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Characterisation of diverse sub-variants of the meningococcal factor H binding protein (fHbp) for their ability to bind fH, mediate serum resistance and induce bactericidal antibodies

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Running title: Functional and immunological analysis of fHbp sub-variants

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Abstract

Neisseria meningitidis is a commensal of the human nasopharynx but is also a major cause of septicemia and meningitis. The meningococcal factor H binding protein (fHbp) binds human factor H (fH), enabling downregulation of complement activation on the bacterial surface. fHbp is a component of two serogroup B meningococcal vaccines currently in clinical development. Here we characterize 12 fHbp sub-variants for their level of surface exposure and ability to bind fH, mediate serum resistance and induce bactericidal antibodies. Flow cytometry and Western analysis revealed that all strains examined expressed fHbp on their surface to different extents and bound fH in an fHbp-dependent manner. However, differences in fH binding did not always correlate with the level of fHbp expression, indicating that this is not the only factor affecting the amount of fH bound. To overcome the issue of strain variability in fHbp expression, the MC58Δ*fHbp* strain was genetically engineered to express different sub-variants from a constitutive heterologous promoter. These recombinant strains were characterized for fH binding and the data confirmed that each sub-variant binds different levels of fH. Surface plasmon resonance revealed differences in the stability of the fHbp-fH complexes ranging over 2 orders of magnitude, indicating that differences in residues between and within variant groups can influence fH binding. Interestingly, the level of survival in human sera by recombinant MC58 strains expressing diverse sub-variants did not correlate with the level of fH binding, suggesting that the interaction of fHbp with fH is not the only function of fHbp that influences serum resistance. Furthermore, cross-reactive bactericidal activity was seen within each variant group, although the degree of activity varied suggesting that amino acid differences within each variant group influences the bactericidal antibody response.

Introduction

Disease caused by *Neisseria meningitidis*, including meningococcal septicemia and meningitis, is a significant health problem worldwide, the control of which is largely dependent on the availability and widespread use of vaccines (45). The majority of meningococcal disease is caused by 5 serogroups, and effective capsular-polysaccharide and conjugate vaccines are available to prevent disease caused by serogroups A, C, W-135, Y (reviewed in 53). However, there is still no broadly protective vaccine to combat serogroup B (MenB) disease, which is the main cause of meningococcal disease in the developed world (36, 44). The capsule polysaccharide of MenB is highly similar to glycoproteins in human neural tissues and as such is poorly immunogenic and an unsuitable vaccine candidate (9, 33). However, several outer membrane proteins have been identified as potential antigens for a MenB vaccine (reviewed in 23).

Factor H binding protein (fHbp) is a meningococcal protein antigen that is currently in vaccine development (previously referred to as GNA1870 (11) and LP2086 (10)). fHbp is a *Neisseria*-specific 29 kDa surface lipoprotein that binds human factor H (fH), a key inhibitor of the complement alternative pathway (14, 25). fH is a cofactor for the factor I-mediated cleavage and inactivation of C3b and also promotes the decay of the alternative pathway C3 convertase C3bBb (reviewed in 30). Therefore, binding of fH to fHbp on the meningococcal surface allows the pathogen to evade complement-mediated killing by the innate immune system. As such, fHbp is important for survival of bacteria in human serum and blood (25, 50, 56). The discovery that binding of fH to *N. meningitidis* is specific for human fH has significant implications for the study of this organism and its species specificity (14). fHbp induces high levels of bactericidal antibodies in mice (11) and humans (12, 18, 40, 42) (Brunelli et al., manuscript submitted), which activate bacterial killing by the classical complement pathway. Furthermore, it has been shown that binding of antibodies to fHbp can block binding of factor H, thus increasing the susceptibility of the bacterium to killing by the alternative complement pathway (25). Several other microorganisms have evolved the ability to bind fH and other complement inhibitors in order to evade complement mediated killing, and many of these proteins have also been studied for vaccine development (30).

fHbp is expressed by almost all *N. meningitidis* strains studied to date (10, 26, 32), although levels of expression vary between isolates (26, 29). Furthermore, amino acid sequence diversity exists for fHbp between strains. Several designations have been used to characterize different variants, which have been found to be antigenically poorly cross-reactive. Three variant groups, fHbp-1, fHbp-2 and fHbp-3, have been described, where fHbp-1 is the most abundant among group B meningococci (4) and sub-variant 1.1 (fHbp-1.1) is present in the multivalent Novartis MenB vaccine (4CMenB) currently in

Phase III clinical trials (1, 11, 42). Another system divides fHbp variants into subfamilies A and B, and the bivalent Pfizer (previously Wyeth) MenB vaccine that is in Phase II clinical trials contains a representative of each of these families, A05 (sub-variant 3.45) and B01 (sub-variant 1.55) (10, 20, 29, 32). Subfamilies A and B correspond to variants 2/3 and 1, respectively. A modular architecture has also been described for fHbp that describes the overall architecture of fHbp as being comprised of combinations of 5 modular variable segments that are flanked by invariable residues, with each of the modular variable segments being derived from either variant 1 or 3 fHbp genes (2).

The purpose of this study was to analyze a selection of the most frequently isolated fHbp sub-variants, to gain a better understanding of the ability of different sub-variants to bind fH, mediate serum resistance, and induce cross protective bactericidal antibodies.

Material and Methods

Bacterial strains and culture conditions

N. meningitidis strains used in this study are described in Table 1. *N. meningitidis* strains were routinely grown on chocolate agar (Biomerieaux), GC (Difco) agar supplemented with Kellogg's supplement I, or on Mueller Hinton (MH) agar (Difco) at 37°C/5% CO_2 overnight. For liquid cultures, colonies from overnight growth were used to inoculate 7 ml cultures (in MH broth supplemented with 0.25% glucose) to an OD_{600} of ~0.05. The culture was incubated for approximately 1.5-2.5 h at 37ºC with shaking until early-log (OD_{600} of ~ 0.25) or mid-log (OD_{600} of ~ 0.5). When required, erythromycin and chloramphenicol were used at final concentrations of 5 μg/ml. *Escherichia coli* strains used for cloning were cultured in Luria-Bertani (LB) broth or on LB agar (Difco). When required, ampicillin, erythromycin and chloramphenicol were used at a final concentration of 100, 100 and 20 μg/ml, respectively.

Phylogenetic analysis and reconstruction of fHbp sub-variants

The *fHbp* gene was amplified and sequenced from different *Neisseria* strains as previously reported (1). fHbp amino acid sequences were aligned using MUSCLE v3.6 (8), with default parameters. The evolutionary distances between pairs of aligned fHbp sequences were computed using the JTT matrix-based method (21) and units were scaled as number of amino acid substitutions per site, standard errors of distances were computed by bootstrap using 500 replicates. The phylogenetic reconstruction based on this distance matrix was inferred using the Neighbor-Joining method (46). The tree branch lengths are proportional to the evolutionary distances used to infer the phylogenetic tree. All sequence

alignment positions containing gaps were eliminated in pairwise sequence comparisons. Phylogenetic analyses were conducted in MEGA4 (54). The amino acid numbering used starts from the first residue (cysteine) of the mature protein.

Cloning and expression of fHbp sub-variants in *E. coli*

Recombinant DNA techniques were routinely performed as described by Sambrook *et al.* (47). Plasmid DNA preparations and purification of DNA fragments from PCR samples were performed using QIAGEN kits according to the manufacturer's instructions. All restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs or Roche. The recombinant proteins were obtained by cloning each gene into the pET-21b+ expression vector (Novagen) and expressing them as C-terminal histidine fusions in an *E. coli* heterologous system (*E. coli* DH5α for cloning and BL21(DE_3) (Invitrogen) for expression).

fHbp genes of sub-variants were amplified from the corresponding strains shown in Table 1. Primers 741v1f and 741v1r were used to amplify *fHbp* sub-variant 1 genes (except for 1.55 primers 741v1.55f and 741v1.55r were used, and for 1.4 primers 741v2,3f and 741v1r were used), primers 741v2,3f and 741v2r for sub-variant 2 genes, primers 741v2,3f and 741v3r for sub-variant 3.28, primers 741v3.13f and 741v3.13r for 3.45, and primers 741v1f and 741v2r for 1-2,3.x (see Table 2 for primer details). The expressed proteins did not contain the leader peptide. For sub-variants 1.4, 2.16, 2.19, 2.22, 2.25 and 3.28, the sequence GPDSDRLQQRRG from the gonococcal fHbp homologue was added to the N-terminal to aid expression in *E. coli.* PCR conditions used to amplify sub-variants are as follow: 1.1 and 1-2,3.x - 5x (94°C 30s, 57°C 30s, 68°C 1 min) then 30x (94°C 30s, 68°C 30s, 68°C 1 min); 2.16 and 3.28 - 5x (94°C 30s, 56°C 30s, 68°C 1 min), then 30x (94°C 30s, 71°C 30s, 68°C 1 min); for the remaining sub-variants, 94°C 2 min, 5x (94°C 30s, 52°C 30s, 68°C), 30x (94°C 30s, 65°C 30s, 68°C 1 min) then 68°C 10 min. PCR were performed on approximately 10 ng of chromosomal DNA using High Fidelity Taq DNA Polymerase (Invitrogen) as per manufacturer's instructions. PCR products were digested with *Nde*I as well as *Xho*I or *Hind*III, and then cloned into the *Nde*I/*Xho*I and *Nde*I/*Hind*III sites of pET-21b+. Recombinant plasmids were transformed into the *E. coli* expression strain. Recombinant strains were grown at 37°C to an OD_{600} of 0.6-0.8 and expression of recombinant proteins was induced with 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG; Sigma).

