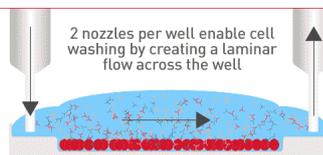


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The Binding of Factor H to a Complex of Physiological Polyanions and C3b on Cells Is Impaired in Atypical Hemolytic Uremic Syndrome¹

Viviana P. Ferreira,* Andrew P. Herbert,[†] Claudio Cortés,* Kristi A. McKee,* Bärbel S. Blaum,[†] Stefan T. Esswein,[†] Dušan Uhrín,[†] Paul N. Barlow,[†] Michael K. Pangburn,* and David Kavanagh^{2†}

Factor H (fH) is essential for complement homeostasis in fluid-phase and on surfaces. Its two C-terminal domains (CCP 19–20) anchor fH to self-surfaces where it prevents C3b amplification in a process requiring its N-terminal four domains. In atypical hemolytic uremic syndrome (aHUS), mutations clustering toward the C terminus of fH may disrupt interactions with surface-associated C3b or polyanions and thereby diminish the ability of fH to regulate complement. To test this, we compared a recombinant protein encompassing CCP 19–20 with 16 mutants. The mutations had only very limited and localized effects on protein structure. Although we found four aHUS-linked fH mutations that decreased binding to C3b and/or to heparin (a model compound for cell surface polyanionic carbohydrates), we identified five aHUS-associated mutants with increased affinity for either or both ligands. Strikingly, these variable affinities for the individual ligands did not correlate with the extent to which all the aHUS-associated mutants were found to be impaired in a more physiological assay that measured their ability to inhibit cell surface complement functions of full-length fH. Taken together, our data suggest that disruption of a complex fH-self-surface recognition process, involving a balance of affinities for protein and physiological carbohydrate ligands, predisposes to aHUS. *The Journal of Immunology*, 2009, 182: 7009–7018.

Atypical hemolytic uremic syndrome (aHUS)³ is a disease characterized by hemolytic anemia, thrombocytopenia, and acute renal failure (1). In 1998, Warwicker et al. (2) described mutations in the complement regulator, factor H (fH), associated with aHUS. This observation was subsequently confirmed in five independent cohorts of aHUS patients (3–7). The majority of these mutations are heterozygous and cluster in the C terminus of fH. Incomplete penetrance is described in all reports, suggesting that a combination of mutations, risk haplotypes, and precipitating factors, such as infection or pregnancy, are required for aHUS to manifest (8–12).

fH, an abundant serum glycoprotein (~500 μg/ml), plays a key role in the homeostasis of the complement system. It accelerates

the decay of C3b,Bb complexes that, in the alternative pathway, proteolytically convert C3 to its activated form, C3b (13). It is also a cofactor for factor I-mediated cleavage and inactivation of C3b (14). fH is composed of 20 complement control protein modules (CCP) that resemble beads on a flexible string in the transmission electron microscope (15, 16). The N-terminal four CCP (i.e., CCP 1–4) encompass the region capable of cofactor and decay accelerating activity (17–20). A second principal C3b binding site lies within the C-terminal pair of CCPs (CCP 19–20) and also interacts with iC3b and C3d (18, 21–23). At least two sites on fH (i.e., CCP 7 and CCP 20) interact with heparin and other polyanions (24–30). The C-terminal site additionally binds sialic acids (24, 28).

In the absence of fH, spontaneous activation of the alternative pathway of complement occurs in plasma, which leads to consumption of complement components C3 and factor B. In addition to its function as a regulator of alternative pathway activation in fluid phase, fH is essential for complement control on many self-surfaces. For example, it binds to human endothelial cells and basement membranes (31) protecting them from complement-mediated attack (32). Indeed, fH has a 10-fold higher affinity for C3b on host cells and other nonactivators of complement vs activators, which is dependent on the presence of sialic acid clusters or other polyanions on the surface (33, 34). The specific chemical nature of human host markers has not been identified, but it is assumed to be polyanionic because sheep cells, which do not activate complement, activate the alternative pathway after surface sialic acid is removed (33, 34). Previous studies have shown that the C3b and polyanion binding sites in CCP 19–20 are key for fH interactions with host surfaces (21, 22, 28, 31, 35–37). Furthermore, a recombinant form of these C-terminal CCPs (rH19–20) has been shown to compete with full-length fH for binding to C3b and host polyanions (35). This competition results in increased complement activation on host surfaces, without affecting control of complement in plasma (35).

