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Mutations in Complement Factor H Impair Alternative Pathway Regulation on Mouse Glomerular Endothelial Cells *in Vitro**

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Complement factor H (FH) inhibits complement activation and interacts with glomerular endothelium via its complement control protein domains 19 and 20, which also recognize heparan sulfate (HS). Abnormalities in FH are associated with the renal diseases atypical hemolytic uremic syndrome and dense deposit disease and the ocular disease age-related macular degeneration. Although FH systemically controls complement activation, clinical phenotypes selectively manifest in kidneys and eyes, suggesting the presence of tissue-specific determinants of disease development. Recent results imply the importance of tissue-specifically expressed, sulfated glycosaminoglycans (GAGs), like HS, in determining FH binding to and activity on host tissues. Therefore, we investigated which GAGs mediate human FH and recombinant human FH complement control proteins domains 19 and 20 (FH19–20) binding to mouse glomerular endothelial cells (mGEnCs) in ELISA. Furthermore, we evaluated the functional defects of FH19–20 mutants during complement activation by measuring C3b deposition on mGEnCs using flow cytometry. FH and FH19–20 bound dose-dependently to mGEnCs and TNF-α treatment increased binding of both proteins, whereas heparinase digestion and competition with heparin/HS inhibited binding. Furthermore, 2-O-, and 6-O-, but not *N*-desulfation of heparin, significantly increased the inhibitory effect on FH19–20 binding to mGEnCs. Compared with wild type FH19–20, atypical hemolytic uremic syndrome-associated mutants were less able to compete with FH in normal human serum during complement activation on mGEnCs, confirming their potential glomerular pathogenicity. In conclusion, our study shows that FH and FH19–20 binding to glomerular endothelial cells is differentially mediated by HS but not other GAGs. Furthermore, we describe a novel, patient serum-independent competition assay for pathogenicity screening of FH19–20 mutants.

The complement system, which consists of the classical, lectin, and alternative pathway, initiates and amplifies inflammatory responses, including proliferative glomerulonephritis (1–3). The three pathways converge in the activation of complement component C3 and lead to the formation of membrane attack complexes that lyse the affected cells. The alternative pathway is initiated by spontaneous hydrolysis of C3 (4), eventually leading to covalent attachment of C3b to both adjacent host and non-host cell surfaces (5) and the release of the proinflammatory anaphylatoxin C3a (6). Deposited C3b binds complement factor B and, after proteolytic cleavage by factor D, forms C3 convertases (C3bBb) on the cell surface, providing localized feed-forward amplification of complement activation (7). To protect host cells from complement-mediated damage, several regulatory proteins disrupt the complement cascade, including the plasma proteins complement factor H (FH)[2] and FH-like protein 1, and membrane-bound regulators like complement receptor 1 (CD35), membrane cofactor protein (CD46), and decay accelerating factor (CD55) (8–12).

FH, a 155-kDa glycoprotein, is the major inhibitor of the alternative pathway both in the fluid phase and on cellular surfaces (13–15). It competes with factor B for C3b (16), acts as a cofactor for complement factor I-mediated proteolytic inactivation of C3b (14), and promotes the dissociation of C3bBb convertases (17). FH consists of 20 complement control protein (CCP, also called short consensus repeats) domains of ~60 amino acids each (18). The N-terminal domains CCP1–4 contain the cofactor and decay accelerating activity (19, 20), whereas CCP7 and CCP19–20 mediate cell surface recognition by binding to sulfated glycosaminoglycans (GAGs) on host cells (21–23). Mutations in the two C-terminal surface recognition

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[2] The abbreviations used are: FH, complement factor H; CCP, complement control protein domain; GAG, glycosaminoglycan; aHUS, atypical hemolytic uremic syndrome; AMD, age-related macular degeneration; DDD, dense deposit disease; HS, heparan sulfate; FH19–20, complement factor H CCP19–20; mGEnC, mouse glomerular endothelial cell; NHS, normal human serum.

domains can result in the rare renal disease atypical hemolytic uremic syndrome (aHUS) (24). Furthermore, a polymorphism in CCP7 (Y402H) has been associated with the ocular disease age-related macular degeneration (AMD) as well as the rare renal disease dense deposit disease (DDD) (25). In turn, the rare SCR20 mutation R1210C is associated with both AMD and aHUS (26). Interestingly, mutations cluster within the cell surface recognition domains, and whereas FH systemically controls complement activation, complement-mediated damage appears restricted to eyes and kidneys.

