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Factor H Facilitates Adherence of Neisseria gonorrhoeae to Complement Receptor 3 on Eukaryotic Cells

Sarika Agarwal,* Sanjay Ram,* Jutamas Ngampasutadol,* Sunita Gulati,* Peter F. Zipfel,†‡ and Peter A. Rice*†

Neisseria gonorrhoeae can engage human complement receptor 3 (CR3) directly or through surface-bound iC3b. Factor H (fH) that binds to bacteria facilitates conversion of C3b to iC3b. fH also binds directly to CR3 on professional phagocytes. Certain nonprofessional phagocytes, such as primary cervical epithelial cells, also express CR3. We hypothesized that fH could bridge bacteria to CR3 and facilitate gonococcal association with host cells. Specificity of the fH–CR3 interaction was confirmed using human CR3-transfected Chinese hamster ovary (CHO-CR3) cells. Using recombinant proteins that comprised contiguous fH domains (fH contains 20 short consensus repeat [SCR] domains) fused to murine Fc, we observed strong binding through SCRs 18–20, whereas weaker binding occurred through SCRs 6–10. Both regions also bound to unsialylated porin (Por) B.1A-expressing N. gonorrhoeae. Accordingly, fH-related protein 1 (three of its five SCRs are highly homologous to fH SCRs 18–20) bound to CHO-CR3 and to unsialylated PorB.1A gonococci. An alternatively spliced variant of fH called fH-like protein-1 (contains fH SCRs 1–7) bound to gonococci but minimally to CHO-CR3. An fH SCRs 6–20 construct enhanced binding of unsialylated PorB.1A gonococci to CHO-CR3. However, a construct that contained only the apparently relevant SCRs (6, 7, and 18–20) bound to CHO-CR3 and to gonococci separately, but did not enhance bacteria–CR3 interactions, suggesting that the intervening SCRs (8–17) may impart a configurational and spatial requirement for fH to bridge gonococci to CR3. These results indicate adherence between fH-coated gonococci and CR3 and may provide a means for gonococci to gain sanctuary into nonprofessional phagocytes. The Journal of Immunology, 2010, 185: 000–000.
also includes fH-like protein 1 (FHL-1) and six additional human fH-related (FHR) proteins (CFHR1, -2, -3, -4A, -4B, and -5; these proteins have also been referred to as FHR-1 through FHR-5) (16, 17). These proteins are immunologically and structurally related and are composed exclusively of SCRs. CFHR1 has been shown recently to inhibit C5 convertase activity and interferes with C5b deposition and C5b-9 formation (18) and is present in the human plasma at a concentration of 70–100 μg/ml (18). FHL-1 consists of the seven N-terminal SCRs of fH plus four unique amino acids at the C terminus (19) and is present in the human plasma at a concentration of 10–50 μg/ml (20). Akin to fH, FHL-1 also possesses complement inhibiting activity. In addition, FHL-1 can facilitate cell adhesion (21).

Gonococci isolated from cervical secretions of infected women are coated with complement components (22). Complement present in cervical secretions (6, 18–21) may be the result of “spill over” into the female genitourinary tract from the systemic circulation or from de novo synthesis by cervical epithelial cells (6, 23). One of these components, fH, binds to gonococci present in secretions in significant quantity (22). In addition to its function as a complement inhibitor both in solution and on cell surfaces, fH is also an adhesion ligand for neutrophils and platelets and may also participate in immune adherence in other host tissues (24–26).

*N. gonorrhoeae* can scavenge CMP-NANA from the host to sialylate the lacto-N-neotetraose structure of their lipo-oligosaccharide (LOS) molecules. We have shown previously that sialylation of gonococcal LOS enhances binding of fH to bacteria (27, 28), via Por molecules (10, 28). However, certain strains of *N. gonorrhoeae* can bind to fH independently of LOS sialylation. The Por molecule plays an important role in enabling gonococci (both sialylated and unssialylated) to bind to fH (10, 29). In *N. gonorrhoeae*, Por is an allelic protein and exists as two forms called PorB.1A and PorB.1B (30). In the unsialylated state, PorB.1A gonococcal strains bind more fH than PorB.1B strains (29). PorB.1A strains usually cause disseminated disease, whereas PorB.1B strains more frequently are the causative agent of gonococcal PID and less symptomatic local disease in women (30, 31). Two regions in fH, one spanning SCRs 18–20 and the other in SCR 6 are involved in binding to gonococci (32).

