.

N. meningitidis is an encapsulated gram-negative bacterium that causes meningitis and sepsis worldwide. *N. meningitidis* colonizes the nasopharyngeal epithelium of 5 to 10% of the population and in susceptible individuals may cross the epithelial barrier into the bloodstream and cause systemic infection. Essentially all isolates of *N. meningitidis* recovered from the bloodstream or cerebrospinal fluid are encapsulated, and expression of the polysaccharide capsule is critical for high-level resistance to complement-mediated killing by normal human serum (NHS) (10). Meningococci employ numerous mechanisms to avoid complement-mediated killing. These include binding of complement regulatory proteins such as C4b binding protein (C4BP) and fH, which regulate the classical and

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TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristic(s)	Reference(s)
H44/76	B:15:P1.7,16:ST-32 (Norway, 1976); variant 1 fHbp; invasive isolate	13, 48
H44/76 Δ fHbp	H44/76 fHbp::ermC	28
RM1090	C:2a:P1.5,2:ST-unknown (U.S., 1985); variant 2 fHbp; invasive isolate	19
M1239	B:14:P1.23,14:ST-437 (U.S., 1994); variant 3 fHbp; invasive isolate	5
Y2220 <i>siaD</i>	Y2220 <i>siaD</i> ::cat; unencapsulated derivative of Y2220; Y:21:P1.19,15-1 ^a (ST-172); low-level expresser of variant 2/3 hybrid fHbp; carriage isolate; LOS sialylated	28
Y2220 <i>siaD</i> fHbp _{H44/76}	Chromosomal replacement of Y2220 <i>siaD</i> fHbp gene with fHbp gene of strain H44/76; high-level variant 1 fHbp expresser; LOS sialylated	28
Y2220 <i>siaD</i> por _{F62}	Chromosomal replacement of <i>porB2</i> of Y2220 <i>siaD</i> with <i>porB.1B</i> gene of gonococcal strain F62; LOS sialylated	27

alternative complement pathways, respectively (22, 28, 46). fH promotes conversion of C3b bound to the bacterial surface to iC3b, a hemolytically inactive form of C3b, and also results in an overall decrease in total C3 deposition that results in decreased opsonization. Binding of fH to meningococci is mediated by fH binding protein (fHbp; also named GNA1870 or lipoprotein 2086), a 28-kDa surface-exposed lipoprotein present in all strains of *N. meningitidis* that have been examined (28). fHbp is a prominent component of an experimental recombinant polyvalent vaccine currently in phase III clinical trials. Based on amino acid sequence comparisons, two different classification systems for fHbp have been proposed. One system divides this protein into subfamilies A and B, while the other classification system divides fHbp into three variant families called variant 1, variant 2, and variant 3 (11, 29). Amino acid identity is 74.1% between variants 1 and 2, 62.8% between variants 1 and 3, and 84.7% between variants 2 and 3 (29). Despite these variations in amino acid sequence, all three variants of fHbp bind to fH (28). fHbp expression varies widely among different strains, and expression levels correlate directly with the level of fH binding; strains that express larger amounts of fHbp bind more fH (28, 29).

N. gonorrhoeae is the causative agent of gonorrhea, a serious health problem worldwide. Host defenses successfully limit most cases of gonorrhea to the genital tract. Invasion of the bloodstream and dissemination occur in fewer than 5% of cases and are highly dependent on the strain attributes, particularly intrinsic resistance to complement-mediated killing (termed stable serum resistance) (40). One way that gonococci evade complement-mediated killing is by binding human fH (41, 42). Gonococci bind fH via the porin molecules PorB.1A and PorB.1B. In vivo, the lacto-*N*-neotetraose (LNT) epitope of neisserial lipooligosaccharide (LOS) is modified with sialic acid (37), and sialylation of the LNT epitope of LOS facilitates the binding of fH to all gonococcal porins and renders gonococci resistant to killing by nonimmune NHS (32). The binding of fH to gonococci that express PorB.1B is dependent upon LOS sialylation (42) and is mediated by fH SCR 18 to SCR 20 (SCR 18–20) (34, 42). Gonococci expressing PorB.1B bind fH very weakly or not at all in the absence of LOS sialylation. Gonococcal strains that express PorB.1A proteins can bind to fH directly, even in the absence of LOS sialylation. LOS sialylation enhances the binding of fH to PorB.1A-bearing gonococci. This interaction involves both fH SCR 6 and SCR 18–20; however, SCR 18–20 is the preferred site of binding (34).

In this communication, we characterized the interaction of

fH with fHbp on *N. meningitidis* by identifying amino acids in fH that are involved in binding to all three variants of fHbp. Our studies revealed salient differences in the way meningococci and gonococci bind fH. We also examined the functional consequences of the different mechanisms that these organisms use to bind fH.