Recent studies (21) provide evidence that the tissue-specific disease manifestation is mediated by the differential expression of heparan sulfates (HS), a class of GAGs. HS are linear, negatively charged polysaccharides that can be extensively modified by sulfation and epimerization to yield highly heterogeneous structures (27). The exact sequence of modifications along the carbohydrate backbone, the "HS code," generates specifically sulfated domains that are recognized by proteins with the corresponding HS binding site. This way, endothelial HS plays a crucial role during inflammation by binding to selectins, integrins, chemokines, cytokines, inflammatory cells, and complement proteins (28–32). Clark *et al.* (21) recently found that HS-mediated FH binding in the glomerulus is primarily mediated by CCP19–20, providing an explanation for the C-terminal localization of aHUS-related FH mutations. Their observations are supported by earlier evidence from FH knock-out mouse models. FH-deficient mice display a phenotype reflecting human DDD, which is associated with systemic loss of complement control in the fluid phase (33). Recently, the model has been refined to a hepatocyte-specific FH knock-out, which results in a phenotype even closer to human C3 glomerulonephropathies that include DDD (34). However, if mice instead express a FH construct lacking the five C-terminal CCPs, the resulting phenotype reflects aHUS, with glomerulospecific, complement-mediated damage despite normal plasma C3 concentrations (35). The strong similarities between human and murine disease phenotypes led us to investigate the genotype-phenotype relation of FH19–20 mutants in the context of mouse glomerular endothelial cells.

In a previous study we showed that aHUS-associated FH CCP19–20 (FH19–20) mutants exhibit impaired binding to C3b/C3d and impaired or enhanced binding to mouse glomerular endothelial cells (mGEnCs) and heparin (36). Our current study aimed to determine the functional effects of FH19–20 mutations and evaluate the role of GAGs in binding of FH to glomerular endothelium, the clinically affected tissue. We demonstrated that binding of FH to mGEnCs is partially mediated by HS, but not chondroitin sulfate, dermatan sulfate or hyaluronan. Furthermore, several aHUS-associated FH19–20 mutants were less able to compete with full-length FH compared with wild type FH19–20 during alternative pathway activation on mGEnCs. The obtained results not only confirm the evaluated mutants' potential role in aHUS etiology but illustrate glomerular pathogenicity screening of FH mutants under highly controlled experimental conditions without requiring access to patient serum.

Experimental Procedures

FH, FH19–20 Mutants, and GAG Preparations—Factor H (FH, Tyr-402 homozygous) was isolated from healthy donors as described (37). Recombinant wild type FH19–20 and aHUS-associated FH19–20 mutants (D1119G, W1183L, T1184R, E1198A, R1210A, R1215Q) (38) were generated and purified as described (39). GAG preparations included heparin, HS from bovine kidney, hyaluronic acid (Sigma), 2-*O*-desulfated heparin, 6-*O*-desulfated heparin, and *N*-desulfated heparin (Neoparin Inc., Alameda, CA).

Cell Culture—Conditionally immortalized mGEnCs with all features of primary glomerular endothelial cells were cultured as described (40). Where indicated, cells were activated by incubation with tumor necrosis factor (TNF)-α (10 ng/ml; Peprotech, Rocky Hill, NJ) for 18 h.

Binding of FH and FH19–20 to Mouse Glomerular Endothelial Cells in Enzyme-linked Immunosorbent Assays (ELISAs)—mGEnCs were grown in 96-well plates (Corning Life Sciences, Schiphol-Rijk, The Netherlands). The cells were washed with phosphate-buffered saline (PBS) and incubated with serial dilutions of FH and FH19–20 (0–80 μg/ml) in PBS containing 2% bovine serum albumin (2% PBA, w/v; Sigma) for 2 h at 37 °C. Binding was detected using polyclonal rabbit anti-human FH antiserum (a kind gift from Dr. J. Hellwage, Hans Knöll Institute, Jena, Germany). The cells were then washed twice with 0.05% Tween 20 in PBS (PBS/Tween; v/v) and subsequently incubated with horseradish peroxidase-conjugated $F(ab')_2$ donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in 2% PBA 1 h at room temperature. Finally, the cells were washed three times with PBS/Tween and incubated with tetramethylbenzidine substrate solution (Bio-Rad). The reaction was stopped after 15 min with an equal volume of 2 M H_2SO_4, and the absorbance at 450 nm was measured using a Bio-Rad Multiplate Reader (Bio-Rad). Full-length FH and wild type FH19–20 were used at their 50% effective concentrations (EC_{50}) for the remaining binding assays.

Effect of GAG-degrading Enzyme Treatment and (Modified) Heparin/HS Competition on FH/FH19–20 Binding to mGEnCs—HS on TNF-α-activated mGEnCs was removed by treatment with 0.25 units/ml heparinase I, II, and III (Sigma) in 0.1 M sodium acetate, 0.2 mM calcium acetate (pH 7.0) for 1 h at 37 °C. Cell surface HS degradation was confirmed by measuring the expression of the HS epitope recognized by the antibody AO4B08 (41). Chondroitin sulfate/dermatan sulfate was removed by treating mGEnCs with 1 units/ml chondroitinase ABC (Sigma) in 25 mM Tris/HCl, 2 mM magnesium acetate (pH 8.0) for 1 h at 37 °C. The activity of chondroitinase ABC was checked with the anti-chondroitin sulfate antibody IO3H10 (42). Hyaluronic acid was removed by 0.1% hyaluronidase (w/v; from bovine testes, type I-S; Sigma) in 0.1 M sodium acetate (pH 6.0) for 1 h at 37 °C, and its activity was confirmed using biotinylated hyaluronic acid-binding protein (Sigma). For the competition assays, FH/FH19–20 were preincubated with the different GAG preparations or the modified heparinoids (50 μg/ml) before being added to the cells. Binding of FH/FH19–20 was then determined using ELISA as described.