In this study, we examined the role of fH and related molecules, CFHR1 and FHL-1, that bind to gonococci in facilitating the interaction between bacteria and CR3 on eukaryotic cells. We reasoned that because CR3 is expressed by cervical epithelial cells and alternative complement components are present in the cervical mucosa, the interaction between fH and CR3 could contribute to binding of gonococci to cervical epithelia. We also characterized the specific regions of fH that play a role in facilitating gonococcal adherence to CR3. We used CR3 transfected Chinese hamster ovary (CHO) cells to capitalize on an existing eukaryotic nonprofessional phagocyte model that isolates events surrounding interactions with CR3. Better understanding of the mechanism(s) of engagement between gonococci and host tissues is important because effective intervention strategies that may interrupt the ability of the organism to secure a foothold and cause infection would be important in disease prevention.

### Materials and Methods

#### Cell lines

CHO cells stably transfected with CR3 (CHO-CR3) (33) and control CHO cells transfected with the “empty” vector that contained the selection marker neomycin phosphotransferase (CHO-Neo) (34) were the gift of Dr. Douglas T. Golenbock (University of Massachusetts Medical School, Worcester, MA). Cell lines were maintained at 37°C in 5% CO₂ in Ham’s nutrient mixture F-12 medium ((Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated FBS and the antibiotic, gentamicin (50 μg/ml). Media was changed to antibiotic-free Ham’s nutrient mixture F-12 medium 12 h before experiments were performed.

#### Sera and complement reagents

Normal human serum (NHS) prepared from 10 healthy volunteers with no history of gonococcal infection were pooled and stored at −70°C until used as a source of active complement. Complement was inactivated by heating NHS to 56°C for 30 min. Purified human fH, iC3b, and C3b were purchased from Complement Technology (Tyler, TX).

#### Gonococcal strains

*N. gonorrhoeae* 252, described previously (29), is a (stable) serum-resistant PorB.1A strain that binds fH (32) in the presence or absence of sialylation. Strain UU1 (PorB.1A) was isolated from an individual with disseminated gonococcal infection (35); and also binds fH, but relatively weakly compared with 252. Strain F62 (PorB.1B) (32) binds barely detectable levels of fH in the unsialylated state. All strains were transformed with plasmid pEG2 [a gift from Dr. Myron Christodoulides (36)] to express GFP and maintained on GC agar media supplemented with 1% isovitalex purified (37) containing fH (50 mg/ml). The primary gonococci were harvested from overnight cultures and inoculated into GC broth (37), supplemented with the equivalent of 1% isovitalex and grown to midlog phase. When required, sialylation of gonococcal LOS was achieved by adding CMP-NANA to the growth media (1 μg/ml). Bacteria were washed and resuspended in HBSS containing 0.15 mM CaCl₂ and 1 mM MgCl₂ (HBSS⁺⁺) for use in binding and cell association assays.

#### Abs and immunochemicals

Expression of CR3 on CHO-CR3 was confirmed using anti-human PE-CD11b (Caltag, Carlsbad, CA) and anti-human PE-Cy5-CD18 (BD Biosciences Pharmingen, Carlsbad, CA) by FACS analysis. Biotin-labeled goat anti-mouse IgG primary Ab, followed by streptavidin-labeled Alexa Fluor 647 (both from Molecular Probes, Carlsbad, CA) were used in FACS experiments (below) to detect fH/Fc fragments bound to CHO cells and to gonococci. Specificity of fH binding to CHO-CR3 was determined by including fH, or experiments using CR3 mutants or C3b (each at a concentration of 250 nm), or mAbs against anti-CD11b and anti-CD18 (Sigma-Aldrich, St. Louis, MO), each at a concentration of 294 and 526 nM, respectively.

To measure binding of human fH, FHL-1, CFHR1, SCRs 6, 7, 18–20 or SCRs 6–20 to CHO-CR3 cells or to gonococci in FACS experiments, we used polyclonal Ab against fH that was made by immunizing goats with purified human fH (Bethyl Laboratories, Montgomery, TX) as the primary Ab and anti-goat IgG conjugated to Alexa Fluor 647 (Molecular Probes) was used as the secondary Ab. mAb against human fH that is specific for an epitope within SCRs 18–20 (Quidel [catalog no. A229], San Diego, CA) was used in capture ELISA to estimate the concentration of recombinant fH constructs SCRs 6, 7, 18–20 and SCRs 6–20 (see below).