Purification of fHbp sub-variants in *E. coli*

Bacterial pellets were resuspended in 10ml of B-PER™ (Bacterial-Protein Extraction Reagent) containing 10 µl of $MgCl_2$ 100 mM, 50 µl of DNAse (Sigma) and 100 µl of lysozyme (Sigma),

incubated for 40 min at room temperature, then centrifuged at 35000 x*g* for 30 min. The supernatant was collected and subjected to 2 serial purification steps using metal affinity chromatography (IMAC) and ionic exchange chromatography with a desalting step in between. All purification steps were performed using an AKTAxpress chromatographic system and monitored at OD_{280}. For the IMAC purification step, filtered supernatants were automatically injected in 1ml Ni^{2+}-HisTrap FF column with a flow rate of 1 ml/min, columns were washed with 20 column volumes (CV) of washing buffer (50mM NaH_2PO_4 (Sigma), 300mM NaCl (Fluka), 30mM of Imidazole (Merck), pH 8.0). Then the His-tag fusion proteins were eluted with 5 CV of elution buffer (50mM NaH_2PO_4, 300mM NaCl, 500mM Imidazole, pH 8.0), and automatically loaded on 3 x 5 ml HiTrap Desalting (GE) columns connected in series and eluted with a flow rate of 5 ml/min. For ionic exchange chromatography, the eluted proteins were automatically loaded on 1ml-HiTrap Q HP, with a flow rate of 1ml/min. Subsequently, the column was washed with 10 CV of 50 mM Tris-HCl, pH8.0. The elution was set up in a linear gradient, between 50 mM Tris-HCl, pH8.0 and 50 mM Tris-HCl, 1.0 M NaCl, pH 8.0 buffer in 10 CV and 1 ml fractions were collected. Protein purity was checked by SDS-PAGE, and protein concentration was estimated using a Bradford assay.

Mice immunization

To prepare anti-fHbp polyclonal antisera, 20 µg of each recombinant protein was used to immunize 8 mice (6-week-old CD1 female mice; Charles River). The recombinant proteins were administered intraperitoneally at days 1, 21 and 35. Prior to immunization, fHbp sub-variants (100µg/ml protein) were adsorbed onto aluminium hydroxide (Alum; 3mg/ml) in 10mM Histidine buffer pH6.5 (Sigma) with NaCl (final osmolarity of 0.308/kg) for bactericidal assays. The solution was incubated for 15 min with stirring at room temperature and than stored overnight at 4°C. For antibodies used in flow cytometry, Freund's complete adjuvant was added to the antigen the day of immunization. Final formulations were isotonic and at physiological pH. Blood samples for analysis were taken on day 49. The treatments were performed in accordance with internal animal ethical committee and institutional guidelines.

Flow cytometry analysis of fH binding and fHbp expression

The ability of meningococci to bind fH was determined using a FACS-Scan flow cytometer. Briefly, approximately $1x10^8$ bacteria (grown in MH broth plus 0.25% glucose for approximately 2.5 h at 37ºC with shaking until mid-log phase (OD_{600} of ~ 0. 5)) were suspended in PBS+1%BSA and incubated with purified human fH (whole molecule, Calbiochem) at the quantity specified in each experiment in a

final reaction volume of 100 µl, for 20 min at 37˚C. fH binding was detected using polyclonal goat anti-human fH antibodies (Calbiochem) and Donkey anti-goat IgG-FITC (Jackson Immunoresearch). For competitive inhibition analysis of fH binding, mice polyclonal antisera specific for each fHbp sub-variant (at 1:100 dilution) was incubated with bacteria for 10 min at 37˚C prior to addition of fH.

The ability of mouse polyclonal anti-fHbp sera to bind to the surface of meningococci was measured using a 1:100 dilution of mouse polyclonal anti-fHbp antiserum specific for each sub-variant. Primary antibody binding was detected using a anti-mouse (whole molecule) FITC-conjugated antibody (Sigma) at a 1:100 dilution.

Immobilization of fH on a CM5 chip and kinetic analysis of fH binding by fHbp sub-variants

Surface Plasmon Resonance (SPR) analyses were performed using a Biacore X100 instrument (GE Healthcare). fH was coupled to a CM5-sensor chip (GE Healthcare) in 2 steps to modulate the total resonance units (RU) of immobilization, using the Biacore Amine Coupling Kit (GE Healthcare). In the first step, the carboxymethylated CM5 dextran layer was activated by a 1:1 (v/v) mixture of 0.4 M *N*-ethyl-*N*'-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.1 M *N*-hydroxysuccinimide (NHS) in MilliQ water with 7 min injection at 10 µL/min. After surface activation, 8 µg/mL fH in 10 mM sodium acetate buffer pH 4.0 was injected in the flow cell 2 with a 9 min pulse and a flow rate of 10 µL/min reaching 925 RU of protein immobilization. In the second step, 10 µg/mL fH was injected as above, to obtain 1500 RU of immobilized fH. The free N-hydroxysuccinimide ester groups were blocked with 3 manual injections (240 s each) of 1.0 M ethanolamine hydrochloride, pH 8.5. Untreated flow cell 1 was used as reference. Kinetic analyses were run as follows: 2-fold dilutions, in running buffer (PBS pH 7.2), of each protein in a concentration range from 15.6 to 500 nM were injected for 2 min at the flow rate of 45 µl/min followed by a 10 min dissociation time with PBS at the same flow rate. Biosensor regeneration was performed using 100 mM glycine, 3M NaCl, pH 2.0 with 1 min contact time and 10 µl/min flow rate. Interaction parameters (k_a, k_d, K_D) were determined by a simultaneous local fitting with a model of equimolar stoichiometry using the BIAevaluation X100 software version 1.0 (GE Healthcare).

Construction of fHbp knockout and complemented strains of *N. meningitidis*

The MC58Δ*fHbp,* 961-5945Δ*fHbp* and M1239Δ*fHbp* mutant strains have previously been described (formerly named Δ*gna1870*) (26). Complementation of the MC58Δ*fHbp* mutant with different fHbp variants was achieved by insertion of the *fHbp* gene, under the control of the constitutively active P_{tac} promoter, into a noncoding chromosomal location between the 2 converging open reading frames

(ORFs) NMB1428 and NMB1429, as previously described (50). The *fHbp* gene was amplified from the appropriate strain in Table 1 (using primers: 741-F2 and 741-R2 for 1.1; EP1For1.1 and EP5RV1.4 for 1.10; EP2For1.4 and EP6RV2.1 for 2.16 and 2.25; EP1For1.1 and EP6RV2.1 for 3.28 (Table 2)) and cloned as a *Nde*I-*Nsi*I fragment into the pComP$_{RBS}$ plasmid (50). This plasmid was linearised with *Spe*I and used to transform MC58Δ*fHbp.* Transformants were selected on chloramphenicol and checked by PCR, and complementation of the mutant strain was verified by flow cytometry. Several phase variable structures were analysed and no differences were identified in these strains for lipooligosaccharide (LOS; controlled by silver staining of LOS preparations as previously described (19)) and Opc (controlled by sequencing the repetitive DNA tracts associated with *opc,* using previously described primers (48)).