MATERIALS AND METHODS

Bacterial strains and mutants. Neisserial strains and their relevant characteristics are listed in Table 1. Bacteria were grown on chocolate agar plates supplemented with an IsoVitalX equivalent at 37°C in an atmosphere enriched with 5% CO₂. For immunological assays, organisms were grown for 10 to 12 h and then suspended in Hanks' balanced salt solution (HBSS) containing 0.15 mM CaCl₂ and 1 mM MgCl₂ (HBSS⁺⁺). *Escherichia coli* strains (Invitrogen, Carlsbad, CA) were cultured in Luria-Bertani (LB) broth or on LB agar.

Serum and complement reagents. Sera from seven healthy adult volunteers with no history of neisserial infections and who had not received a meningococcal vaccine were pooled and stored at –70°C. Complement activity in the sera was abrogated by heating at 56°C for 30 min. Purified human fH was purchased from Advanced Research Technologies (Tyler, TX).

Recombinant fH fragment/Fc fusion proteins. fH/murine Fc fusion constructs were used to examine the binding of fH to fHbp. These constructs contained contiguous fH SCR domains (SCR 1–5, 1–6, 5–8, 6–10, 11–15, or 16–20) fused in frame at their C-terminal ends to the N terminus of the Fc fragment of murine immunoglobulin G2a (IgG2a) (fH/Fc fusion proteins). The construction of these chimeric fH/murine Fc fusion proteins and the estimation of the concentration of the fusion proteins in concentrated cell culture supernatants have been described in detail previously (34). These constructs permit use of the Fc region as a detection site (tag) for equal detection of each fusion molecule, using anti-mouse IgG. Collectively, the fH fragments spanned the entire length of the fH molecule.

Site-directed mutagenesis of SCR 6. Amino acids in fH SCR 6 important for binding to each variant of fHbp were identified by replacing individual amino acids in human SCR 6 with the corresponding rhesus macaque SCR 6 amino acids in the background of the human SCR 5–8/Fc construct. Point mutations were created using a QuikChange site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). The sequences of all constructs were validated by DNA sequencing. The quality and quantity of the constructs were analyzed as discussed above. Based on the sequence dissimilarity between human and rhesus fH SCR 6 (see Fig. 2A) and the lack of binding of rhesus fH to *Neisseria* spp. (15), the following SCR 6 point mutations were constructed: T321S, K323R, H337Y, N339S, A348P, Y352H, Y353F, H360P, and H371Y. Similarly, we created three double mutants (H337Y H360P, H337Y H371Y, and H360P H371Y).

Recombinant FHL-1. Recombinant fH-like protein 1 (FHL-1) was generated by PCR, using pBluescript containing the cloned human fH cDNA (a gift of Michael K. Pangburn, University of Texas Health Science Center, Tyler, TX) as a template. The fH SCR 1–7 fragment was cloned into the KpnI and NotI sites of the eukaryotic expression vector pcDNA3 (Invitrogen Life Technologies), using the forward primer FHL-1 KpnI Forward (CGGGGTACCAAAAAATGAGACTTCTAGCAAA) and reverse primer FHL-1 NotI Reverse (GCGGC CGCTTAGAGTGTAATACTGACACGGATACATCTTGGAGTA). The reverse primer adds four C-terminal amino acids (SFTL) found in FHL-1 that are not present in fH. All clones were confirmed by DNA sequencing. Chinese hamster ovary (CHO) cells were transfected with the FHL-1 construct, using

FIG. 1. Binding of fH/Fc fusion proteins to *N. meningitidis*. H44/76, RM1090, and M1239 represent meningococcal strains that express variant 1, 2, or 3 fHbp proteins, respectively. All three fHbp variants bound only to fH/Fc fusion constructs that contained SCR 6 (SCR 1–6/Fc and SCR 6–10/Fc) of fH. In all graphs, the x axis represents fluorescence intensity on a log₁₀ scale, and the y axis represents the number of events. No fusion protein is present in the control tube. The results for one experiment representative of at least three independent experiments are shown.

lipofectin (Invitrogen Life Technologies) according to the manufacturer's instructions. Media from transfected cells were collected over a 2-day period, and FHL-1 was purified from culture supernatant by passage over a polyclonal anti-fH column. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting were used to assess the purity of the FHL-1. Protein concentrations were determined by absorbance at 280 nm and by using a bicinchoninic acid protein assay kit (Pierce).