#### Recombinant complement proteins

We constructed five fH/murine Fc fusion proteins that contained contiguous fH SCR domains (SCRs 1–5, 6–10, 11–15, 16–20, and 18–20) fused in frame at their C-terminal ends to the N terminus of Fc fragment of murine IgG2a (fH/Fc fusion proteins) as previously described (38). This allowed us to use the Fc region as a tag for symmetric detection of each fusion protein using anti-mouse IgG. Deletion mutants in the SCRs 16–20 domain were also constructed where SCRs 16, 17, 18, 19, and 20 were each individually removed. To construct deletion mutants, pBluescript that contained cloned human fH SCRs 16–20 was used as a template to construct the exon encoding the desired recombinant protein. Overlapping PCR was used to delete either SCRs 17, 18, or 19 (primers listed in Table I). To delete SCR 16, the forward primer was designed at the 5’ of SCR 17 and to delete SCR 20 the reverse primer was designed at the 3’end of SCR 19 (Table I). Each fH fragment was cloned into the Ascl and NotI site of pcDNA3 and the sequence was confirmed by automated DNA sequencing. CHO cells were transfected with the resulting construct using lipofectin (Invitrogen Life Technologies, Carlsbad, CA). Proteins were expressed, purified, and quantified as described previously (38).

CFHR1 is composed of five SCR domains that bear 36, 45, 100, 100, and 97% amino acid identity to SCRs 6, 7, 18, 19, and 20 of human fH, respectively (17, 39). CFHR1 was expressed and purified as described previously (17). FHL-1, an alternatively spliced variant of fH that contains the seven N-terminal (fH SCR) plus four unique amino acids at the C-terminal end (39) was expressed and purified as described previously (40).

We also constructed a recombinant fH fragment containing (from the N to the C terminus) a FLAG tag, followed by SCRs 6, 7, 18, 19, and 20, that...
were called SCRs 6, 7, 18–20. In addition, we constructed an fH fragment spanning SCRs 6–20. Briefly, overlap extension PCR with plExscript vector containing either human fH SCRs 6–10 or SCRs 16–20 as templates for SCRs 6,7,18–20 and whole fragment fH (SCRs 1–20; a gift from Dr. Michael K. Pangburn, University of Texas Health Science Center, Tyler, TX) was used (primers listed in Table I). The final PCR product encoding SCRs 6–7 fused to SCRs 18–20 and separately, SCRs 6–20, were digested with Ascl and XhoI and cloned into pCDNA3 (38). The resulting PCR products and clones were verified by DNA sequencing. These plasmids were used to transiently transfet CHO cells. SCRs 6,7,18–20 secreted in cell culture supernatants was purified by affinity chromatography using EZview Red ANTI-FLAG M2 affinity gel (Sigma-Aldrich). Protein purity was confirmed by Coomassie-stained SDS-PAGE gels and protein concentration was determined by BCA assay (Pierce Chemicals, Rockford, IL). Cell culture supernatant containing SCRs 6–20 was spin-concentrated using Amicon Ultra-15 Centrifugal Filter Units (Millipore, Billerica, MA). Protein concentration was estimated by ELISA using Quidel anti-fH mAb (catalog no. A229) (32) as the capture Ab and goat polyclonal anti-human fH, followed by anti-goat IgG-alkaline phosphatase as the detecting Ab. Purified fH (Complement Technology, Tyler, TX) was used to generate a standard curve.

**FIGURE 1.** Binding of human fH to CHO-CR3 cells. CHO cells transfected with human CR3 (CHO-CR3 cells) and CHO cells transfected with a vector containing only the neomycin marker (CHO-NEO control cells) were incubated with purified human fH at a concentration of 67 nM. fH binding to cells was detected by flow cytometry using goat polyclonal anti-human fH as the primary Ab and Alexa Fluor 647 conjugated (rabbit anti-goat IgG as the disclosing Ab. The thick black line represents fH binding to CHO-CR3 cells; the gray shaded graph, fH binding to CHO-NEO cells. The broken black and gray lines represent the controls for CHO-CR3 and CHO-NEO, respectively (no fH added). One representative experiment is shown of three independently performed experiments.

**FIGURE 2.** Specificity of fH binding to CHO-CR3 cells. Specificity of fH binding to CR3 was determined by incubating CHO-CR3 cells with purified iC3b (220 nM), a ligand for CR3, followed by addition of purified human fH at a concentration of 67 nM. Purified C3b (220 nM) known to bind minimally to CR3 served as a negative control. The thick black line represents fH binding to CHO-CR3 cells incubated with fH alone, the gray shaded graph, binding of fH to CHO-CR3 cells after preincubation with: iC3b (A) or C3b (B). The broken lines represent the controls (no fH added).

**Flow cytometry**

Binding of complement proteins and recombinant proteins to N. gonorrhoeae was performed as described above and previously (32). Briefly, 10⁶ bacteria suspended in HBSS ++ were incubated with 67 nM fH or other complement-related proteins and detected with anti-human fH (Bethyl Laboratories, Montgomery, TX) at a dilution of 1:400, followed by anti-goat IgG conjugated to Alexa Fluor 647. Bacteria were similarly incubated using 80 nM fH/Fc fusion proteins in final reaction volumes of 100 μl for 30 min at 37°C and fH/Fc constructs detected using anti-mouse IgG-biotin, followed by streptavidin-Alexa Fluor 647.