Alternative Pathway Activation on mGEnCs and FH19–20 (Mutant) Competition—TNF-α-activated mGEnCs were grown in 48-well plates (Corning Life Sciences). After washing twice with PBS, the cells were incubated with 20% pooled normal human serum (NHS; Complement Technology, Inc., Tyler, TX) in veronal-buffered saline (15 mM veronal, 145 mM NaCl, 3 mM $MgCl_2$, 5 mM EGTA, 0.025% NaN_3 (pH 7.3)) at 37 °C. Twenty percent heat-inactivated NHS (30 min at 56 °C) in veronal-buffered saline was used as the negative control. Convertase formation was stopped after 60 min by adding EDTA to a final concentration of 10 mM. The cells were then detached by vigorous pipetting, washed with 0.5% PBA, and incubated with rabbit anti-human C3/C3b (1 μg/ml; clone H-300; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in 0.5% PBA for 30 min at 4 °C. After washing and incubating with a goat anti-rabbit IgG: Alexa488 (Life Technologies) for 30 min at 4 °C, C3b deposition on mGEnCs was detected using a Beckman Coulter FC500 flow cytometer and evaluated using CXP2.2 software. To evaluate the functional effects of aHUS-associated mutations, 10 μg/ml wild type or mutant FH19–20 were added to NHS to compete with full-length FH during the activation step.

Statistical Analysis—Titration data were fitted using nonlinear regression with a "log(agonist) *versus* response" model using GraphPad Prism version 5.03 (GraphPad Software Inc., San Diego, CA). Values are expressed as the means ± S.E., and significance was evaluated by Student's *t* test or analysis of variance using GraphPad Prism. Post hoc comparison of individual means was performed using Tukey's method. Binding experiments were performed four times in duplicate. Results of the activity assays represent data from three separate experiments.

Results

TNF-α Treatment Increases Full-length FH and FH19–20 Binding to Glomerular Endothelial Cells—Complement FH binds endothelial cells (43), and recently, we demonstrated the differential binding of FH19–20 and FH19–20 mutants to mGEnCs (36). In the current study we investigated the ability of mGEnCs to bind FH and FH19–20 using ELISA and found that both FH and FH19–20 exhibited specific and dose-dependent binding to mGEnCs (Fig. 1*a*). Activation of mGEnCs with TNF-α to simulate the inflammatory conditions of aHUS significantly increased binding of both full-length FH and FH19–20 ~1.5-fold and ~1.3-fold, respectively (Fig. 1*b*). To take advantage of the observed increase in signal, we investigated binding of FH and FH19–20 using TNF-α-activated mGEnCs for the subsequent experiments.

Binding of Full-length FH and FH19–20 to Glomerular Endothelial Cells Is Differentially Mediated by HS but Not Chondroitin Sulfate or Dermatan Sulfate—Because endothelial glycocalyx composition and modifications differ between different tissues, we investigated the role of different types of GAGs in regulating binding of FH and FH19–20 to glomerular endothelium. Therefore, mGEnCs were either treated with a mixture of glycosidases to degrade specific GAGs before measuring FH and FH19–20 binding, or the proteins were preincubated with soluble GAGs to compete for cell surface GAGs on mGEnCs. Although removal of chondroitin sulfate using chondroitinase ABC (*CSase ABC*) had no effect on binding of either full-length FH (Fig. 2*a*) or FH19–20 (Fig. 2*b*), digesting HS with heparinase I, II, and III (*HepI-II-III*) significantly reduced binding ~1.7-fold and ~1.2-fold, respectively. Treatment with hyaluronidase, which removes hyaluronic acid, decreased binding of FH19–20 to TNF-α-activated mGEnCs, whereas the binding of full-length FH was unaffected. However, binding of both proteins was significantly decreased after treating mGEnCs with both hyaluronidase and HepI-II-III, although not below the observed effect of HepI-II-III treatment alone. Accordingly, soluble hyaluronic acid did not effectively compete with mGEnC-associated hyaluronic acid for full-length FH and FH19–20 (Fig. 3). In contrast, preincubation with heparin or HS significantly inhibited the binding of full-length FH and FH19–20 to TNF-α-activated mGEnCs.