Flow cytometry. Bacteria grown overnight on chocolate agar plates were washed with HBSS⁺⁺ and suspended to a final concentration of 3×10^8 cells/ml; 10^8 organisms were used in each fluorescence-activated cell sorter (FACS) assay. To detect the binding of recombinant fH/Fc fusion proteins, bacteria were incubated with concentrated tissue culture supernatant containing 0.5 μ g of recombinant fH/Fc protein (as determined by enzyme-linked immunosorbent assay) in a final reaction volume of 100 μ l for 30 min at 37°C. After a washing, fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Sigma-Aldrich) diluted 1:100 in 1% bovine serum albumin-HBSS⁺⁺ was used to detect bacterium-bound fH/Fc fusion proteins. To detect the binding of fH, bacteria were incubated with either purified human fH (0.5 μ g or as indicated) or heat-inactivated (HI) NHS (at the concentrations indicated in Fig. 5). HI serum does not support the activation of C3 to C3b and was used to prevent fH-C3b interactions that would confound measurements intended to detect direct binding of fH in serum to bacterial surfaces. Bound fH was detected by using monoclonal antibody 90X (MAb 90X; specific for SCR 1; detects both full-length fH and FHL-1) and FITC-conjugated anti-mouse IgG (Sigma) as previously described (34). The binding of FHL-1 was detected similarly, following the incubation of bacteria with 0.5 μ g of purified FHL-1. The binding of C3 to bacteria was detected as previously described, using anti-human C3c-FITC (Bioscience/Meridian Life Science, Inc.) (34). Flow cytometry was performed using a FACS-Calibur instrument (Becton Dickinson), and data analysis was performed using the FlowJo data analysis software package (Tree Star, Inc.).

Serum bactericidal assay. The susceptibility of meningococci to complement-mediated killing was determined by using a serum bactericidal assay as described previously (30). Bacteria from an overnight culture on chocolate agar plates were inoculated onto fresh chocolate agar and allowed to grow for ~6 h at 37°C in 5% CO₂. Briefly, ~2,000 CFU of meningococci were incubated with sera (concentrations specified for each experiment in Fig. 6) in a final reaction volume of 150 μ l. Aliquots of 25 μ l were plated in duplicate at the start of the assay (t_0) and after the reaction mixture was incubated at 37°C for 30 min (t_{30}). The percent survival was calculated as the number of viable colonies at t_{30} relative to the baseline colony counts at t_0 . Each experiment was repeated a minimum of three times.

RESULTS

fH SCR 6 binds to all three variant fHbp families. Full-length human fH binds to all three variant families of fHbp (28). To determine the fH SCRs that are involved in binding to

fHbp, we constructed fusion proteins that contain contiguous fH SCRs fused at their C termini to the Fc portion of IgG2a. We examined the ability of six fH/Fc fusion constructs (SCR 1–5/Fc, SCR 1–6/Fc, SCR 5–8/Fc, SCR 6–10/Fc, SCR 11–15/Fc, and SCR 16–20/Fc) to bind to meningococcal strains H44/76, RM1090, and M1239, which express variant 1, 2, or 3 fHbp proteins, respectively. Previously, we established that none of these strains bind fH in the absence of fHbp (28). As seen in Fig. 1 and 2 (SCR 5–8/Fc shown in Fig. 2), only those fH/Fc proteins that contained SCR 6 (SCR 1–6/Fc, SCR 5–8/Fc, and SCR 6–10/Fc) bound to all three strains. These data suggest that SCR 6 is important for the binding of fH to all fHbp variant proteins. These data do not unequivocally exclude the possibility that other fH SCRs, for example, SCR 7 and SCR 8, may also play a role in the binding of fH to fHbp. Furthermore, another recombinant fH molecule that contained SCR 8–20 was tested using the variant 1 fHbp meningococcal strain H44/76, and it did not bind (data not shown). Attempts to construct an fH/Fc fusion molecule containing SCR 7–10 were not successful.

Histidine residues in human SCR 6 are important for fH binding to meningococci. Site-directed mutagenesis was employed to examine the amino acids in human fH SCR 6 that are required for binding to fHbp. Nine SCR 6 amino acids were selected for mutagenesis after the following considerations were addressed. The recently solved crystal structure of human fH SCR 6–8_{402H} (His 402 is a polymorphism in SCR 7 that is associated with age-related macular degeneration) in complex with the polyanion sucrose octasulfate (SOS) (38) identified six surface-exposed amino acid residues in SCR 6 that are important for binding to polyanions (His 337, Arg 341, Tyr 352, Ser 354, His 360, and His 371) (Fig. 2A). Binding to SCR 6 was pH dependent, supporting a major role for the histidine side chains in glycan coordination (38) and prompting our speculation that these amino acids might be important for the binding of fH to fHbp. In addition, a comparison of the amino acid sequence of human SCR 6, which binds fHbp, with rhesus fH SCR 6, which does not bind *N. meningitidis* (15), revealed

FIG. 2. Site-directed mutagenesis of human fH SCR 6. (A) Amino acid sequence comparison of human and rhesus fH SCR 6. Identical amino acid residues are denoted by dots. Asterisks indicate amino acids in SCR 6 that are involved in binding to the polyanion SOS, identified by Prosser et al. (38). The histidine residues mutated and examined below in panel B are boxed. (B) Binding of human SCR 5-8/Fc (wild-type) and human SCR 5-8/Fc constructs containing point mutations in the indicated histidine residues (mutant protein) to meningococcal strains H44/76, RM1090, and M1239. No fusion protein is present in the control tube. The *x* axis represents fluorescence on a log₁₀ scale, and the *y* axis represents the number of events. The results of one experiment representative of at least three independent experiments are shown.

differences in 9 amino acids (Fig. 2A). We hypothesized that one or more of these species-specific amino acids may also be involved in the binding of human SCR 6 to fHbp. A similar species-specific approach was used successfully to define the amino acids in C4BP that are required for binding to gonococci (21, 33). Four of the nine species-specific amino acids (His 337, Tyr 352, His 360, and His 371) were also identified in the crystal structure as important for SOS binding. Of note, two of the amino acids (Arg 341 and Ser 354) are conserved in both rhesus and human fH SCR 6, and these amino acids were not investigated further.