Binding of complement proteins and recombinant proteins to CHO-CR3 or CHO-NEO cells was also performed. Briefly, 10⁶ CHO cells were suspended in antibiotic-free Ham’s Nutrient mixture F-12 medium and incubated with fH, or fH-related proteins or constructs; fH, CFHR1, FHL-1, and SCRs 6, 7, 18–20 (each protein/construct was used at 10 μg/ml, which is equivalent to 67, 268, 231, and 268 nM, respectively). CHO cells were similarly incubated for 30 min at 37°C using 80 nM fH/Fc fusion proteins in final reaction volumes of 100 μl. Detection of fH, fH-related proteins/constructs and fH/Fcs to CHO cells was identical to that used for detection to gonococci (see above). In experiments with CHO-CR3 or CHO-NEO cells, 50,000 events were recorded.

**Association of gonococci with CHO cells**

CHO cell lines were seeded in 12-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) at a concentration of 1.25 × 10⁶ epithelial cells/well and grown to confluency. Twelve hours prior to the addition of bacteria to cells, the media was changed to antibiotic-free Ham’s Nutrient mixture F-12 medium (Ham’s/F-12 media). Cells were then released using enzyme-free PBS based cell dissociation buffer (Invitrogen). GFP-expressing gonococci grown to midlog phase were suspended in antibiotic-free Ham’s/F-12 media and preincubated for 30 min with purified human fH, fH-related proteins (fH, CFHR1, FHL-1, or fH-related constructs (SCRs 6, 7, 18–20 and SCR 6–20) and added to cells at a multiplicity of infection of 100 (100 gonococci per CHO cell). Gonococci were preincubated initially with 67 nM of each of the proteins or constructs. If enhanced gonococcal association was not observed at these equimolar concentrations using any individual protein or construct, we increased the concentration of the protein under examination to 10 μg/ml, which is equivalent to 67, 268, 231, and 89 nM, respectively, and reported that experiment. We also tested: 1) nonpreincubated GFP-expressing gonococci added to CHO-CR3 cells, which instead had been preincubated with fH (67 nM) and gonococci added to CHO-CR3 cells simultaneously (no preincubation involved), and 3) fH (67 nM) preincubated goncocci added to CHO-CR3 cells that had also been preincubated with fH (67 nM). Infected cell lines were incubated at
FIGURE 3. Defining the SCR regions in human fH that bind to CHO-CR3 cells. A. Binding of fH derived SCR domains/mouse Fc fusion proteins to CHO-CR3 cells. The SCR fragments used spanned the entire length of the fH molecule. B. Binding to CHO-CR3 cells of SCRs 16–20 lacking individual SCRs (16, 17, 18, 19, or 20), each fused to mouse Fc. Biotin-labeled goat anti-mouse IgG Ab was followed by streptavidin-labeled Alexa Fluor 647 to detect binding of each SCR/Fc to CHO-CR3 cells. SCRs 18, 19, and 20 together were required for binding of the C-terminal region of fH to CR3. Each Fc fusion protein was added at a concentration of 80 nM. The histogram marked “Control” lacked any fH-derived SCR/Fc fusion protein. Numbers in the histogram represent the median fluorescence of the bound SCR/Fc fusion protein to CHO-CR3 cells. The x-axis represents fluorescence on a log 10 scale, the y-axis the number of events. One representative experiment is shown of three independently performed experiments.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
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<tr>
<td>SCRs 16–20Δ16 F AscI</td>
<td>GGCAGGCGAAAAACAGATACTTTCCACGTTAAC</td>
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<tr>
<td>SCRs 16–20Δ16 R NotI</td>
<td>GGCAGGCGTTCATTAGCTAAGATGATATTT</td>
</tr>
<tr>
<td>SCRs 16–20Δ17 R</td>
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<tr>
<td>SCRs 16–20Δ17 F</td>
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<tr>
<td>SCRs 16–20Δ18 R</td>
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<tr>
<td>SCRs 16–20Δ18 F</td>
<td>CCACATCTGCAAGATTCTACAGATTCTACAGG</td>
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<tr>
<td>SCRs 16–20Δ19 R</td>
<td>TACACACAGATTCTGATGAGGTGGTTCCGTC</td>
</tr>
<tr>
<td>SCRs 16–20Δ19 F</td>
<td>CTCATCTGCAAGATTCTACAGATTCTACAGG</td>
</tr>
<tr>
<td>SCRs 16–20Δ20 R AscI</td>
<td>GGCAGGCGCTTCCATTAGCTAAGATGATATTT</td>
</tr>
<tr>
<td>SCRs 16–20Δ20 F NotI</td>
<td>GGCAGGCGTTCATTAGCTAAGATGATATTT</td>
</tr>
<tr>
<td>SCRs 6, 7, 18–20 SCR 6 F AscI</td>
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</tr>
<tr>
<td>SCRs 6, 7, 18–20 SCR 20 R XhoI</td>
<td>GAGGAGATGTTTGCATTGAGGTGGTTCCGTC</td>
</tr>
<tr>
<td>SCRs 6, 7, 18–20 SCR 7, 18 F</td>
<td>CCCAGAATGCTGACACCTCCCTGTTGGAATC</td>
</tr>
<tr>
<td>SCRs 6, 7, 18–20 SCR 18, 7 R</td>
<td>ACCAGAATGCTGACACCTCCCTGTTGGAATC</td>
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</table>