***Ex vivo* human serum models of meningococcal infection.**

Bacteria were grown in MH broth plus 0.25% glucose and 0.02mM Cytidine-5'-monophospho-N-acetylneuraminic acid sodium salt (CMP-NANA) for approximately 2.5 h at 37ºC with shaking until mid-log phase (OD_{600} of ~ 0. 5) then diluted in MH broth to approximately 10^5 CFU/ml. The assay was started by addition of 90µl serum to 10µl of the bacterial suspension. A sample from this mix was taken immediately and used as time 0 min. Cultures were incubated at 37°C with gentle agitation, and at various time points an aliquot of the sample was removed and the number of viable CFU were determined by plating serial dilutions onto MH agar. Experiments were performed in triplicate on several occasions. The Students' t-test was used to determine the statistical significance of survival of each complemented strain with respect to either the isogenic wild type strain or the *fHbp* mutant, with a p-value <0.05 considered to be significant. For preparation of human serum, whole blood was collected from healthy individuals (unimmunized against *N. meningitidis* and with no history of disease), coagulated at 25˚C for 30 min, centrifuged at 1,000 x*g* for 10 min at 4˚C and the supernatant retained.

Complement-mediated bactericidal activity

Serum bactericidal activity against *N. meningitidis* strains was evaluated as previously described with pooled baby rabbit serum (Pel-Freeze) (13), or human complement obtained from volunteer donors under informed consent (3). Complement donations were screened before use to ensure they lacked endogenous bactericidal activity at concentrations of both 25% and 50% in the assay and that they supported bactericidal activity. Bacteria were grown in MH broth plus 0.25% glucose for approximately 1.5 h at 37ºC with shaking until early-log phase (OD_{600} of ~ 0.25) then diluted in MH broth to approximately 10^5 CFU/ml. Serum bactericidal titers were defined as the serum dilution

resulting in 50% decrease in CFU/ml after 60 minutes incubation of bacteria with reaction mixture, compared to control CFU/ml at time 0. Typically, bacteria incubated with the negative control antibody in the presence of complement showed a 150 to 200% increase in CFU/ml during the 60 min incubation. The titers obtained with the sera anti-fHbp 1.1, 2.16 and 3.28 were an average of 4 different experiments.

Results

Characterization and phylogenetic analysis of fHbp sub-variants

Sequence analysis of fHbp in a worldwide collection of meningococcus B clinical isolates has allowed the identification of more than 330 peptide sub-variants (see the Neisseria Multi Locus Sequence Typing (MLST) website [http://pubmlst.org/neisseria/fHbp/]). A set of 12 diverse fHbp sub-variants were selected in order to characterize the influence of fHbp sequence diversity on surface expression, and the ability of the protein to bind human factor H (fH) and to induce cross-bactericidal antibodies (Table 1). These 12 sub-variants were identified in strains belonging to different clonal complexes and sequence types and isolated in different geographic regions in different years, and include members of each of the 3 variant groups. The sub-variants most frequently found on the basis of variability studies in different strain panels worldwide were included in the analysis (sub-variants 1.1, 1.4, 1.14, 2.16, 2.19) (1, 17, 24). The nomenclature used in this study is in accordance with the public fHbp database (http://neisseria.org) (4), in which new protein sub-variants are assigned a sequential numerical identifier, alongside a prefix corresponding to the Novartis variant designation (variant 1, 2, or 3) (26); e.g., fHbp 1.1 refers to Novartis variant 1, Neisseria.org protein sub-variant 1.

The phylogenetic tree constructed with the amino acid sequences of the 12 fHbp sub-variants used in this study (Fig. 1A) resembles the phylogenetic tree already described for the 3 variant classification system. One exception is fHbp 1-2,3.x, which is intermediate between fHbp-1 and fHbp variants 2 and 3. The alignment of the amino acid sequences of the 12 fHbp sub-variants analyzed (Fig. 1B) shows the chimeric nature of some fHbp sub-variants. The fHbp-2 forms have previously been described by Beernink *et al.* (2) as possible chimeric proteins between fHbp-1 and fHbp-3, based on a different classification of 5 modular variable segments that are derived from either a variant 1 or 3 *fHbp* gene. In fact, the sequence alignment highlights that the fHbp-2 sub-variants are composed of a fHbp-1 N-terminal (residues 1-97) and a fHbp-3 C-terminal (residues 98 to the stop codon). However, in this paper we refer to fHbp 1-2,3.x as a chimeric form between fHbp-1 and variants 2 and 3, since the N-terminal (from residues 1 to 191) is more similar to proteins of variant group 1 (96% identity to

1.1 in this region), and the C-terminal (from residue 192 to the stop codon) is more similar to variant groups 2 and 3 (95% identity to both 2.16 and 3.28, 56% identity to 1.1 in this region). fHbp 1.55 can also be considered as another a chimeric form, with the N-terminal (from residues 1 to 49) containing a group of amino acids typical of variant 3 molecules (96% identity to 3.28, 80% identity to 1.1 in this region), while the C-terminal (from residues 50 to the stop codon) is more similar to the variant 1 group (90% identity to 1.1). Due to the higher homology to the variant 1 group, 1.55 clusters with the variant 1 group. In general, while amino acid variations are scattered along the entire fHbp sequences, higher diversity is seen in the C-terminal part of the molecule where the majority of the bactericidal epitopes are located (13).

N. meningitidis strains carrying different fHbp sub-variants differ in their level of surface expression and fH binding

The level of expression and surface-exposure of each fHbp sub-variant in the panel of selected strains was evaluated by flow cytometry analysis using mice polyclonal fHbp homologous antisera (i.e., antisera raised against each sub-variant). As shown in Figure 2A, all strains express fHbp on their surface regardless of the sub-variant, but the level of expression varies between strains and can be considered as high (1.1, 1-2,3.x, 2.19, 3.28), medium (1.14, 1.55, 2.16, 2.22, 2.25, 3.45), or low (1.4, 1.10) for the sub-variants expressed by this panel of strains on the basis of fluorescence units being $\geq 10^2$ (high/medium) or $<10^2$ (low). Similar variability of fHbp expression has been described previously (26, 28). Expression of each sub-variant in the different strains was confirmed by western blot analysis of meningococcal whole cell extracts (data not shown).

Binding of human fH to the surface of each of the meningococcal strains was assessed by flow cytometry using anti-fH antibodies. All 12 strains studied bind fH at the highest concentration of fH used (50µg/ml). By titrating the fH concentration used (5 and 0.5 µg/ml), differences in binding were detected, suggesting that each sub-variant has different affinity for fH. Using 0.5µg/ml fH, the highest level of binding was seen for strains carrying sub-variants 1.1, 2.16, 2.19 and 3.45, relatively low binding was observed for strains carrying sub-variants 1.55, 1-2,3.x, 2.25 and 3.28, while no binding was seen for strains carrying sub-variants 1.4, 1.10, 1.14 and 2.22 (Fig. 2B). No fH binding was observed when the analysis was performed using the 3 *fHbp* knock out mutants, MC58Δ*fHbp*, 961-5945Δ*fHbp* and M1239Δ*fHbp*, used as controls for each of the 3 variant groups, indicating that for these strains the majority of fH binding is due to fHbp.

Furthermore, we evaluated whether anti-fHbp antibodies were able to inhibit the binding of fH (using the highest fH concentration, 50µg/ml) to the surface of each bacterial strain. The results show

that antibodies against each of the homologous fHbp variants have the ability to decrease the binding of fH to the cell surface (Fig. 2C), demonstrating that in this panel of strains fHbp is responsible for the majority of fH binding.

MC58 strains engineered to express equivalent levels of fHbp sub-variants differ in their level of fH binding

In order to investigate fH binding by each fHbp sub-variant, independently of differences in expression level or strain genotype, an isogenic panel of strains was generated to express a subset of 5 of the fHbp variants described above. The isogenic panel consists of the MC58Δ*fHbp* host strain into which the gene encoding different fHbp sub-variants was inserted *in trans* under the control of the constitutive P_{tac} promoter, to enable similar levels of gene expression. Incubation of these strains with human fH, as described above, revealed that despite there being a similar level of fHbp expression between the strains (Fig. 3A), there is a different level of binding between the variants (Fig. 3B). The small differences seen in surface reactivity for the anti-fHbp sera are most likely a result of different immunogenicity of the proteins used to raise mouse antisera, and/or the difference in affinity of the antibodies used, rather than differences in expression. Sub-variant 1.10 binds the least amount of fH, consistent with the data obtained with the strains naturally expressing this sub-variant. Sub-variants 1.1, 2.16 and 3.28 have a medium level binding, while sub-variant 2.25 has the highest level of binding (relative to 1.1, the approximate relative levels of fH binding were: 0.3 for 1.10; 0.9 for 2.16 and 3.28; 2.6 for 2.25). These data suggest that while the ability of a strain to bind fH is influenced by the expression level of fHbp as previously reported, differences in the sequence of the sub-variants also affect fH binding.