A concern when making point mutations is the structural integrity of the resultant mutant protein. Taking into account that through evolution, the structure-function relationships of complement molecules are likely to have been preserved, we believe that mutating the amino acids in human fH to their counterparts in rhesus fH would be less likely to cause major alterations in the tertiary structure of the protein. We therefore constructed human-to-rhesus point mutations in human SCR 6 in the background of human fH SCR 5-8/Fc to study the effects of amino acid alterations of fH-fHbp binding. Here we present data on the mutation of His 337, His 360, and His 371, amino acids that are important for binding to polyanions and for species specificity. Mutation of the remaining six amino acids (T321S, K323R, N339S, A348P, Y352H, and Y353F) did

not impact the binding of the mutated human fH SCR 5–8/Fc construct to fHbp-bearing *N. meningitidis* (data not shown).

Each of the three His residues (H337Y, H360P, and H371Y) was mutated individually and in combination with the other two His residues. Binding of the mutated human SCR 5–8/Fc constructs to each of three meningococcal strains, each representative of one of the three fHbp variant families, was examined by flow cytometry. Replacement of H337 with the rhesus counterpart (H337Y) resulted in decreased binding to strains expressing fHbp variants 2 and 3 but had no impact on binding to the fHbp variant 1-expressing strain (Fig. 2). A single mutation of H360 or H371 did not impact binding to any fHbp variant. The H337Y H360P double mutant decreased binding of the human SCR 5–8/Fc construct to variant 2 and 3 strains (consistent with the H337Y mutation alone) but not to the variant 1 strain. Binding of this construct to fHbp variant 3 was completely abolished. The H337Y H371Y mutation resulted in a reduction in the binding of the fusion construct to all three fHbp variant proteins; of note, this is the only construct with decreased binding to the variant 1 fHbp. Binding of the H337Y H371Y construct to the variant 1 and 3 fHbp-bearing strains was completely lost; partial binding to the variant 2 protein was detected. The H360P H371Y construct bound well to all three fHbp variants, consistent with the single-mutation studies. Together, these data indicate that His 337 is important for the

FIG. 3. Binding of *N. meningitidis* to FHL-1. (A) Binding of purified recombinant FHL-1 to *N. meningitidis* strains that express variant 1, 2, or 3 fHbp. In all graphs, the x axis represents fluorescence on a log₁₀ scale, and the y axis represents the number of events. A representative isotype control (using variant 1 fHbp strain H44/76) in which purified FHL-1 was omitted from the reaction mixture is shown (broken line).

binding of human SCR 5–8 to fHbp from variant 2 and 3 families. Furthermore, His 337 in human SCR 5–8 acts cooperatively with His 371 to bind to the variant 1 protein. These results suggest that the manner in which human SCR 5–8 interacts with fHbp variant 1 differs slightly from the interactions with variant 2 and variant 3 proteins.

The binding (or partial binding) of mutated human SCR 5–8/Fc constructs to at least one fHbp variant family suggests that the approach of making human-to-rhesus point mutations was successful and did not result in disruption of the tertiary structure of the protein.

Meningococci bind FHL-1. Human serum contains both fH (~550 µg/ml) and FHL-1 (~10 to 50 µg/ml) (17, 26). FHL-1 is an alternatively spliced variant of fH. The first seven N-terminal SCRs of fH are also present in FHL-1. FHL-1 differs from fH in that SCR 7 of FHL-1 is spliced to a separate exon that encodes four unique C-terminal amino acids (SFTL) (53). FHL-1 has both complement regulatory and cell attachment properties, and binding of FHL-1 may contribute to serum resistance and attachment to host cells (17). The ability of meningococci to bind to fH via SCR 6 raises the possibility that meningococci might also bind to FHL-1. We examined the binding of purified recombinant FHL-1 to meningococci expressing variant 1, 2, or 3 fHbp by FACS, using Mab 90X. Mab 90X recognizes fH SCR 1 and therefore can detect both full-length fH and FHL-1. As seen in Fig. 3A, all three meningococcal strains bind FHL-1. Consistent with its high expression levels of fHbp, the variant 1 strain H44/76 exhibited the highest level of binding to FHL-1.