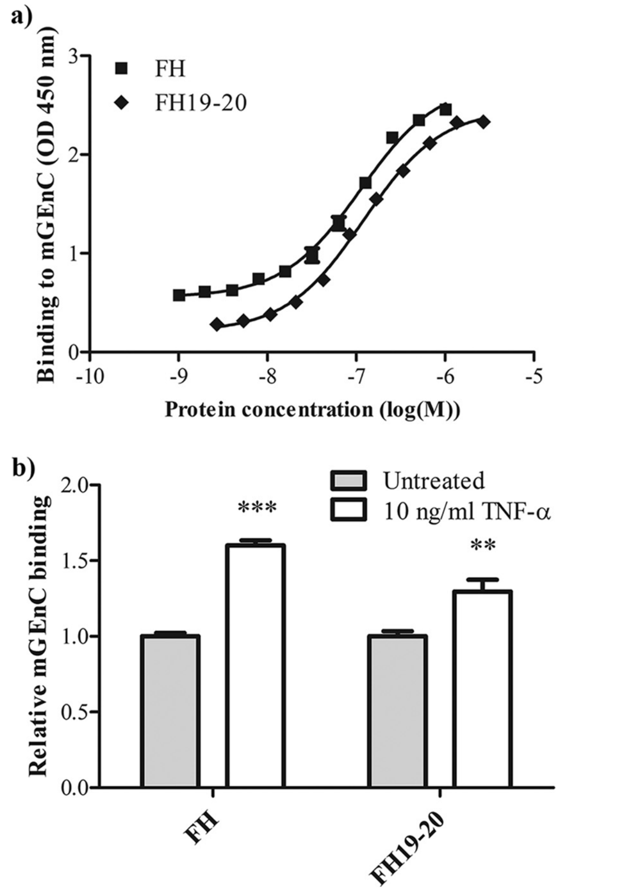

FIGURE 1. **Full-length FH and FH19–20 bind differentially to mGEnCs.** *a*, serial dilutions of FH and recombinant FH CCP19–20 (*FH19–20*) were added to mGEnCs, and binding was detected using ELISA. Full-length FH bound mGEnCs at a lower EC_{50} concentration than FH19–20, suggesting the presence of additional binding sites for ligands on the cell surface. *b*, TNF-α activation of mGEnCs resulted in an increased binding of full-length FH and FH19–20. The results were normalized on binding of the complement factors to untreated mGEnCs. **, $p < 0.01$ *versus* untreated mGEnCs; ***, $p < 0.001$ *versus* untreated mGEnCs.

Because the interaction between HS and FH depends on the sulfation of the GAG backbone, we evaluated the effect of several selectively desulfated heparinoids in competition with HS in the glomerular endothelial glycocalyx. None of the modified heparinoids affected the binding of full-length FH when compared with heparin as the competitor (Fig. 3*a*). In contrast, preincubation with 2-*O*- and 6-*O*-desulfated heparin significantly