Recombinant fHbp sub-variants differ in their ability to bind human fH

Surface Plasmon Resonance (SPR) analysis was used to investigate the interaction between fH and the purified recombinant fHbp sub-variants, in terms of binding affinity and association/dissociation kinetics. All fHbp sub-variants bound fH and kinetics of association (k_a) with fH were comparable for all variants tested (within a range of ~3 fold above or below the k_a for sub-variant 1.1; Table 3). However, larger differences were seen in the dissociation kinetics (k_d), which describes the stability of the complex. The most stable binding to fH was observed by sub-variants 2.25 and 3.45 (lowest k_d, approximately 10 fold lower than 1.1), while the least stable fH binding is seen by sub-variants 1.14 and 1.10 (highest k_d, approximately 10 fold higher than 1.1). The corresponding thermodynamic dissociation constants (K_D) are in agreement with these differences (Table 3). The sensorgrams for sub-

variants 1.1, 1.10 and 2.25 are shown as representatives of the different stabilities of binding (Fig. 4). These results support the finding described above for fH binding by MC58 expressing different sub-variants at equivalent levels, where 1.10 was the lowest binder and 2.25 the highest binder.

The differences seen in kinetics analysis suggest that fHbp sequence variability influences the stability of binding between fH-fHbp. In principle, residues specifically found in sub-variants with lower affinity for fH could be considered to be responsible for higher dissociation rates. On the contrary, residues specifically observed in sub-variants with higher affinity could increase the stability of the fHbp-fH complex. Although the sequence comparison of the different sub-variants did not lead to an unambiguous identification of single amino acids responsible for increased or decreased affinity for fH, a collection of residues has been identified that could affect the stability of the complex (Fig.1B).

Serum survival of MC58 strains expressing fHbp sub-variants does not correlate with the level of fH binding

To determine if higher levels of fH binding lead to increased resistance to complement mediated killing in human serum, MC58 strains expressing different fHbp sub-variants were evaluated in an *ex vivo* human serum assay (Fig. 5). The MC58Δ*fHbp* strain is highly sensitive to killing by human serum due to the decreased binding of fH and thus the decreased inhibition of complement activation, as previously reported (26, 50). Resistance to serum killing was restored by complementation of this knockout with each of the 5 sub-variants examined, but to different degrees. The sub-variants 1.1 and 1.10 provided the greatest resistance, while sub-variants 2.16, 2.25 and 3.28 displayed 3-20 fold less survival, depending on the serum donor. This suggests that there is not a direct correlation between the amount of fH bound to the bacterial surface and the level of resistance to complement-mediated killing. In fact, variant 1.10, which has the lowest level of fH binding, provided one of the highest levels of survival in serum to the recombinant MC58 strain.

fHbp sub-variants induce bactericidal antibodies that differ in their level of cross-protection within and between variant groups

The established correlate of protection for meningococcal disease is the serum bactericidal antibody (SBA) assay, which measures complement-mediated bacterial killing by serum antibodies. To evaluate the ability of each fHbp sub-variant to induce bactericidal antibodies against MenB strains carrying homologous or heterologous sub-variants, postimmunization sera from mice injected with each sub-variant formulated with aluminium hydroxide were tested against 12 natural strains expressing the

fHbp sub-variants used in this study. As shown in Table 4A in SBA assays using rabbit complement, all sub-variants induced functional bactericidal antibodies against most of the strains in the same variant group. Bactericidal titers were generally higher against strains carrying the homologous sub-variant with the exception of sub-variant 1.55, which was negative for the homologous M1573 strain. However, strain M1573 was highly resistant to killing by all polyclonal mouse antisera tested (this strain was sensitive to the anticapsular monoclonal antibody SEAM 12). However, when the anti-1.55 serum was analyzed in the bactericidal assay against a recombinant MenB strain that was engineered to express the homologous sub-variant, a bactericidal titer of 8192 was obtained, suggesting that the negative titers against the homologous strain reflects an intrinsic resistance of this strain to SBA. fHbp 1.1, 1.4, 1.10 and 1.14 sub-variants elicited high bactericidal titers against strains carrying fHbp from variant group 1. Similarly, fHbp 2.16, 2.19, 2.22, 2.25 and 3.28 elicited high bactericidal titers against strains expressing variant 2 and 3 fHbp. As expected there is some cross coverage between groups 2 and 3 (26). However, sub-variant 3.45 was less effective in terms of inducing sera able to provide cross-coverage. Sub-variant 1.55, described as a chimeric form (Fig. 1), did not elicit bactericidal antibodies against MC58 (expressing fHbp 1.1) possibly because of the sequence distance between these 2 sub-variants. In addition, it had no bactericidal activity against strains expressing variant 3 fHbp suggesting that the region of sequence identity does not include bactericidal epitopes. In contrast, the 1-2,3.x chimera provided a particularly broad range of coverage against strains carrying each of the 3 variant groups, which is likely due to the more pronounced hybrid nature of the protein and the presence of conserved epitopes from all 3 groups. The SBA assays were also performed using human complement (Table 4B) and, as expected, there is a general decrease in the bactericidal titers and the cross-reactivity seen. However, the trend of cross-coverage within the same variant and between variants 2 and 3 is maintained. Cross-coverage between the 3 variants is induced only by fHbp-1-2,3.x.

Discussion

fHbp has been the focus of increasing interest in recent years due to its important role in meningococcal survival and species specificity, as well as its inclusion in 2 investigational MenB vaccines currently in clinical trials (reviewed in 39). Despite fHbp having a conserved function in *N. meningitidis*, with fH binding providing the ability to down regulate killing by the human complement pathway, several genetically and antigenically diverse variants of fHbp have been identified (1, 32). It has been proposed that these variants have a mosaic architecture, like many meningococcal proteins, that may result from the high degree of horizontal gene transfer that is common in pathogenic Neisseria

(2, 32). In this study we have characterized a selection of fHbp sub-variants in terms of their expression, ability to bind fH, role in serum resistance and their immunogenicity and ability to induce cross protection in SBA assays, in order to better understand the functional significance of fHbp sequence variability. This selection of sub-variants includes fHbp 1.1 that is present in the Novartis MenB vaccine, as well as fHbp 1.55 (B01) and fHbp 3.45 (A05) that are present in the Pfizer MenB vaccine. Two particular chimeric forms of fHbp are present in the panel of sub-variants analysed, *i.e.* 1-2,3.x and 1.55.

Through a detailed analysis of fH binding to 12 different sub-variants, using several approaches with live bacteria (natural and recombinant strains) or purified recombinant proteins, we have demonstrated that different fHbp sub-variants have different fH binding characteristics. In particular, we found that a panel of natural strains, or a single recombinant strain expressing various fHbp sub-variants, bound different levels of fH. Differences in the level of binding of fH to the bacterial surface did not always correlate with the expression level of fHbp, rather the stability of binding seems to be an important factor. SPR analysis of fH binding to recombinant fHbp proteins showed a range of binding affinities between the sub-variants varying over 2 orders of magnitude for the dissociation rate constant (k_d) and more than 1 order of magnitude for the dissociation constant (K_D). These results are supported by the findings on a different panel of sub-variants that was published by Dunphy *et al.* while this manuscript was under review (7). It has been elucidated that fHbp binds to short consensus repeat 6 (SCR6) of fH (51). Moreover, the NMR structure of the C-terminal portion of fHbp 1.1 (5) and the crystal structure of fHbp 1.1 in complex with fH SCR67 (49) has led to identification of residues important for binding of anti-fHbp antibodies and fH, respectively. High affinity binding was previously seen between the fH SCR67 fragment and sub-variant 1.1 (K_D of 5 nM) (49). We also saw high affinity binding in our experiments using the entire fH molecule (SCR1-20) with a K_D of 45 nM for sub-variant 1.1, while the K_D of the sub-variants ranged from 7-350 nM. This variation in binding affinity was evident in flow cytometry using whole cell bacteria and a titration of the fH concentrations, with some variants having binding even at the very low concentrations of fH (0.5µg/ml), while others had no binding. The most variable residues between the 3 variant groups, as well as those involved in fH binding or those that are targeted by mAbs, were found to be in the upper exposed surface of the molecule (C domain), which most likely has the greatest exposure to the immune system (5, 49). It was proposed that an extended recognition site for fH exists across the entire surface of fHbp, with numerous electrostatic interactions, H-bonds and salt-bridges with both ß-barrels of fHbp (49). Twenty amino acid residues of fHbp have been identified that interact with fH, and substitution of 2 glutamate residues led to a loss of fH binding (49). Since one of these glutamate

residues is not conserved in all the variants analyzed in this study, we speculate that other residues may be involved in fH binding. Indeed, there are several amino acid residues that are conserved in the sub-variants with higher binding affinity. Residues specifically present in higher or lower affinity binders tend to cluster in three regions that contain residues known to be involved in interaction with fH (49), namely residues 115-119, 126-134 and 241-248. In particular, residues 119, 128 and 130 have substitutions that are associated either with higher (H119/K, Q128/S, R130/L) or lower (H119/D, Q128/R L130/K) affinity binding to fH. In all of these cases, a difference in charge can be observed between the sequences of stable and less stable binders, confirming the hypothesis that electrostatic effects could play a critical role in modulating the interaction of fHbp with fH. Further sequence comparison and analysis of binding capabilities of mutants may help confirm the role of these residues.