F, Forward primer from 5’ to 3’ direction. Incorporated AscI sites are underlined. R, Reverse primer from 5’ to 3’ direction. Incorporated NotI or XhoI sites are underlined.

37°C, 5% CO₂ for 2 h. Following incubation, the reaction mixture was washed twice with Dulbecco’s PBS containing Ca ++ and Mg ++ (D-PBS ++), resuspended in 600 μl D-PBS ++ containing 0.5% BSA, and flow cytometry performed immediately to enumerate bacteria associated with cells. Binding of gonococci to CHO-CR3 and CHO-NEO was detected by monitoring GFP expression of the gonococci by flow cytometry performed immediately to enumerate bacteria associated with cells.

Results

fH binds to CR3

We incubated CHO-CR3 cells with purified fH (67 nM) and detected cell-bound fH by flow cytometry. CHO cells transfected with the empty vector that contained the neomycin selection marker (CHO-NEO cells) were used as the negative control. We measured a 4-fold increase in fH binding to CHO-CR3 cells compared with CHO-NEO cells (Fig. 1). We also used 10% heat-inactivated NHS as a source of fH and observed similar results (data not shown).

Binding specificity of purified human fH to CR3 was examined using as inhibitors: 1) purified human iC3b, a known ligand for CR3 (33, 41, 42) and 2) mAbs specific for: i) CD11b, the unique α subunit of CR3 and ii) CD18, the noncovalently associated β subunit (43). Purified C3b, known to possess diminished binding to CR3 (41, 42), was used as a “negative” control. iC3b completely blocked fH binding to CHO-CR3 cells (Fig. 2A; fluorescence decreased to control levels); C3b produced less inhibition (Fig. 2B). mAbs specific for CD11b and CD18 each inhibited fH binding to CHO-CR3 cells almost to isotype control levels (Fig. 2A; fluorescence decreased to control levels); C3b produced less inhibition (Fig. 2B). mAbs specific for CD11b and CD18 each inhibited fH binding to CHO-CR3 cells almost to isotype control levels (Fig. 2C).

Collectively, these results suggest a specific interaction between fH and CR3 on CHO-CR3 cells.

Defining regions in fH that bind to CR3

We used contiguous domains of human fH fused to the Fc fragment of mouse IgG2a [fH/Fc fusion proteins (32)] to determine SCR region(s) in fH that bound to CR3. SCRs 1–5/Fc, SCRs 6–10/Fc, SCRs 11–15/Fc, SCRs 16–20/Fc and SCRs 18–20/Fc that collectively spanned all 20 fH SCRs were examined for binding to CHO-CR3 cells. SCRs 16–20/Fc and SCRs 18–20/Fc showed the greatest binding to CHO-CR3 (∼15- and ∼30-fold increase in fluorescence over control levels; Fig. 3A). SCRs 6–10/Fc also showed increased binding; however, the fluorescence intensity was only ∼3-fold greater than control levels.

In an attempt to further narrow the region in SCRs 18–20 that bound to CR3, we deleted individual SCR domains in the background of SCRs 16–20/Fc (Table I). As seen in Fig. 3B, deleting either SCR 16 or 17 from the SCRs 16–20/Fc construct had little effect on binding to CHO-CR3 cells. However, deleting either SCRs 18, 19, or 20 singly abrogated binding and suggested that all three SCR domains were necessary for interaction of the C-terminal region of fH with CR3 (Fig. 3B).