Comparison of fH binding to meningococcal fHbp and gonococcal porin in an isogenic background with sialylated LOS. The binding of fH to meningococcal fHbp involves SCR 6 and is independent of LOS sialylation. In contrast, the binding of fH to gonococci involves the porin molecules (PorB.1A and PorB.1B) and is enhanced by sialylation of LNT LOS (42). The binding of fH to sialylated gonococci involves SCR 18–20 (34).

To compare the functional consequences of fH binding to meningococcal fHbp via SCR 6 with fH binding to gonococcal PorB.1B via SCR 18–20 in an isogenic background, we employed meningococcal strains Y2220 *siaD* fHbp_{H44/76} (see below), which expresses a variant 1 fHbp, and Y2220 *siaD* *por*_{F62}, which expresses a gonococcal PorB.1B (27, 28). We did not include a comparison of fH binding to PorB.1A, as PorB.1A mediates binding to C4BP, which would confound interpretation of the results of functional tests such as serum bactericidal assays (40). Meningococcal porins in the context of intact bacteria do not bind to fH (27, 28, 46) or to C4BP when physiological isotonic buffers are used (22). Meningococcal strain Y2220 *siaD* is an unencapsulated (*siaD::cat*) serogroup Y strain that expresses its own variant 2/3 hybrid fHbp molecule in very small amounts, resulting in the barely detectable binding of fH (27) (Fig. 4A, upper panel). Consistent with its inability to bind the full-length fH molecule, Y2220 *siaD* also does not bind fH/Fc fusion proteins (Fig. 4A, upper panel). To study fH-fHbp interactions, we used a variant of Y2220 *siaD* in which the native fHbp (fHbp_{Y2220}) is replaced with fHbp from strain H44/76 (fHbp_{H44/76}). This allelic replacement results in high-level expression of the variant 1 protein in the background of a meningococcus with a sialylated LNT LOS (28). This mutant binds full-length fH and, as expected, also binds SCR 6 (Fig. 4A, middle panel). To study fH-gonococcal PorB.1B interactions in an isogenic background, we used a variant of Y2220 *siaD* in which the native *porB2*_{Y2220} (meningococcal porins do not bind to fH in the context of intact bacteria) is replaced with *porB.1B* from *N. gonorrhoeae* strain F62 (Y2220 *siaD* *por*_{F62}). Expression of Por_{F62} in the Y2220 *siaD* background results in increased fH binding (27). We have shown previously that sialylation of neisserial LNT LOS results in enhanced fH binding only when gonococcal Por is concomitantly expressed. This mutant binds the full-length fH and SCR 16–20/Fc (Fig. 4A, lower panel), which is consistent with our prior observations that the C-terminal region of fH binds to sialylated gonococci (34). These two isogenic strains allowed us to make comparisons between the properties of fH binding to *Neisseria* spp. through these two distinct receptors.

Qualitative differences in the binding of fH to meningococcal fHbp and gonococcal porin. The binding of fH to Por_{F62} is mediated through an interaction with SCR 16–20, while the interaction of fH with fHbp_{H44/76} is mediated through an interaction with SCR 6. The nature of the binding of fH to each of these ligands (Y2220 *siaD* *por*_{F62} and Y2220 *siaD* fHbp_{H44/76}) was examined in the presence of buffers of increasing ionic strength. The binding of fH to Y2220 *siaD* *por*_{F62} decreased as the ionic strength of the assay buffer was increased (Fig. 4B). This finding is consistent with an ionic interaction between these two molecules. In contrast, the binding of fH to Y2220 *siaD* fHbp_{H44/76} was minimally affected by the increase in ionic strength, suggesting that this interaction is nonionic and possibly hydrophobic. While the most likely cause of this difference is charge neutralization or a change in the charge of the substrate and/or receptor, we cannot rule out the possibility of conformational changes to Por or the sialylated LOS in the high salt environment. Nevertheless, these data highlight differences in the binding of fH SCR 6 to fHbp_{H44/76} and fH SCR 16–20 to Por_{F62}.

FIG. 4. Binding of fH to fHbp_{H44/76} and Por_{F62} in an isogenic background. (A) Binding of the full-length fH or the indicated fH/Fc fusion proteins to *N. meningitidis* Y2220 *siaD* expressing either fHbp_{H44/76} (Y2220 *siaD* fHbp_{H44/76}; middle row) or Por_{F62} (Y2220 *siaD* por_{F62}; bottom row). Y2220 *siaD* expresses small amounts of a variant 2/3 hybrid fHbp and does not bind fH (top row). In all graphs, the x axis represents fluorescence on a log₁₀ scale, and the y axis represents the number of events. (B) The influence of ionic strength on the binding of full-length fH to Y2220 *siaD* fHbp_{H44/76} and Y2220 *siaD* por_{F62} was studied by flow cytometry. The binding of fH to Y2220 *siaD* por_{F62} decreased as the ionic strength of the assay buffer was increased. The binding of fH to Y2220 *siaD* fHbp_{H44/76} was minimally diminished at NaCl concentrations of 0.4 M and 1 M. Control binding of these strains to full-length fH in HBSS⁺⁺ is also shown. The x axis represents fluorescence on a log₁₀ scale, and the y axis represents the number of events.