In terms of the functional significance of the different levels of fH binding displayed by the sub-variants and strains, the level of serum resistance of a strain did not correlate with the level of fH binding or the affinity of binding. This was particularly evident for *N. meningitidis* strain MC58Δ*fHbp* that was complemented with sub-variant 1.10; this strain had one of the lowest levels of fH binding (~3 fold less than to 1.1) but had equivalent survival to the strain complemented with sub-variant 1.1. Sub-variant 1.10 also had one of the lowest binding stabilities in SPR analyses. It is important to note that fH is present at high levels in serum (110-615 μg/ml; 0.71-3.9 μM (43)), which is in excess of the concentrations used in these studies and binding of fH to fHbp is most likely saturated even for strains with sub-variants with low binding or stability. However, fH is also present in other sites relevant to meningococcal colonization and disease, including the nasopharynx (37, 55) and the CSF (16). The levels of fH in these sites are not well characterized, but concentrations at mucosal surfaces are expected to be ~10% of serum levels, and it has been reported that levels in CSF are <1% of those found in serum (16). Hence, the different levels of fH binding by sub-variants may be more relevant in different niches during colonization or disease, rather than during disease in the blood. Also, it has been reported that for strain 2996, which has very low fHbp expression and negligible fH binding, that deletion of the *fHbp* gene leads to increased serum sensitivity (25). These data suggest that either small levels of fH binding are sufficient to provide serum resistance and/or that other host factors also play a role in fHbp dependent survival in human serum. Indeed, *N. meningitidis* Δ*fHbp* mutant strains (both sub-variant 1.1 and 1.10 strains) have been shown to be sensitive to killing by the antimicrobial peptide LL-37, indicating an additional role of fHbp (50). This may be relevant for survival in serum, given that the precursor of LL-37, hCAP-18, is present in plasma at levels of approximately 1.2 μg/ml, which is more than 20% of the amount in circulating neutrophils (52). The importance of various factors of the immune system in preventing meningococcal disease is indicated by the increased incidence of

disease in people with deficiencies of fH or complement factors (i.e., factor I, C5 to C9) (15, 27, 35, 41). Work is underway to determine if different sub-variants mediate different levels of resistance to LL-37 and/or additional host defenses. It was recently reported that Neisserial Surface Protein A (NspA) also binds fH, particularly in unencapsulated strains expressing lipopolysaccharide with truncated heptose I chains, and enhances meningococcal resistance to complement (22), indicating that additional factors may influence the level of fH binding by the panel of strains examined. Since more than one meningococcal protein can interact with fH it is a possibility that a specific fHbp sub-variant may be best suited to a particular strain if it has evolved a certain combination of protein variants to ensure optimal fitness in the host. This could be relevant to the level of serum resistance seen for the MC58 strains engineered to express different sub-variants; for example complementation of MC58 (normally expresses fHbp-1.1) with a sub-variant 2 or 3 protein may be less effective in mediating serum resistance than complementation with a more closely related sub-variant 1 protein.

In terms of the immunogenicity of fHbp and its ability to induce bactericidal antibodies in mice, the sub-variants analysed displayed different levels of cross-reactive bactericidal activity within each variant group in the panel of strains tested in SBA assays using both rabbit and human complement. The majority of sub-variants were unable to induce antibodies that were cross-protective between the 3 variant groups, supporting previously published data (26). However, of particular interest was the finding that the natural 1-2,3.x chimera induces high SBA titers against sub-variants in each of the 3 variant groups. This broad coverage is likely due to the hybrid nature of the protein sequence and the presence of epitopes from both the variant 1, 2 and 3 groups. However, sub-variant 1.55 is also a hybrid between fHbp-1and fHbp-3, but a similar broad protection is not seen by this sub-variant in our experimental conditions. The lower titers and cross-reactivity seen in the SBA assays using human complement may be due to the interactions of human fH with the meningococcus and the down-regulation of complement activation. However, we have shown that binding of anti-fHbp antibodies to meningococcal strains decreased the amount of fH binding to the bacterial surface, and therefore it is not expected that complement activation would be fully down regulated in the presence of human complement and anti-fHbp antibodies. It has been suggested that the best predictor for killing by anti-fHbp antibodies is the surface expression level of fHbp (26). However, the variability of cross-reactive bactericidal activity within a variant group suggests that amino acid variations within each sub-variant also have an important influence on the bactericidal activity.

Many microbial surface proteins, in addition to fHbp, have been identified that have extensive sequence variability and yet retain a conserved ligand binding function. For example, C4BP binding by the hypervariable region of *Streptococcus pyogenes* M protein, sialic acid binding by heamagglutinin

(HA) of influenza, and CD4 binding by gp120 of HIV-1. HA and gp120 both have regions that are relatively well conserved between variants that are required for ligand binding (6, 34, 57), whereas M protein variants lack conserved amino acid motif or residues to explain binding (38). *N. meningitidis*, as a strictly host adapted pathogen, is adept at varying its surface structures to avoid host defenses (31). As such, fHbp seems to have evolved to maintain conserved regions that are involved in fH recognition that enable activation of the alternative complement pathway to be decreased at the bacterial surface, but also contain a large degree of variability, which may enable bacteria to escape classical complement activation.

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Table 1. Meningococcal strains used in this study.

Strain	Clonal complex	ST	Year	Country	Serogroup: serotype: serosubtype	fHbp variant*
MC58	32	74	1985	UK	B:15:P1.7,16b	1.1
M01-0240149	41/44	41	2001	UK	B:4:P1.7,4	1.4
M0-01240185	11	11	2001	UK	B:2a:P1.5,10	1.10
NZ98/254	41/44	42	1998	NZ	B:4:P1.4	1.14
M1573	41/44	44	1995	USA	B: P7-1,1	1.55
NL096	41/44	110	1960	The Netherlands	B:14:NT	1-2,3.x
961-5945	8	153	1996	AUS	B:2b:P1.21,16	2.16
M3153	41/44	5906	1996	USA	B:4,7:P1.4	2.19
C11	NA	345	1965	Cuba	C:NT:P1.1	2.22
M2552	103	103	1996	USA	B:NT:NT	2.25
M1239	41/44	437	1994	USA	B:14:P1.23,14	3.28
M0-01240320	213	213	2001	UK	B:1:P1.22,14	3.45

*fHbp is named in terms of the translated (protein) sequence as 'variant_class.protein_ID', in accordance with the public fHbp database (http://neisseria.org), in which new protein sub-variants are assigned a sequential numerical identifier, alongside a prefix corresponding to the Novartis variant designation (variant 1, 2, or 3); e.g., fHbp 1.1 refers to Novartis variant 1, Neisseria.org protein sub-variant 1. *Neisseria* strain NL096 expresses a hybrid natural chimera of fHbp, hence the designation 1-2,3.x (the peptide number has not yet been assigned). ST, sequence type as determined by MLST. NA, not assigned.