Defining regions in fH that bind to N. gonorrhoeae strain 252

We used N. gonorrhoeae strain 252 (expressing the PorB.1A molecule and manifesting stable serum-resistance) that binds to fH...
The Fc fusion protein was added at a concentration of 80 nM. The “Control” 6–10 and SCRs 16–20, sialylated strain 252 bound (only) SCRs 16–20. Each the entire length of the fH molecule. Unsialylated strain 252 bound SCRs

FIGURE 4. Binding of human fH/murine Fc fusion proteins to N. gonorrhoeae. Binding of fH-derived SCR regions/mouse Fc fusion proteins to unsialylated and sialylated N. gonorrhoeae strain 252. SCR regions spanned the entire length of the fH molecule. Unsialylated strain 252 bound SCRs 6–10 and SCRs 16–20, sialylated strain 252 bound only SCRs 16–20. Each Fc fusion protein was added at a concentration of 80 nM. The “Control” lacks any fusion protein. Detection was performed as described in Fig. 3. The x-axis represents fluorescence on a log10 scale, the y-axis the number of events. Numbers in the histogram represent the median fluorescence of the bound Fc fusion proteins to N. gonorrhoeae strain 252. One representative experiment is shown of three independently performed experiments.

Binding of CFHR1 and FHL-1 to CR3

The binding of SCRs 18–20/Fc and to a lesser extent SCRs 6–10/Fc to CR3 raised the possibility that two other members of the fH family of proteins, CFHR1 (44) and FHL-1 (19) may also bind CR3. The relative homologies of CFHR1 and FHL-1 with homologous regions in fH are shown schematically in Fig. 5A, 5C. We measured strong binding of CFHR1 to CHO-CR3 cells and weaker binding to CHO-NEO cells (Fig. 5B). In contrast, FHL-1 bound minimally to CHO-CR3 cells even when the concentration of FHL-1 was increased from 67 nM to 231 nM; no binding was seen to CHO-NEO cells (Fig. 5D). These data are consistent with greater binding of SCRs 18–20/Fc compared with SCRs 6–10/Fc (Fig. 3A).

FIGURE 5. Binding of CFHR1 and FHL-1 to CHO-CR3 and CHO-NEO cells. A and C are schematic representation of CFHR1 and FHL-1. CFHR1 is the product of a unique gene, and the two N-terminal and the three C-terminal SCRs bear amino acid homology to fH SCRs 6–7 and 18–20 as indicated (39). FHL-1 is an alternatively spliced variant of fH that contains the seven N-terminal fH SCRs and is spliced to a separate exon that encodes for four C-terminal amino acids that are unique to FHL-1; S, F, T, and L (as indicated). CHO-CR3 cells and CHO-NEO cells were incubated either with purified human CFHR1 or FHL-1, each at a concentration of 268 and 231 nM (B and D, respectively). The thick black lines represent controls for the CHO-CR3 cells in each of the histograms and the gray shaded graphs, binding to CHO-NEO cells. The broken black line represents controls for the CHO-CR3 cells (no CFHR1 or FHL-1 added). CFHR1 and FHL-1 binding was detected by flow cytometry using polyclonal goat anti-human fH, followed by Alexa Fluor 647 conjugated anti-goat IgG. The x-axis represents fluorescence on a log10 scale, the y-axis the number of events. One representative experiment is shown of three independently performed experiments.

fH enhances the association of gonococci with CR3-expressing CHO cells

Next, we examined whether fH and its derivatives, CFHR1 and FHL-1, that had bound to strain 252 enhanced binding of gonococci to CHO-CR3 cells. GFP-expressing N. gonorrhoeae 252 (either unsialylated or sialylated) were used and FACS analysis was performed by gating on CHO cells. Intrinsic binding of 252, unsialylated or sialylated, to CR3 expressing CHO (CHO-CR3) cells, occurred even in the absence of fH or its derivatives affixed to the strain, compared with binding to CHO-NEO cells (Fig. 7B–D compared with Fig. 7A), consistent with previous observations that gonococci can bind directly to CR3 (2). Full-length fH used at 67 nM concentration further enhanced binding of unsialylated bacteria selectively to CHO-CR3 (Fig. 7B). Preincubation of sialylated strain 252 with 67 nM fH did not enhance binding to CHO-CR3 (Fig. 7B, rows 1 and 2). No differences were found when 1) nonpregment GFP-expressing gonococci were added to CHO-CR3 cells, which instead had been preincubated with fH (67 nM), 2) fH (67 nM) and gonococci...
were added to CHO-CR3 cells simultaneously (no preincubation involved), and 3) fH (67 nM) preincubated gonococci were added to CHO-CR3 cells that had also been preincubated with fH (67 nM) (data not shown). We also examined another PorB.1A strain of N. gonorrhoeae UU1 (35) that binds fH in the unsialylated state, but with weaker fluorescence intensity than 252 and a third strain, F62, that does not bind detectable amounts of fH in the unsialylated state (32). fH (67 nM) increased binding of unsialylated strain UU1 to CHO-CR3 cells but did not increase binding of unsialylated strain F62 (data not shown).