Meningococci with sialylated LOS that express gonococcal porin bind more fH than meningococci with sialylated LOS that express fHbp. Porin is the most abundant protein found in the outer membrane of gonococci (23, 35). The amount of fHbp expressed varies among strains, but it is expressed at a much lower level than porin, even in strains that express high levels of fHbp (29). The effects of copy number on fH binding

were compared by measuring the amount of fH bound to Y2220 *siaD* por_{F62} and Y2220 *siaD* fHbp_{H44/76}, using a FACS assay and a range of fH concentrations. Strains that expressed Por_{F62} bound more fH at all concentrations tested (Fig. 5). This binding trend was observed in assays using both purified fH (1 to 10 µg/ml) and HI NHS (5 to 100%) as sources of human fH. Human fH is present in serum at a concentration of

FIG. 5. The amounts of fH bound to Y2220 *siaD* por_{F62} and Y2220 *siaD* fHbp_{H44/76} were compared, using a FACS assay over a range of fH concentrations. Representative histogram plots of full-length fH binding to Y2220 *siaD* fHbp_{H44/76} and Y2220 *siaD* por_{F62} when the strains were incubated with either HI NHS (upper panel) concentrations of 5, 10, 25, 50, or 100% or purified fH (lower panel), the latter at concentrations of 1.25, 2.5, 5, or 10 µg/ml. Median values for each histogram are indicated. HI-NHS and pure fH were omitted from controls (dashed lines). The x axis represents fluorescence on a log₁₀ scale, and the y axis represents the number of events. The results of an experiment representative of at least three independent experiments are shown.

FIG. 6. fH that binds to Por_{F62} regulates the alternative pathway of complement more efficiently than fH that binds to fHbp_{H44/76}. C3 binding to Y2220 *siaD por*_{F62} is diminished compared to C3 binding to Y2220 *siaD fHbp*_{H44/76} or the control Y2220 *siaD*. Organisms were incubated in 10% NHS, and C3 was measured by FACS, using FITC-conjugated anti-human C3c. Y2220 *siaD* meningococci that expressed Por_{F62} showed diminished C3 deposition (at both 3 and 30 min) compared to strains that expressed fHbp_{H44/76}. The x axis represents fluorescence on a log₁₀ scale, and the y axis represents the number of events. The numbers represent the median fluorescence intensity of C3 binding. The results of an experiment representative of at least three independent experiments are shown.

~550 μ g/ml. These data suggest that gonococcal Por may be able to bind more fH. This finding is likely to be biologically relevant because gonococci reside in the genital tract, an environment with a low concentration of fH (6).

fH bound to Por_{F62} on *Neisseria* with sialylated LOS regulates the AP of complement more efficiently than fH bound to fHbp. fH controls activation of the AP by dissociating the AP C3 and C5 convertases and by serving as a cofactor for the inactivation of C3b to iC3b (7, 36, 51, 52). C3 deposition can be used as a measure of fH function. We have demonstrated previously that fH possesses functional activity when it is recruited by fHbp and Por/sialylated LOS to the surfaces of meningococci and gonococci, respectively (28, 42). Above we have shown that Por_{F62} expression results in increased binding of fH to Y2220 *siaD por*_{F62} compared to fH binding to fHbp_{H44/76} on Y2220 *siaD fHbp*_{H44/76}. To determine the functional consequences of these differences, we compared C3 deposition at different time points after the exposure of organisms to NHS. The amounts of C3 binding to Y2220 *siaD por*_{F62} (binds fH through SCR 16–20) and Y2220 *siaD fHbp*_{H44/76} (binds fH through SCR 6) were compared after bacteria were incubated with NHS. The expression of Por_{F62} more effectively limited deposition of C3 than the expression of fHbp_{H44/76} (Fig. 6).

Correlation of the degree of serum resistance with the mechanism of fH binding. The binding of fH to Por_{F62} is mediated through an ionic interaction with SCR 16–20, while the interaction of fH with fHbp_{H44/76} is most likely mediated through a nonionic/hydrophobic interaction with SCR 6. The expression of Por_{F62} allows for quantitatively more fH binding than the expression of fHbp_{H44/76} and is associated with less C3 deposition. To determine if these differences in fH binding translated into differences in protection from complement-dependent killing, we measured the relative percent survival of the

FIG. 7. Y2220 *siaD por*_{F62} is more serum resistant than Y2220 *siaD fHbp*_{H44/76}. Serum bactericidal assays were performed using Y2220 *siaD*, Y2220 *siaD por*_{F62}, or Y2220 *siaD fHbp*_{H44/76} and 3.3 and 6.6% NHS as indicated. The x axis represents the strain, and the y axis represents the percent survival. Values represent average percent survival calculated from three or more independently performed experiments.

recombinant strains in NHS. We observed that Y2220 *siaD por*_{F62} was more serum resistant than Y2220 *siaD fHbp*_{H44/76} (Fig. 7). The levels of IgG and IgM binding to the two mutant strains were similar, indicating that differences in antibody binding did not account for differences in survival in NHS (data not shown). These data suggest that Por_{F62}, in association with sialylated LOS, is more effective at regulating complement activation on Y2220 *siaD* than is fHbp_{H44/76}.