*Department of Biochemistry, Center for Biomedical Research, University of Texas Health Science Center, Tyler, TX 75708; and [†]Edinburgh Biomolecular NMR Unit, Schools of Chemistry and Biological Sciences, University of Edinburgh, Edinburgh, United Kingdom

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² Address correspondence and reprint requests to Dr. David Kavanagh, Edinburgh Biomolecular NMR Unit, Schools of Chemistry and Biological Sciences, Joseph Black Chemistry Building, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, United Kingdom. E-mail address: davidkavanagh@doctors.org.uk

³ Abbreviations used in this paper: aHUS, atypical hemolytic uremic syndrome; CCP, complement control protein domain; dp6, hexasaccharide; E_H, human erythrocyte; E_sC3b, C3b-coated sheep erythrocyte; fH, factor H; GAG, glycosaminoglycan; GMSA, gel mobility shift assay; HSQC, heteronuclear single quantum coherence; NHS, normal human serum; rH 19–20, recombinant domains 19–20 of factor H; RU, response unit; SA, streptavidin; SPR, surface plasmon resonance.

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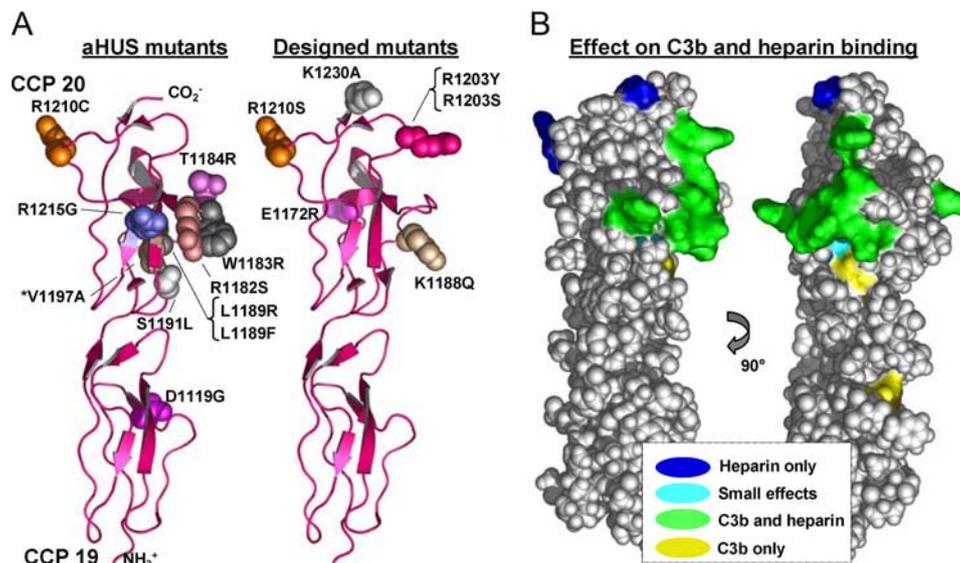


FIGURE 1. Summary of mutagenesis study mapped onto 3D structure of fH CCP 19–20. *A*, Side-chain heavy atoms of disease-linked mutations (*left panel*) or nondisease linked mutations are drawn (in various colors for clarity) on cartoon representations of fH CCP 19–20. *V1197A was made in the context of the double mutant—V1197A;S1191L. *B*, A surface representation of mutated residues is superimposed on the structure (orthogonal views are shown; *left view* is identical to that in *A*) and color-coded (see key) according to the predominant impact of the mutation on ligand binding. Positive and negative effects were not discriminated—“heparin only” implies that the effect on C3b-binding was small while “C3b only” implies a small effect on heparin binding. The cyan color indicating “small effect” refers to that of L1189F on C3b and heparin binding. L1189R had significantly increased binding to both C3b and heparin.

The C-terminal module of fH is the most commonly mutated one in individuals with aHUS and functional analysis to date has demonstrated an associated impairment of binding to C3b and/or glycosaminoglycans (GAGs) (38). Moreover, a transgenic mouse line expressing fH missing its C-terminal region regulated C3 activation in plasma but spontaneously developed aHUS (39). The truncated murine fH failed to bind to endothelial cells. Thus, in aHUS, the mutant form of fH is believed to regulate the alternative pathway of complement in the fluid phase but fails to be recruited by glomerular endothelial cells and/or the glomerular basement membrane.

Controversy has arisen over the interpretation of the limited structure-function studies that attempted to assign disease-linked mutations in the fH C terminus to binding sites on the fH C terminus for specific ligands. Jokiranta et al. (40) concluded that aHUS-linked mutations lie in a C3d/b binding site, whereas the polyanion binding site is not affected. On the other hand, Herbert et al. (41) presented evidence that shows that mutations cluster within an electropositive polyanion binding site. Moreover, none of the disease-linked mutants have been analyzed with respect to their structural integrity; thus, it remains possible that the mutations are destabilizing or result in unfolded protein. In the current work, we set out to resolve these issues and to thereby address the question: why do aHUS-linked mutations prevent fH from functioning properly?