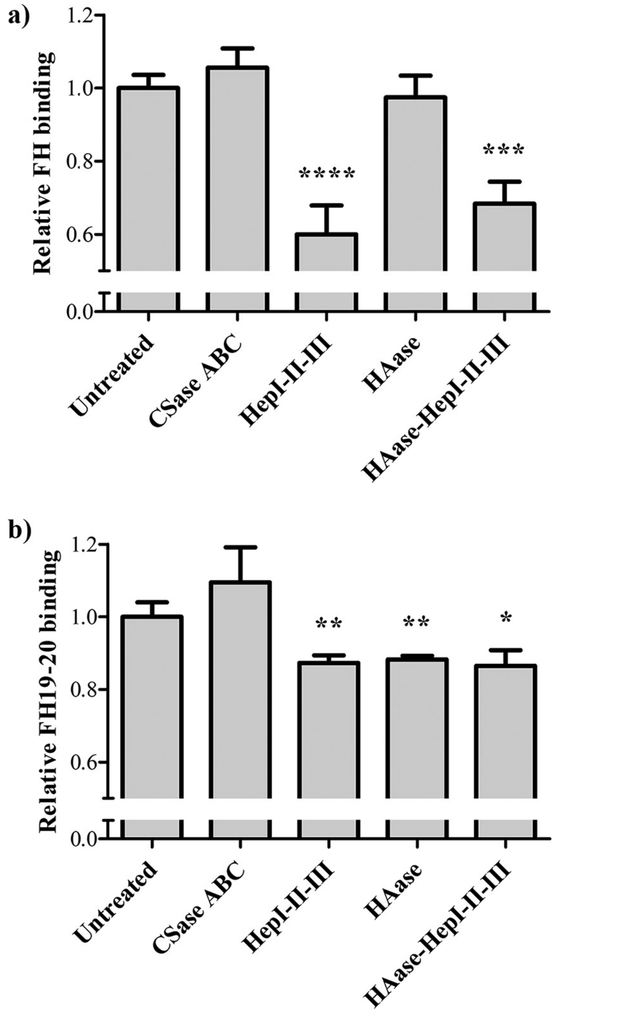

FIGURE 2. **Heparinase treatment reduces the binding of full-length FH and FH19–20 to mGEnCs.** Monolayers of TNF-α-activated mGEnCs were treated with chondroitinase ABC (*CSase ABC*), a mixture of heparinase I, II, and III (*HepI-II-III*), hyaluronidase (*HAase*), or a combination of hyaluronidase and heparinase I, II, and III (*HAase-HepI-II-III*). The binding of full-length FH (*a*) and FH19–20 (*b*, both at EC_{50} concentrations) was measured by ELISA. The results were normalized on binding of full-length FH and FH19–20 to untreated mGEnCs. *, $p < 0.05$ *versus* untreated mGEnCs; **, $p < 0.01$ *versus* untreated mGEnCs; ***, $p < 0.001$ *versus* untreated mGEnCs; ****, $p < 0.0001$ *versus* untreated mGEnCs.

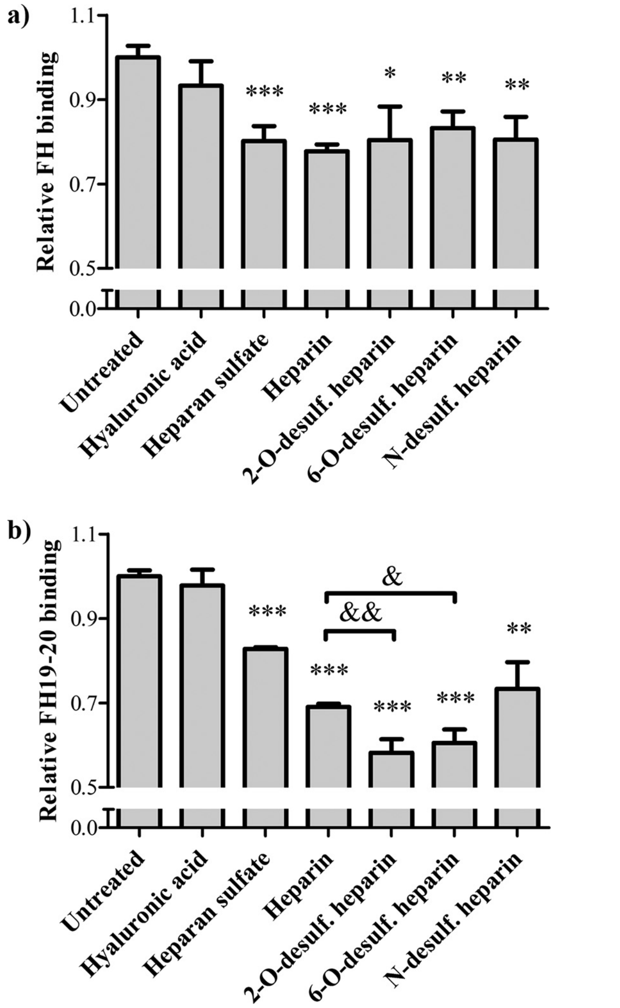

FIGURE 3. **Competition with soluble heparin and HS reduces the binding of full-length FH and FH19–20 to mGEnCs.** FH and FH19–20 were preincubated with 50 μg/ml hyaluronic acid, HS, heparin, or 2-*O*-, 6-*O*-, and *N*-desulfated (*desulf.*) heparin before adding the mixtures to monolayers of TNF-α-activated mGEnCs. The binding of full-length FH (*a*) and FH19–20 (*b*) to the cells was then evaluated by ELISA. Preincubation with heparin and HS significantly reduced binding of both proteins, whereas 2-*O*- and 6-*O*-desulfation selectively increased the inhibitory effect compared with heparin on FH19–20 but not FH binding to mGEnCs. The results were normalized on binding of full-length FH and FH19–20 to mGEnCs in the absence of competitors (*Untreated*). *, $p < 0.05$ *versus* untreated; **, $p < 0.01$ *versus* untreated; ***, $p < 0.001$ *versus* untreated; &, $p < 0.05$ *versus* heparin; &&, $p < 0.01$ *versus* heparin.

reduced the binding of FH19–20 to mGEnCs compared with heparin (Fig. 3*b*), indicating that these modifications could be involved in the self-recognition of glomerular endothelium by FH19–20. *N*-Desulfated heparin in turn did not affect binding of FH19–20 to mGEnCs. Thus, 2-*O*- and 6-*O*-desulfated heparin showed differential inhibitory effects on the binding of full-length FH and FH19–20 to mGEnCs. Finally, GAG digestion appeared more effective in reducing full-length FH binding compared with FH19–20 (Fig. 2), whereas the opposite was observed during the competition experiments (Fig. 3).

aHUS-associated FH19–20 Mutants Are Less Able to Compete with Full-length FH for Ligands on mGEnCs Compared with Wild Type FH19–20 during Alternative Pathway Activation—We previously identified three types of defects caused by mutations in FH19–20, *i.e.* decreased binding to C3b/C3d and decreased or increased binding to heparin and mGEnCs (36). However, the functional consequences of these mutations for complement control on glomerular endothelium were still unexplored. For the current study we selected FH mutants to include all defined defects as well as their combinations and evaluated their ability to compete with full-length FH on mGEnCs during alternative pathway activation.