CFHR1 and FHL-1 did not increase binding of unsialylated strain 252 to CHO-CR3 cells (Fig. 7C, 7D) even when concentrations were each increased from 67 nM to 268 and 231 nM, respectively. Although CFHR1 contains both potential binding sites (within SCRs 6–7 and 18–20) to CR3 and gonococcal strain 252, the sequence homologies of the two N-terminal SCRs of CFHR1 are only 36 and 45% compared with SCRs 6 and 7 in fH and may therefore not have been effective in binding to CR3 via one site and strain 252 via the other.

We speculated that unsialylated gonococci may engage CR3 in a cooperative fashion through fH using one of the individual binding regions, SCRs 6–10 or SCRs 16–20 that also bind gonococci (shown in Fig. 4, left panel), or alternatively that other sites on CR3 may be used for direct binding to N. gonorrhoeae. To more faithfully isolate the SCRs possibly involved in bridging CR3 and gonococci to enhance binding to CHO-CR3 cells, we constructed a mutant fH molecule that comprised (from the N to C terminus) fH SCRs 6, 7, 18, and 19 and 20 (SCRs 6, 7, 18–20) did not enhance the association of strain 252 with CHO-CR3 (Fig. 8B) (concentration increased to 268 nM) although it maintained its ability to bind to unsialylated (and sialylated) gonococci (Fig. 8A, left and middle panels) and to CHO-CR3 (Fig. 8A, right panel). These data suggested that in addition to the mere presence of both the binding sites for bacteria and CR3, spatial orientation of these domains provided by the intervening SCRs (SCRs 8–17) present in the full-length fH molecule was required for enhanced binding of fH-coated unsialylated gonococci to CR3-expressing cells (Fig. 7B). To examine this possibility, we constructed and used an fH recombinant protein, SCRs 6–20, which in addition to containing CR3 and bacterial binding SCRs 6–7 and 18–20, also contained the intervening SCRs 8–17. Incubation of N. gonorrhoeae strain 252 with SCRs 6–20 (67 nM) enhanced the association of bacteria with CHO-CR3 cells by both unsialylated and sialylated gonococci (Fig. 8C) suggesting a key role for the SCRs 8–17 in providing a “spacer” for bridging gonococci and CR3-expressing cells. This result precluded the need to test SCRs 6–20 for separate binding to gonococci and CR3. In addition, SCRs 6–20 bridging of sialylated gonococci to CHO-CR3 was modestly enhanced compared with full-length fH (Fig. 8C) compared with Fig. 7B suggesting improved binding of SCR 6 when it is located in a terminal position. The requirement for the simultaneous presence of two distinct binding regions in fH (SCRs 6, 7 and SCRs 18–20) for CR3 and N. gonorrhoeae and the intervening domains (SCRs 8–17) that serve as a spacer to facilitate bridging of bacteria with cells is illustrated schematically in Fig. 9.

Discussion
In this study, we have characterized the interactions between fH, FHL-1 and CFHR1 with human CR3 and have shown that fH, but not FHL-1 or CFHR1, can serve to bridge gonococci with CR3-expressing cells. Thus, in addition to playing an important role in
gonococcal pathogenesis by enhancing the ability of bacteria to resist killing by human complement (10), fH may also enhance the association of gonococci with CR3 expressing cells. The redundancy of cell adhesion mechanisms used by gonococci would enable it to bind to a variety of cell types that it may encounter in vivo. Using primary cervical epithelial cells, Edwards et al. (6, 45) have shown that gonococcal binding to CR3 is mediated through a cooperative interaction that involves gonococcal Por and pilus and iC3b deposited on the bacteria by alternative pathway activation (6, 45, 46). Our data indicate that gonococci also use fH to associate with CR3.

Using a model system (CHO cells), we demonstrated that expression of CR3 augmented binding of fH. The specificity of the interaction was confirmed by inhibition of binding using mAbs against CR3 and soluble iC3b, the primary ligand for CR3. It is noteworthy that although iC3b at a ~3-fold molar excess (Fig. 2A) blocked fH binding to CHO-CR3, fH at a ~3-fold molar excess over iC3b resulted in only a ~20% decrease in the fluorescence of iC3b binding to CHO-CR3 (data not shown). Thus, fH is not likely to interfere with iC3b–CR3 interactions in vivo. Although our studies have been performed in a model cell line, the ability of fH to bind to CR3 expressed on human cells such as PMNs (25) and facilitate adhesion to microbes (47) indicates that our findings are likely to be pathophysiologically relevant.