To avoid difficult-to-interpret complications likely to arise from the presence of additional C3b and heparin binding sites that reside within the N-terminal 18 CCPs of fH, we mutated and analyzed a recombinant construct containing only the CCPs 19 and 20 (rH19–20). We measured the affinity of each of 16 rH19–20 mutants for C3b and, separately, for polyanions. Because C3b and host polyanions reside together on surfaces, we also used assays designed to measure the interactions with these ligands simultaneously in a physiological setting on the cell surface.

Materials and Methods

RBC and buffers

Blood from sheep and from two healthy human adults was collected by venipuncture and the red cells were frozen at -80°C (42). The University

of Texas Health Science Center institutional review board approved protocols, and written informed consent was obtained from all human donors. The buffers used were as follows: PBS (10 mM sodium phosphate, 140 mM NaCl, and 0.02% NaN_3 (pH 7.4)); Veronal-buffered saline (5 mM veronal, 145 mM NaCl, and 0.02% NaN_3 (pH 7.3)); GVB (Veronal-buffered saline containing 0.1% gelatin); GVBE (GVB containing 10 mM EDTA); DGVB (half-physiological ionic-strength buffer prepared by diluting GVB 2-fold with 5% dextrose in water); and MgEGTA (0.1 M MgCl_2 , and 0.1 M EGTA (pH 7.3)).

Purified proteins

Complement proteins fH (43), C3 (44, 45), factor B (46), and factor D (47) were purified from normal human plasma as described previously. A fragment consisting of C-terminal residues 1107–1231 of fH (domains 19–20, i.e., rH19–20) was cloned, expressed, and purified as described previously (41). Site-directed mutagenesis was used to generate sixteen mutant versions of rH19–20, using the Quickchange mutagenesis system (Stratagene). Ten of these are associated with aHUS (D1119G, R1182S, W1183R, T1184R, L1189F, L1189R, S1191L, R1210C, R1215G, and double-mutant S1191L;V1197A) (48); the remaining six mutants (E1172R, K1188Q, R1203S, R1203Y, R1210S, and K1230A) have not so far been observed in aHUS patients but were generated to probe C3b and polyanion binding sites. The residues chosen for the design of these mutants lie in putative C3b and polyanion binding sites (40, 41) and cover a wider surface region within domain 20, which is where most aHUS-associated mutations in fH lie. The proteins were cloned and expressed using the *Pichia pastoris* strain KM71H as described previously (41). Mass spectra of recombinant proteins (purified by cation-exchange chromatography and stored at -80°C in PBS as previously described (41)) were consistent with predicted molecular weights. A ^{15}N -labeled sample of each mutant was prepared by using $(^{15}\text{NH}_4)_2\text{SO}_4$ (Isotec) as the sole source of nitrogen during cell growth. Protein concentrations were determined (280 nm) using theoretical $\text{E}^{1\%}_{1\text{cm}}$ values (49). fH and an aliquot of wild-type rH19–20 (100 μg) were radiolabeled with ^{125}I to specific activities of 4–6 $\mu\text{Ci}/\mu\text{g}$ as described previously (35).

Nuclear magnetic resonance (NMR) methods

For NMR, samples of ^{15}N labeled proteins were 100 μM in 20 mM deuterated sodium acetate, 10% D_2O , pH 4.5. In each case a $[^{15}\text{N},^1\text{H}]$ heteronuclear single quantum coherence (HSQC) spectrum was collected at 37°C on a Bruker AVANCE 800 MHz spectrometer fitted with a 5-mm CPDCl cryoprobe with Z gradients. On the basis of existing spectral assignments for wild-type rH19–20 (41), analyses were conducted to reveal any