Incubating mGEnCs with normal human serum resulted in significant C3b deposition on the cell surface compared with heat-inactivated human serum as measured in flow cytometry (Fig. 4*a*). Competing with full-length FH for ligands on mGEnCs using wild type FH19–20 further increased the observed C3b deposition by ~1.7-fold, suggesting an increased susceptibility to alternative pathway activation. Interestingly, several of the screened aHUS-associated FH19–20 mutants show a decreased ability to compete with full-length FH compared with wild type FH19–20 (Fig. 4*b*). The effect is statisti-

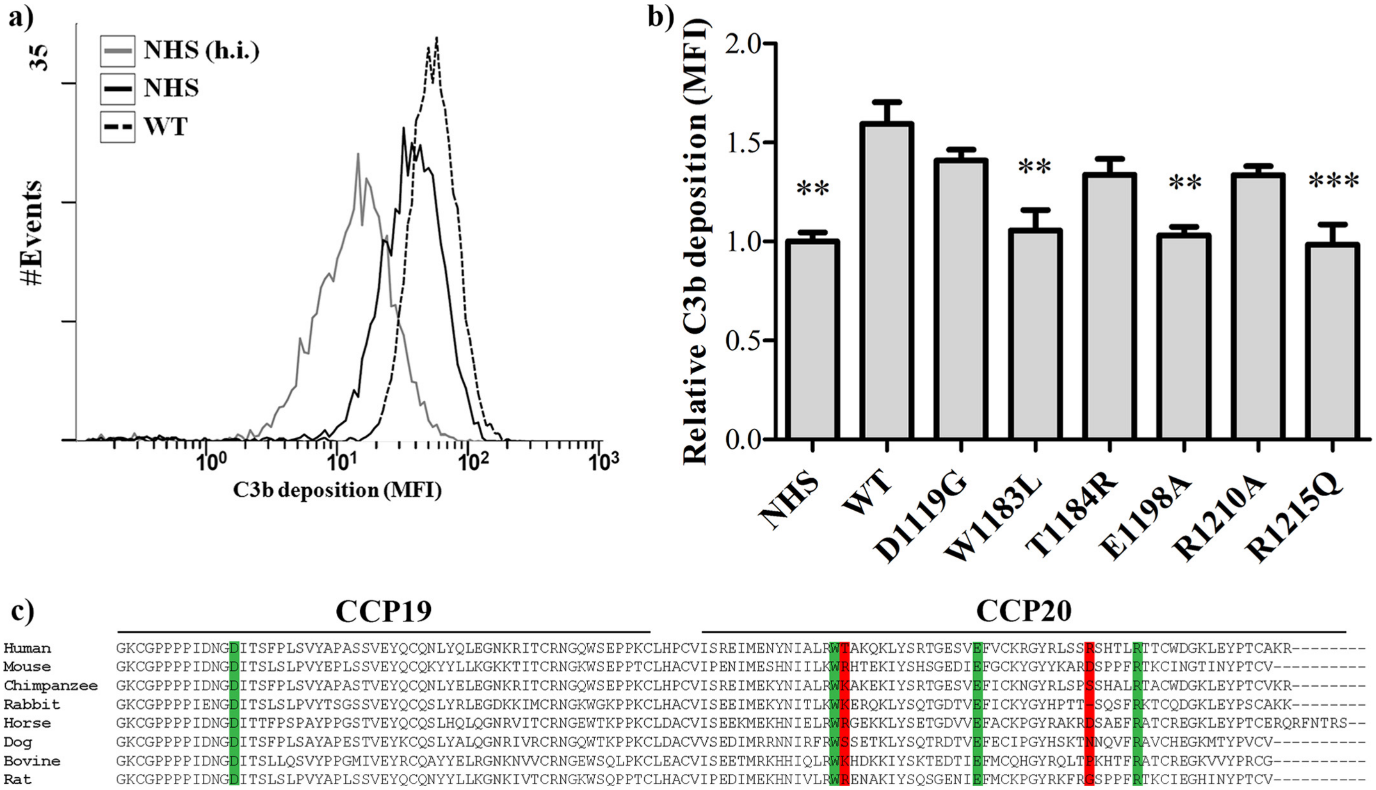

FIGURE 4. **aHUS-associated FH19–20 mutants are less able to compete with full-length FH during alternative pathway activation on mGEnCs.** *a*, TNF-α-activated mGEnCs were incubated with 20% heat-inactivated and normal human serum (*NHS (h.i.)* and *NHS*, respectively) and C3b deposition on the cell surface was evaluated using flow cytometry. *b*, adding 10 μg/ml wild type FH19–20 (*WT*) to compete with FH for ligands in the cellular microenvironment during alternative pathway activation significantly increases C3b deposition compared with incubation with NHS alone. Interestingly, several screened FH19–20 mutants are less able to compete with full-length FH during activation, with the mutations W1183L, E1198A, and R1215Q resulting in an almost complete loss of competitive ability. *c*, sequence alignment of mammalian FH CCP19–20 amino acid sequences. With the exception of D1119G, the loss of inhibitory ability compared with wild type FH19–20 reflects the conservation of the mutated amino acid in mammalian species (*green*, highly conserved; *red*, not conserved). **, $p < 0.01$ *versus* WT; ***, $p < 0.001$ *versus* WT. *MFI*, mean fluorescence intensity.

cally significant for the mutants W1183L, E1198A, and R1215Q, which appear unable to compete with full-length FH at all, indicating a complete loss of function of the C-terminal cell-surface recognition domains. Although the remaining mutants, D1119G, T1184R, and R1210A, are not significantly less able to compete with full-length FH compared with wild type FH19–20, they all display a trend toward decreased function.

Discussion

Previous *in vitro* studies on the interaction between FH and endothelium or the effects of FH mutations on cell-surface complement control used non-renal endothelial cells, which might lack tissue-specific disease-determining factors. Therefore, we characterized the GAG ligands for full-length FH and FH19–20 on glomerular endothelial cells. Furthermore, we explored the effect of aHUS-associated FH19–20 mutations on alternative pathway inhibition in the context of the physiologically affected tissue.