Table 2. Oligonucleotides used in this study

Name	Sequence[a]	Site[b]
U741-F1	gctctagaCCAGCCACGGCGCATAC	*XbaI*
U741-R1	tccccgggGACGGCATTTTGTTTACAGG	*SmaI*
D741-F1	tccccgggCGCCAAGCAATAACCATTG	*SmaI*
D741-R1	cccgctcgagCAGCGTATCGAACCATGC	*XhoI*
741-F2	ggattccatatgGTGAATCGAACTGCCTTC	*NdeI*
741-R2	ccaatgcatTTATTGCTTGGCGGCAAG	*NsiI*
EP1For1.1	CGCggatcccatatgGTGAATCGAACTGCCTTC	*BamHI, NdeI*
EP5RV1.4	TGCATGCATTTACTGCTTGGCGGCAAG	*NsiI*
EP2For1.4	CGCggatcccatatgGTGAACCGAACTGCCTTC	*BamHI, NdeI*
EP6RV2.1	TGCATGCATCTACTGTTTGCCGGCGAT	*NsiI*
741v1f	cgcggatcccatatgGTCGCCGCCGACATCG	*NdeI*
741v1r	cccgctcgagTTGCTTGGCGGCAAGGC	*XhoI*
741v1.55f	cgcggatcccatatgAGCAGCGGAGGCGGCGG	*NdeI*
741v1.55r	cccgctcgagCTGCTTGGCGGCAAGACC	*XhoI*
741v2,3f	cgcggatcccatatg*GGCCCTGATTCTGACCGCCTGCAGC AGCGGAGG*GTCGCCGCCGACATCGG	*NdeI*
741v2r	cccgctcgagCTGTTTGCCGGCGATGCC	*XhoI*
741v3r	gcccaagcttCTGTTTGCCGGCGATGCC	*HindIII*
741v3.13f	cgcggatcccatatgAGCAGCGGAAGCGGAAGC	*NdeI*
741v3.13r	cccgctcgagCTGTTTGCCGGCGATGCC	*XhoI*

[a] Capital letters correspond to nucleotides of the meningococcal *fHbp* sequence (the gonococcus sequence moiety is reported in italics) and small letters correspond to nucleotides added for cloning reasons (underlined letters indicate sequences of restriction enzyme sites used for cloning PCR fragments). [b] Enzymes for which the restriction sites are present in the sequence of the primer, added for cloning reasons.

Table 3. Kinetic analysis of fH binding to fHbp sub-variants by surface plasmon resonance.

fHbp variant	k_a	k_d	Relative k_d *	K_D (nM)
1.1 [I]	1.43×10^5	0.65×10^{-2}	1.0	45
1.4	3.38×10^5	1.86×10^{-2}	2.9	55
1.10	2.85×10^5	5.09×10^{-2}	7.8	178
1.14 [II]	2.23×10^5	7.16×10^{-2}	11.0	350
1.55	0.76×10^5	0.78×10^{-2}	1.2	102
1-2,3.x	0.87×10^5	1.65×10^{-2}	2.5	190
2.16	0.96×10^5	0.42×10^{-2}	0.7	44
2.19	0.54×10^5	0.21×10^{-2}	0.3	40
2.22	0.45×10^5	0.22×10^{-2}	0.3	48
2.25 [III]	0.60×10^5	0.04×10^{-2}	0.1	7
3.28	1.66×10^5	0.26×10^{-2}	0.4	16
3.45	0.59×10^5	0.09×10^{-2}	0.1	16

k_a, association rate constant ($M^{-1}.s^{-1}$); k_d, dissociation rate constant (s^{-1}), lower k_d corresponds to higher affinity binding; K_D, equilibrium dissociation constant (k_d/k_a). M=molar and s= seconds. *The k_d of fH binding for each sub-variant relative to sub-variant 1.1. See Table 1 for fHbp sub-variant details.

Replicate measurements for representative sub-variants are:

[I] Medium affinity fH binder, fHbp-1.1: k_a (1.43±0.21)x10^5, k_d (0.65±0.13)x10^{-2}, K_D 45±5.

[II]Low affinity fH binder, fHbp-1.14: k_a (2.23±0.28)x10^5, k_d (7.16±1.39)x10^{-2}, K_D 350±115.

[III]High affinity fH binder, fHbp-2.25: k_a (0.60±0.08)x10^5, k_d (0.04±0.01)x10^{-2}, K_D 7±0.5.

P-values calculated using a Student's t-test, compared to fHbp-1.1, are: k_a 0.08, k_d 0.01 and K_D 0.06 for fHbp-1.14; k_a 0.06, k_d 0.03 and K_D 0.01 for fHbp-2.25.

Table 4. Serum bactericidal activity induced by fHbp sub-variants

Complement source and *N. meningitidis* strain (fHbp subvariant)	Bactericidal titer[a] for anti-fHbp sera against:											
	Variant 1				Chimeric		Variant 2				Variant 3	
	1.1	1.4	1.10	1.14	1.55	1-2,3.x	2.16	2.19	2.22	2.25	3.28	3.45
Rabbit complement												
MC58 (1.1)	[>8,192]	>8,192	4,096	>8,192	<16	>8,192	<16	64	<16	16	64	<64
M01-0240149 (1.4)	512	[2,048]	1,024	1,024	1,024	2,048	64	<16	<16	<16	<16	<32
M01-0240185 (1.10)	256	128	[2,048]	2,048	1,024	2,048	<16	<16	<16	<16	<16	<16
NZ98/254 (1.14)	128	512	2,048	[>8,192]	256	512	<16	32	<16	<16	<16	<16
M1573 (1.55)	<16	<16	<16	<16	[<16[b]]	<16	<16	<16	<16	<16	<16	<16
NL096 (1-2,3.x)	<16	32	<16	<16	<16	[8,192]	<16	<16	<16	<16	<16	<16
961-5945 (2.16)	32	128	64	256	<32	>8,192	[>8,192]	>8,192	2,048	2,048	4,096	512
M3153 (2.19)	<16	32	128	256	<16	2,048	8,192	[8,192]	2,048	1,024	1,024	64
C11 (2.22)	16	<16	<16	<16	<16	128	256	2,048	[4,096]	256	2,048	<16
M2552 (2.25)	16	<16	<16	<16	<32	128	512	4,096	8,192	[512]	128	<32
M1239 (3.28)	<16	<16	<16	<16	<32	128	512	1,024	2048	512	[8,192]	64
M01-0240320 (3.45)	<32	<32	<32	<32	<32	2,048	2,048	>8,192	1,024	4,096	4,096	[4,096]
Human complement												
MC58 (1.1)	[2,048]	1,024	16	16	<8	32	<8	<8	<8	<8	<8	<8
M01-0240149 (1.4)	128	[1,024]	256	1,024	<8	256	<8	<8	<8	<8	<8	<8
M01-0240185 (1.10)	<8	512	[1,024]	512	512	512	<8	<8	<8	<8	<8	<8
NZ98/254 (1.14)	64	32	256	[1,024]	256	128	<8	<8	<8	<8	<8	<8
M1573 (1.55)	<8	<8	<8	<8	[<8[b]]	<8	<8	<8	<8	<8	<8	<8
NL096 (1-2,3.x)	<8	<8	<8	<8	<8	[1,024]	<8	<8	<8	<8	<8	<8
961-5945 (2.16)	<8	<8	<8	<8	<8	32	[2,048]	512	16	<8	<8	16
M3153 (2.19)	<8	<8	<8	<8	<8	256	128	[512]	64	128	32	<8
C11 (2.22)	<8	<8	<8	<8	<8	64	128	128	[512]	<8	256	<8
M2552 (2.25)	<8	<8	<8	<8	<8	<8	128	128	128	[128]	<8	<8
M1239 (3.28)	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	[512]	<8
M01-0240320 (3.45)	<8	<8	<8	<8	<8	512	512	512	256	512	256	[2,048]

Bactericidal titers are expressed as the reciprocal of the serum dilution of mouse polyclonal antibodies yielding ≥50% bactericidal killing. Antigens were formulated in aluminium hydroxide, and assays were performed with (A) rabbit complement (rSBA) or (B) human complement (hSBA); rSBA titers ≥128 and hSBA titers ≥16 (four-fold increase of baseline) are shown in grey. The black boxes show the SBA titres obtained for each fHbp sub-variant against the strain expressing the homologous sub-variant. *A bactericidal titer of 8192 was seen for sub-variant 1.55 using a recombinant MenB strain that was engineered to express the homologous sub-variant, demonstrating its immunogenicity. Negative results were obtained for strain M1573, which was highly resistant to killing by all antisera.