DISCUSSION

In this study, we have shown that fH SCR 6 contains a binding site for all three variants of fHbp. We utilized the available crystal structure of fH SCR 6–8_{402His} and the species-specific binding of fH to meningococci to map the amino acids in fH SCR 6 that are important for binding to fHbp (15, 34, 38). Specifically, His 337 and His 371 were found to be important for the binding of fH to fHbp. The mutation of His 337 was sufficient to decrease the binding of fH to the variant 2 and 3 fHbp proteins, while mutations of both His 371 and His 337 together were required to abolish binding to the variant 1 fHbp on H44/76. Our findings provide further understanding of the molecular basis of human-specific binding of fH to meningococci. His 337 and His 371 were also identified by Prosser et al. as key residues of SCR 6 involved in coordinating binding of the polyanion SOS (38). The SCR 6 polyanion binding site contains a central positively charged arginine residue (Arg 341) flanked by two histidine side chains (His 337 and His 371) that directly coordinate sulfate groups in the ligand. Binding was pH dependent, implicating a crucial role for these histidine residues. Of note, heparin also binds to SCR 6, and the mutation of His 337 decreases the affinity of SCR 6 for heparin. His 337 not only plays an important role in mediating heparin binding to fH SCR 6 but also plays an important role in the fH SCR 6-fHbp interaction; the glycosaminoglycans heparin and SOS were able to inhibit the binding of fH to meningococci

expressing all three variants of fHbp (see Fig. S1 in the supplemental material), suggesting that the points of contact between fH and fHbp are coincident with the SCR 6-polyanion interaction sites.

His 360 appears to play only a minor role in the binding of fH to fHbp. Although the mutation of His 360 in conjunction with the His 337 mutation further decreased the binding of fH to variant 3 fHbp compared to that of His 337 alone, the addition of the His 360 mutation to the His 337 mutation did not further impact the binding of fH SCR 6 to the variant 1 or 2 proteins. This result is also consistent with the studies of Prosser et al., who found that His 360 was important for the binding of polyanions only to the polymorphic disease-associated fH variant that contains His at position 402 (38). In that model, His 402 from SCR 7 and His 360 from SCR 6 formed a histidine clamp around the sulfate groups of SOS. Wild-type fH contains tyrosine at position 402 (Tyr 402) in SCR 7, and a polyanion binding site would not be predicted to form with Tyr 402 (38).

Schneider et al. recently solved the crystal structure of fH SCR 67 in complex with a variant 1 fHbp (47). Their findings are consistent with ours and corroborate that the variant 1 fHbp interacts with fH SCR 6. The SCR 6-fHbp complex is held together by numerous electrostatic interactions, including many hydrogen bonds and salt bridges; only minor contact with SCR 7 was noted (47). The fHbp binding site was found to overlap with the SOS binding site in fH SCR 6, and both SOS and heparin inhibited the interaction of SCR 67 with fHbp (47). In agreement with our findings, the fH-SCR 67 interaction was resistant to 1 M NaCl, a finding that may possibly reflect the high-affinity interaction (dissociation constant [K_d] of ~5 nM) reported by this group (47).

We found that both His 337 and His 371 were important for the binding of SCR 6 to the variant 1 fHbp. These two SCR 6 amino acids are located in the interaction surface described by Schneider et al. (47). His 371 is proposed to be involved in a salt bridge with fHbp, while His 337 is likely involved in a hydrogen bond with fHbp. Arg 341 of SCR 6, which is important for SOS binding (as described above), was also reported to form a salt bridge with fHbp (47). A double mutation (R341A and H337A) in SCR 6 abolished binding to the variant 1 fHbp (47). The phenotypes of H337A and R341A single mutations were not reported in the Schneider study; however, our data indicate that a His 337 single mutation is not sufficient to abolish binding of SCR 6 to the variant 1 fHbp. Taken together, these data suggest that Arg 341 also plays a key role in the binding of SCR 6 to variant 1 fHbp. These findings are in agreement with the polyanion binding site of SCR 6 as described by Prosser et al.

Three variant families of fHbp have been described. The three alleles elicit little immunologic cross-bactericidal response; sequence similarity ranges from 63% between variants 1 and 3 to 74% between variants 1 and 2 and 85% between variants 2 and 3 (29). Subtle differences in the amino acids of fH SCR 6 that interact with variant 1 fHbp versus variant 2 and variant 3 fHbps were apparent, which is seemingly consistent with the overall sequence variability among these proteins.