We found that both full-length complement FH and FH19–20 bound dose-dependently to mGEnCs. TNF-α activation of mGEnCs resulted in a significant increase in binding of full-length FH and FH19–20 to the endothelial monolayer. This might reflect a cellular response to provide additional protection from complement-mediated damage during glomerular inflammation. We previously showed that TNF-α activation increases the expression highly sulfated HS domains (recognized by the antibodies AO4B08, EW3D10, and EW4G2) as well as lowly sulfated HS (recognized by 10E4) (29). Total HS expression in the endothelial glycocalyx increased ~3-fold in response to TNF-α, which could result in an increased binding of FH and FH19–20.

It is important to note that the interaction studies and pathogenicity screening of FH19–20 mutants were performed using murine instead of human glomerular endothelial cells. However, FH-deficient mouse and pig models suggest that the tissue-specific determinants of FH-associated diseases are conserved within mammals (33–35, 44). The animals develop a DDD-like renal phenotype, and aged FH-deficient mice have been used as the model for AMD (45). Furthermore, the DDD-like phenotype converts to aHUS in FH-deficient mice expressing a transgenic FH variant lacking CCP16–20, as the model for FH mutants with C-terminal loss of function (35). The renal disease phenotype can be reversed by injecting FH-deficient mice with human FH, suggesting cross-reactivity between the human and murine factors involved in complement regulation (46).The removal of HS from the endothelial glycocalyx significantly reduced binding of both full-length FH and FH19–20, confirming that HS mediates FH binding to glomerular endo-

TABLE 1

Functional characterization of aHUS-associated FH19–20 mutants

Arrows indicate an increase (↑), decrease (↓), or no significant change (↔) in binding/activity.

FH19–20 mutant	C3b/C3d binding	Heparin binding	mGEnC binding	Functional activity
D1119G[a]	↔	↔	↔	↔
W1183L[a]	↓	↔	↔	↓
T1184R	↓/↔	↑	↑	↔
E1198A[a]	↔	↑	↑	↓
R1210A	↓	↓	↓	↔
R1215Q[a]	↔	↓	↔	↓

[a] Conserved amino acid; binding data adapted from Lehtinen *et al.* (36).

thelium via CCP19–20. In contrast, removal of chondroitin and dermatan sulfate had no effect on the interaction between mGEnCs and either of the proteins. Removal of hyaluronic acid by hyaluronidase decreased binding of FH19–20, whereas the addition of hyaluronic acid in soluble form had no effect on binding. This apparent discrepancy could arise because hyaluronidase treatment disturbs the integrity of the endothelial glycocalyx, which includes GAGs and short branched *N*- and *O*-linked oligosaccharides with negatively charged terminal sialic acid residues (47).