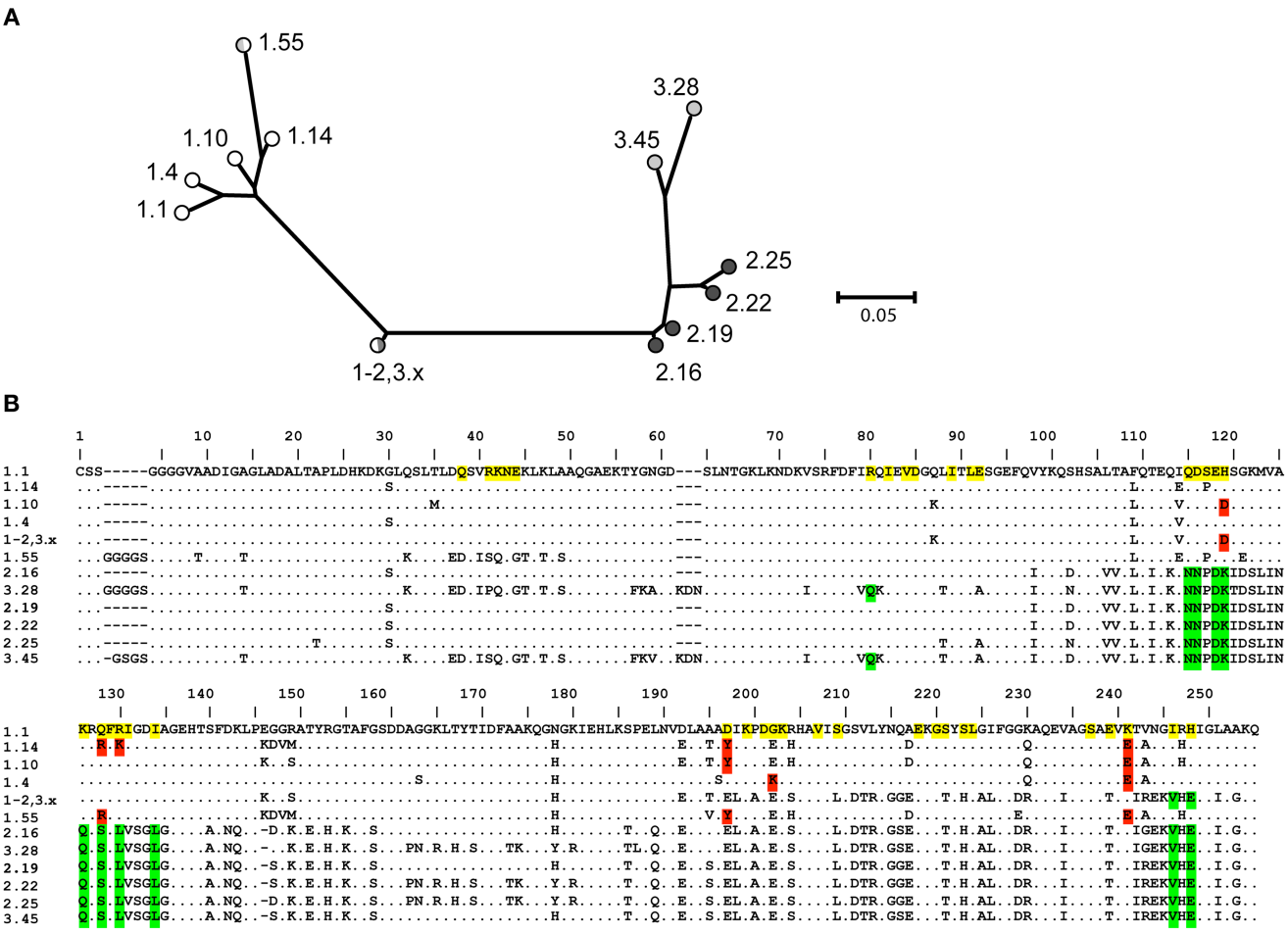

Figure 1. Alignment and phylogenetic analysis of the fHbp sub-variants. (A). Phylogenetic reconstruction of the 12 fHbp sub-variants used in this study based on distance matrix using the Neighbor-Joining method, where the tree branch lengths are proportional to the evolutionary distances used to infer the phylogenetic tree. Sequences belonging to fHbp variant groups 1, 2 and 3, are shown in white, dark grey and light grey, respectively. fHbp 1-2,3.x and 1.55 are highlighted as chimeric sub-variants. Measuring the rate of amino acid mutations per position, the average distance of sub-variants within the 1, 2 and 3 variant groups was 0.077 (± 0.012), 0.041 (± 0.010) and 0.070 (± 0.016), respectively. The average distance between groups 1-2, 1-3 and 2-3 was 0.384 (± 0.042), 0.470 (± 0.046) and 0.123 (± 0.019), respectively. fHbp 1-2,3.x is in an intermediate position between variants 1, 2 and 3, with distances of 0.174, 0.205 and 0.307, respectively. **(B).** Alignment of the amino acid residues of the fHbp sub-variants, using sub-variant 1.1 as a reference. Residues identical to sub-variant 1.1 are shown as dots. Residues previously described to be involved in interaction with fH are colored in yellow on the 1.1 sequence. Amino acids typical of less stable fH binders (deleterious for affinity) are colored in red. Amino acids typical of more stable fH binders (potentially increasing the affinity) are colored in green (see Table 3). Except 1.1, used as reference sequence, the sub-variants are ordered in the alignment for increasing fH affinity, according to the surface plasmon resonance dissociation rate constant (sub-variants 1.1, 1.4, 1.10, 1-2,3.x and 1.55 are classified as lower affinity binders, while 2.16, 3.28, 2.19, 2.22, 2.25 and 3.45 are considered as higher affinity binders).

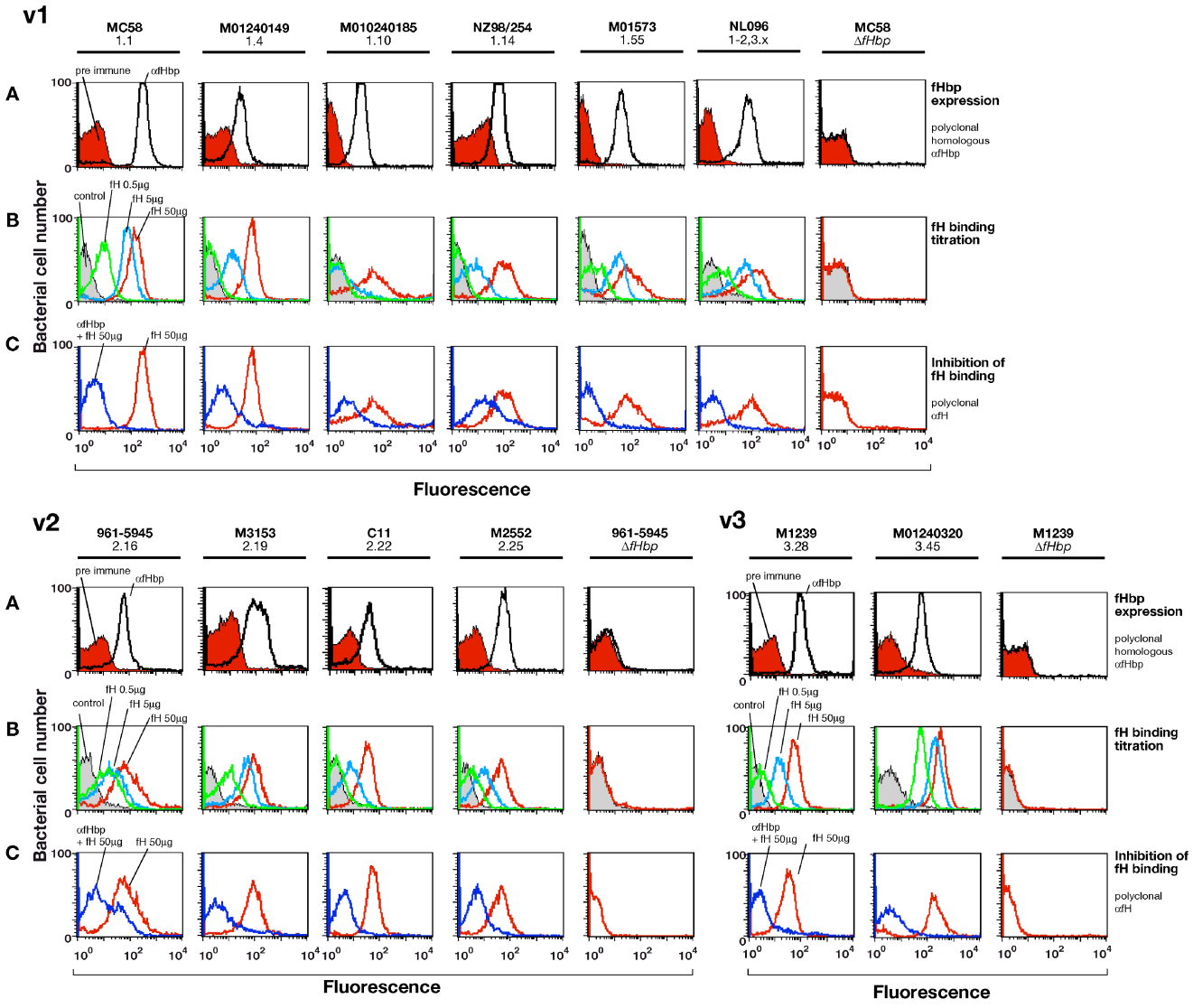

Figure 2. Flow cytometry analysis of fHbp surface expression and fH binding of *N. meningitidis* strains.