In this study, we also identified FHL-1 as a human complement regulatory protein that binds to *N. meningitidis*. Meningococcal strains bearing all three variants of fHbp bound to

FHL-1. This observation is consistent with our finding that human fH SCR 6 is involved in the fH-fHbp interaction. Similarly, gonococcal strains bearing PorB.1A that bind fH via SCR 6 and SCR 18–20 bind to FHL-1, while gonococci with sialylated LOS expressing PorB.1B that bind fH via SCR 18–20 (but not SCR 6) do not bind FHL-1 (34). Although the binding of FHL-1 to PorB.1A-expressing gonococci is weak, FHL-1 can rescue PorB.1A gonococci from killing by primate sera; rescue requires an approximately fivefold molar excess of FHL-1 compared to fH (34). Binding of FHL-1 to surface ligands has been reported for several other pathogens, including *Streptococcus pyogenes*, *Treponema pallidum*, and numerous *Borrelia* species (25). *Borrelia burgdorferi* expresses a number of complement regulator-acquiring surface proteins, or CRASPs, that bind to members of the fH family of proteins. CRASP-1 and CRASP-2 bind both fH and FHL-1 and are associated with serum resistance; CRASP-1 preferentially binds fH, while CRASP-2 preferentially binds FHL-1 (16). It has been speculated that in *Borrelia* species, differential binding to host complement regulators such as fH and FHL-1 may modulate escape from host immune attack (16). Both *N. meningitidis* expressing fHbp and *N. gonorrhoeae* expressing PorB.1A interact with fH SCR 6; however, the binding of FHL-1 to meningococci is superior to that seen with PorB.1A-expressing gonococci (see Fig. S2 in the supplemental material). The role of FHL-1 in serum resistance and/or cell adhesion of meningococci and PorB.1A-expressing gonococci remains to be determined.

Using isogenic mutant strains, we have shown that differences in fH binding to bacterial surfaces are correlated with differences in protection against complement-mediated bacterial killing. Binding of fH to meningococci is mediated by the relatively low-density receptor fHbp and is independent of LOS sialylation. We have shown that SCR 6 of fH is involved in the binding of fH to fHbp. In the context of intact bacteria, meningococcal porins do not bind to fH (27, 28, 46). In contrast, gonococci bind to fH through an interaction with gonococcal porin, which is the most highly expressed protein in the gonococcal outer membrane. Binding of fH to one of the two main types of porin, PorB.1B, requires LOS sialylation and involves SCR 18 to 20 of fH (34, 42). It is not known if a ternary complex between fH SCR 18–20, PorB.1B, and sialylated LOS is formed or if LOS sialylation in some way facilitates an fH-PorB.1B interaction that cannot otherwise occur when LOS is not sialylated. Our studies indicate that fH binding to gonococci in this context is more efficient in thwarting complement-mediated killing than fH binding to meningococcal fHbp; this increased binding correlated with decreased C3 deposition and increased serum resistance.

These results underscore the importance of fH for the serum resistance of *N. gonorrhoeae*. Meningococci normally reside on the nasopharyngeal epithelium and enter the bloodstream to cause disease. Although the expression of fHbp contributes to the ability of meningococci to resist complement-dependent killing, it is not sufficient to confer serum resistance. Expression of capsular polysaccharide is necessary for high-level serum resistance and invasion of the bloodstream (24, 50). Unencapsulated meningococcal mutants are susceptible to killing by NHS despite the expression of fHbp and their ability to bind to fH via this interaction. In addition, sialylation of meningococcal LOS contributes only marginally to serum resistance

(12, 49). In contrast, *N. gonorrhoeae* colonizes epithelial cells of the genital tract and LOS sialylation leads to serum resistance (32). Sialylation of LOS enhances the binding of fH and allows gonococci to effectively resist complement-dependent killing (42). fH bound to gonococci functions to increase the conversion of C3b to iC3b (inactive C3b) and to decrease C3 deposition by inactivating the AP C3 convertase. *N. gonorrhoeae* does not produce a capsule, and strains that invade the bloodstream are stably serum resistant (not dependent on LOS sialylation) by virtue of their ability to also bind C4BP and regulate the classical pathway (39).

A balance between complement activation and the efficiency of complement regulation determines whether a bacterium survives or is killed by serum. Our studies indicate that the differences in the interactions of these pathogens with fH and the localization of these organisms to specific niches may reflect their needs. The ability of meningococci to invade the bloodstream is dependent on the expression of a polysaccharide capsule. fH is abundant in the bloodstream (~550 µg/ml), and the fHbp-fH interaction may be optimized to meet the needs of a serum-resistant organism growing in an environment with high concentrations of fH. In contrast, gonococci must scavenge fH efficiently, as this may be critically important to keep C3 deposition to a minimum in the genitourinary tract, a niche where fH is less abundant.

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