In accordance with the GAG digestion results, competition with soluble heparin and HS significantly diminished the interaction between mGEnCs and full-length FH or FH19–20. Interestingly, 2-*O*- and 6-*O*-desulfated heparin significantly decreased binding of FH19–20 to mGEnCs even further. Although this effect is not observed for full-length FH, it could be masked by the presence of additional non-HS ligands on mGEnCs. These results suggest that FH19–20 preferentially binds to heparan sulfate with low levels of 2-*O*- and 6-*O*-sulfation, contradicting previous observations that the interaction between FH19–20 and heparin depends on high levels of sulfation (21). However, these experiments either measured binding of FH to heparin directly or competed with soluble heparinoids for immobilized heparin. Our experiments in turn used modified heparins in solution to compete for HS in its physiological environment, the endothelial glycocalyx. Therefore, 2-*O*- and 6-*O*-desulfated heparin could be more effective in competing for the physiological HS ligands of FH19–20 compared with unmodified heparin. Both in the GAG digestion and competition experiments, complete reduction of FH and FH19–20 binding could not be achieved, suggesting that other, non-GAG ligands indeed contribute to the interaction between FH and glomerular endothelium. As full-length FH is glycosylated, contrary to FH19–20, interactions between cell surface receptors and glycans in FH could contribute to the interaction as well.

We previously characterized three different functional defects of FH19–20 mutants, *i.e.* decreased ability to compete with wild type FH19–20 for C3b/C3d (W1183L, T1184R, and R1210A), decreased (R1210A and R1215Q), or increased (T1184R and E1198A) binding to heparin and mGEnCs (Table 1) (36). Although we originally found none of these defects in D1119G, later studies revealed a decreased affinity for C3b/C3d (48). Because the D1119G mutant still efficiently competes with wild type FH19–20 for C3b/C3d, the mutation might affect the self-association characteristics of the two C-terminal CCPs, preventing binding to C3b/C3d. Recently, the aHUS/AMD-associated R1210C mutation has been shown to introduce a gain of function defect, as it enables the protein to form covalent bonds with serum albumin (49, 50). The R1210A mutant used in the current study lacks the surface-exposed cysteine; therefore, the observed effects can likely be attributed to a decrease in electrostatic interactions with FH19–20 ligands.

However, the influence of these C-terminal FH mutations on complement control on glomerular endothelium had not yet been explored. Therefore, we designed a competition assay to explore the effect of mutations in FH19–20 on glomerular complement control. The alternative pathway activated spontaneously on mGEnCs as measured through a significant increase in C3b deposition compared with heat-inactivated serum. This represents an advantage of using mGEnCs compared with human endothelium, which is protected from complement attack from normal human serum. We observed significant functional defects in three of the mutants, which are likely caused by altered interactions with both complement proteins and cell surface ligands. W1183L has a decreased affinity for C3b/C3d and is apparently unable to compete with full-length FH for the protein during complement activation. E1198A shows an increased affinity for heparin and mGEnCs, whereas binding of R1215Q to heparin is decreased. The loss of a negatively and positively charged amino acid, respectively, likely changes the specificity of the proteins for FH19–20 binding HS domains, leaving sufficient HS unoccupied to efficiently inhibit complement activation on mGEnCs despite the competition. Note that competition using mutants with two of the defined defects (T1184R and R1210A) did not have the largest effect on alternative pathway inhibition on mGEnCs. Instead, the observed decrease in competitive ability of the screened FH19–20 mutants reflects the conservation of the affected amino acid in mammalian species, with the exception of D1119G (Fig. 4*c*). All other mutations in highly conserved amino acids (W1183L, E1198A, and R1215Q) completely abolish the protein's ability to compete with full-length FH for ligands on mGEnCs. However, additional FH19–20 mutants will have to be screened to expand this observation.

Because recombinant proteins were used to evaluate the functional effects of mutations in FH19–20, the experiments can be performed without access to limited available aHUS patient serum. Therefore, our assay is unaffected by variable complement concentrations in patient sera, current disease state, or improper handling of serum samples, as the same human serum pool is used as a source of complement for all FH19–20 mutants. However, potential pathogenic contributions of other serum factors, such as FH-like 1 and FH-related proteins 1–5, will not be reflected in the obtained results.

In conclusion, this study is the first to demonstrate the role of HS in binding of the full-length FH and FH19–20 to (TNF-α-activated) glomerular endothelial cells. Furthermore, we showed the pathogenic effect of several aHUS-associated FH19–20 mutations on FH-mediated complement control on the clinically affected tissue. Finally, we have described a competition assay that enables pathogenicity screening of uncharacterized FH mutations without requiring access to limited available patient serum.

Author Contributions—M. A. L., A. L. R., M. B., and M. J. L. conceived and performed the experiments and data analysis. M. R. D. advised during the development of the alternative pathway activation and FH mutant competition assay. T. H. v. K. provided the GAG-specific single chain antibodies. M. A. L, A. L. R., R. J. S., J. H. M. B., and J. v. d. V. wrote the manuscript. T. S. J., T. J. R., and J. v. d. V. acquired funding. J. v. d. V. supervised the study.

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Mutations in Complement Factor H Impair Alternative Pathway Regulation on Mouse Glomerular Endothelial Cells *in Vitro*

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