Panel (**A**) shows expression of fHbp on the meningococcal cell surface as detected by binding of polyclonal antiserum raised against the homologous fHbp sub-variant for each strain. Panel (**B**) shows binding of human fH (0.5, 5 or 50 µg/ml) to the cell surface, detected by binding of polyclonal antiserum raised human fH. Panel (**C**) shows inhibition of fH binding after incubation of bacteria with the polyclonal homologous fHbp antiserum. The strain name and the fHbp sub-variant expressed by each strain is shown above panel A.

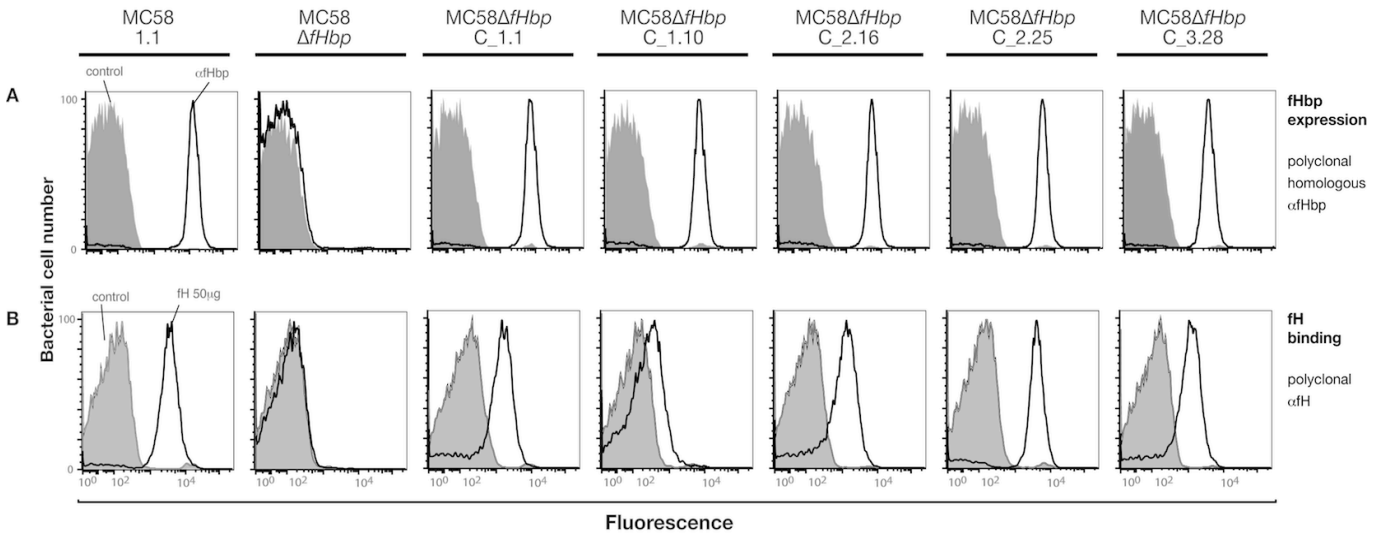

Figure 3. Flow cytometry analysis of fHbp surface expression and fH binding of *N. meningitidis* MC58 strains engineered to express different fHbp sub-variants.

Panel (A) shows expression of fHbp on the meningococcal cell surface as detected by binding of polyclonal antiserum raised against the homologous fHbp sub-variant for each strain. White profiles show reaction with immune sera. Panel (B) shows binding of human fH (50 µg/ml) to the cell surface, detected by binding of polyclonal antiserum raised against human fH (white profiles; grey profiles represent the negative control where no fH was added to the sample).

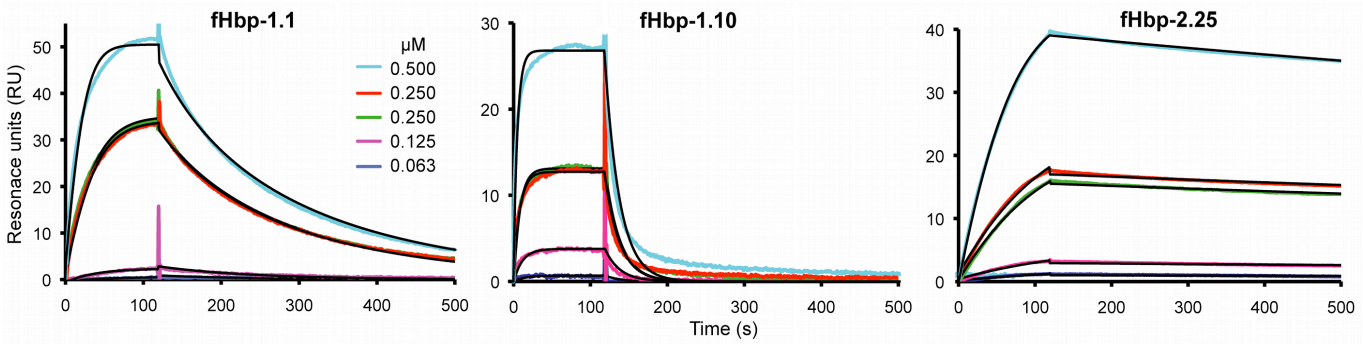

Figure 4. Surface Plasmon resonance kinetic analyses.

SPR kinetic analysis of recombinant fHbp sub-variants 1.1, 1.10 and 2.25 flowed over the fH-bound sensor chip at different concentrations. 0.250 µM samples (green and red lines) were run in duplicate. Interaction parameters were determined by a simultaneous local fitting (black lines) with a model of equimolar stoichiometry using the BIAevaluation X100 software version 1.0.

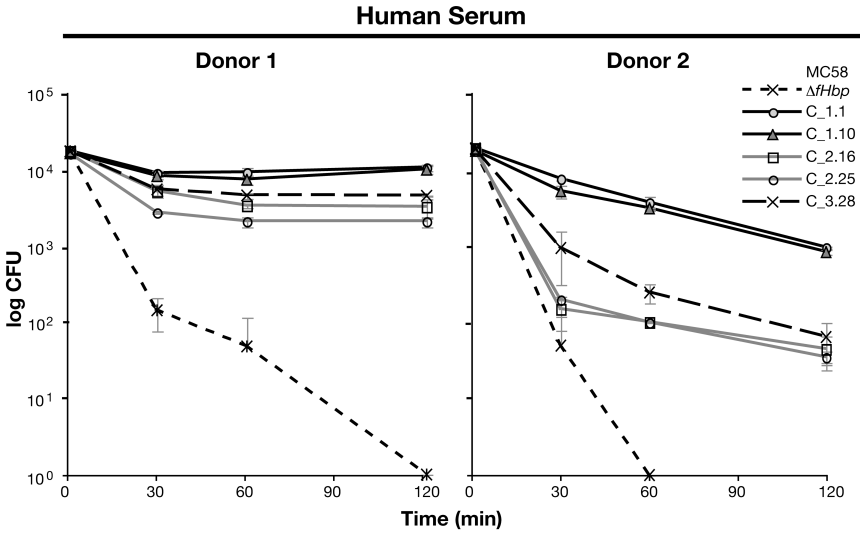

Figure 5. Survival of *N. meningitidis* MC58 strains engineered to express fHbp sub-variants in an *ex vivo* human serum model of meningococcal infection.

Experiments were performed in triplicate on several occasions and representative results are shown for serum from 2 different donors. Error bars indicate ±1 standard deviation of the mean. P-values using Student's t-test for survival at 120 min: between Δ*fHbp* and Δ*fHbp*C_1.1 (C_1.1), < 0.004; sub-variants in group 1 compared to sub-variants in group 2, < 0.02; between sub-variants within group 1, or within groups 2 and 3, > 0.1.