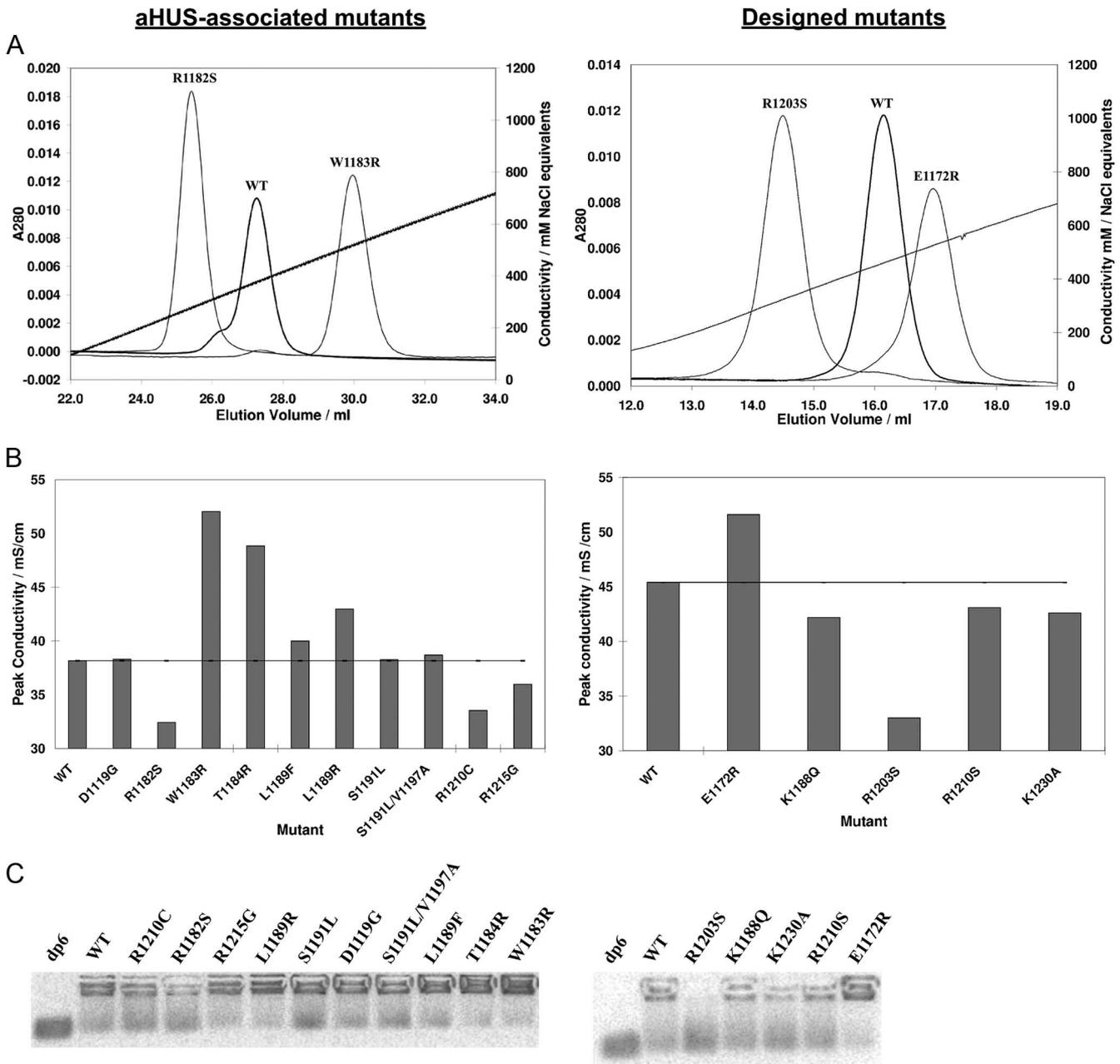


FIGURE 2. Affinities of wild-type and mutant rH19–20 for GAGs. Variants of rH19–20 were eluted from a HiTrap heparin-affinity chromatography column, and protein elution was monitored using absorbance at 280 nm (A). *A, left panel.* For clarity, only the elution profile of the aHUS-associated mutant with the highest (W1183R) and the lowest (R1182S) affinity for heparin are shown in comparison to wild-type rH19–20. The conductivity values at which the aHUS-associated mutants eluted are summarized in *B, left panel.* *A, right panel.* Only the elution profile of the designed mutant with the highest (E1172R) and the lowest (R1203S) affinity for heparin are shown in comparison to wild-type rH19–20. The conductivity values at which all the designed mutants eluted are shown in *B, right panel.* *C,* GMSA results. For these assays, 2-aminoacridone-tagged heparin-derived hexasaccharides were combined with rH19–20 or its mutants to give 34 μ M protein and 40 μ M oligosaccharide in 12 μ l of PBS containing 5 mM EDTA. To these samples were added 0.5 μ l of glycerol and a trace of phenol red before incubation (15 min, room temperature). Samples were then loaded on a 1% agarose gel in 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. Electrophoresis was performed (120 mV, 5 min) in a horizontal agarose electrophoresis system, using an electrophoresis buffer (40 mM Tris/acetate and 1 mM EDTA (pH 7.4)). Immediately thereafter, the fluorescent oligosaccharides were visualized. The *left* and *right panels* show the data obtained with the aHUS-associated mutants or the designed mutants, respectively. All results are representative of two separate experiments.

changes in the ^{15}N and ^1H chemical shifts of each backbone amide consequent upon mutagenesis.