Based on results of binding experiments with recombinant mutant fH fusion proteins, FHL-1 and CFHR1, two regions in fH (SCRs 6–7 and SCRs 18–20) were found to bind to CR3. Interestingly, the same two regions bound to unsialylated PorB.1A-expressing gonococci. SCRs 6–7 and SCRs 18–20 appear to be critical for binding to several other microbes and cell surfaces (reviewed in references 48, 49). The importance of appropriate spatial orientation of the two binding regions in fH to enable it to bridge gonococci to CR3 was illustrated by observing that loss of the intervening SCRs (8 through 17) prevented enhancement of adhesion of bacteria to CHO-CR3 cells (Fig. 8B). A newly proposed functional structure of fH suggests that the SCRs 12–14 region is the site of a bend or a hinge (50) and substantiates the proposed folded-back conformation of monomeric fH (51). Such a bend or hinge may permit SCRs 6 and 7 tethered organisms to establish binding to CR3 via nearby SCRs 18–20 that are folded back. This resultant increase in proximity of SCRs 6–7 to...
SCRs 18–20 in fH-mediated binding of N. gonorrhoeae to CR3-expressing cells (modeled in Fig. 9).

Another noteworthy finding was that fH enhanced the association of unsialylated bacteria to CHO-CR3 cells; binding of sialylated bacteria to CHO-CR3 was only marginally increased. Two possibilities, not mutually exclusive, could be invoked. First, that sialylation of LOS is associated with enhanced negative surface charge of the gonococcal surface and could contribute to increased forces of repulsion between the bacteria and the negatively charged cell surface. A second consideration is that only those fH molecules that bind to bacteria through SCRs 6–7 contribute to adhesion to CR3—that is, a stable bridge is formed only when SCRs 18–20 engages CR3. Binding of fH SCRs 6–7 containing molecules to strain 252 is greatly decreased when LOS was sialylated (Figs. 4, 6). Therefore, in this instance binding of most fH molecules to bacteria would occur through SCRs 18–20 leaving only SCRs 6–7 (bonds weakly to CR3) available. We observed that the SCRs 6–20 construct facilitated bridging of sialylated gonococci over that produced by full-length fH, prompting the suggestion that terminally located SCR 6 may be more effective in binding to gonococci and allowing SCRs 18–20 to bridge to CR3. Male urethral cells that posses the asialoglycoprotein receptor bind to unsialylated gonococci directly via the lecto-N-neotetraose structure of gonococcal LOS (52); however, LOS sialylation does not affect gonococcal invasion into primary endo- or ectocervical cells (45). Primary cervical cells secrete all components of the alternative pathway and deposition of iC3b on bacteria may overcome the potential inhibitory effects of LOS sialic acid on bacteria–cell adhesion to facilitate invasion (6). The inhibitory effects of LOS sialylation is also cell specific; sialylation inhibits gonococcal opacity protein mediated binding of gonococci to Chang epithelial cells and ME-180 endocervical cells (53), but not to HEC-1B and PC-3 cells (53). Because adherence and invasion of gonococci to host cells is a complex process (54, 55), we used a reductionist approach to investigate one of these variables.

Strains that cause disseminated gonococcal infection and other strains that may cause asymptomatic gonococcal infection often express the PorB.1A molecule (56–58); these strains bind fH directly (29) and do not require sialylation. Sialylation and desialylation of other gonococci in the genital tract are dynamic processes; neuraminidases (sialidases) elaborated by vaginal microflora (54) and by cervical epithelia (59, 60) may result in desialylation of gonococci. In this study, we have shown that PorB.1A strains that are not sialylated and bind fH adhere more efficiently to CR3-expressing cells. The present study may contribute an explanation of why PorB.1A-expressing gonococci invade epithelial cells in the genital tract, often without producing clinical signs or symptoms, and from there sometimes disseminate.

In conclusion, this study has shown that fH plays an important role in enhancing binding of unsialylated gonococci to eukaryotic cells that express CR3. Unsialylated gonococci with fH attached, in addition to other complement components, may be ubiquitous in the lumen of the genital tract and be adept in binding female epithelial cells, in particular via the CR3 receptor. Further, the regions in fH that interact with CR3 have been characterized and are in accordance with recent observations using PMNs. These studies could pave the way for future studies that examine the functional consequences of fH bound to gonococci when they interact with CR3-expressing nonphagocytic as well as phagocytic cells. These data also provide another ligand (CR3) for select members of the fH family of proteins to target at sites of inflammation.

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References