Heparin affinity chromatography

Protein samples (3 μ M, 1 ml) in 20 mM potassium phosphate buffer (pH 7.4) at 20°C were loaded individually onto a HiTrap heparin-affinity chromatography column (7 \times 25 mm; GE Healthcare), equilibrated with the same buffer, and subsequently eluted with a 20-column volume gradient from 0 to 1 M NaCl in 20 mM potassium phosphate buffer (pH 7.4). Protein

elution was monitored using absorbance at 280 nm. Similar experiments were conducted for the aHUS-associated mutants (0.6 μ M, 5 ml) in 20 mM HEPES (pH 7.0) at 4°C and eluted with a 20 column volume gradient from 0 to 1 M NaCl in 20 mM HEPES (pH 7.0).

Gel-mobility shift assays (GMSA)

Oligosaccharides were prepared for GMSA from low-m.w. heparin by partial digestion with heparinase I followed by size fractionation on a BioGel P10 gel-filtration column (Bio-Rad) (50). In the case of the tetrasaccharide,

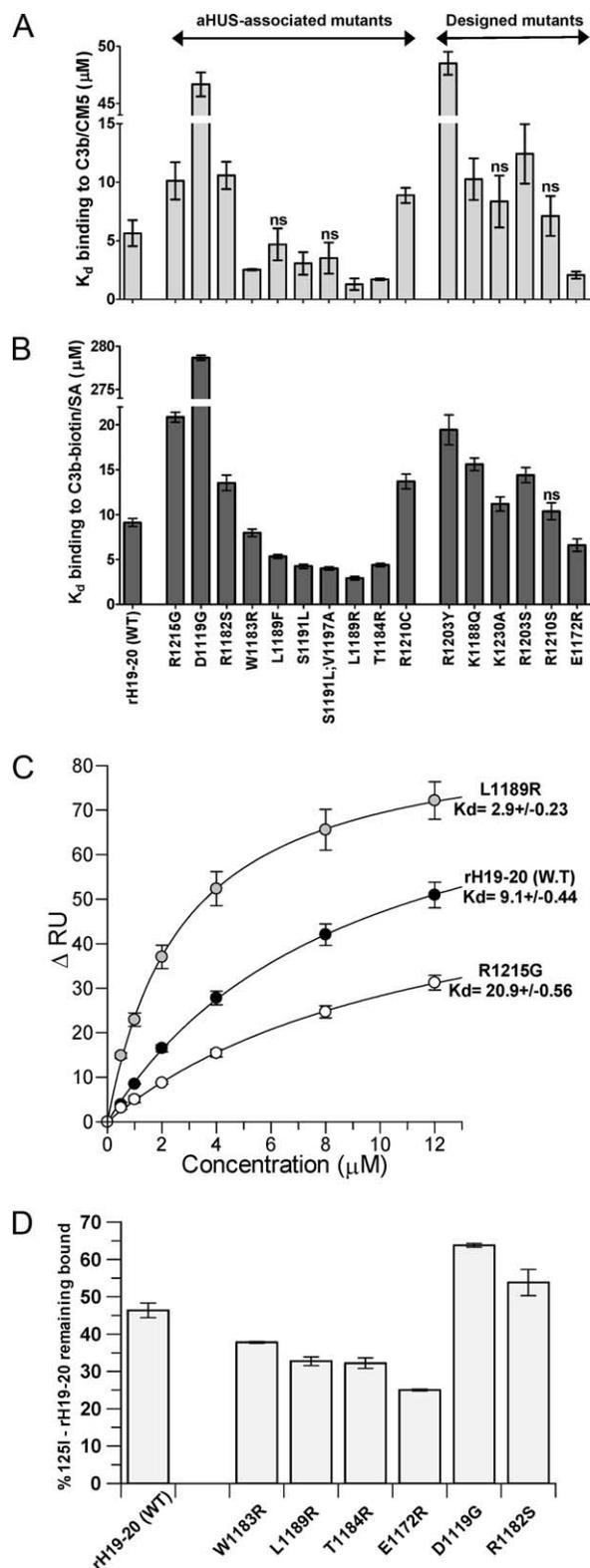


FIGURE 3. Binding affinity of the rH19–20 mutants for C3b. *A*, K_d values for the binding of rH19–20 to C3b were measured on a CM5 sensor chip using SPR. Assays were performed on a BIAcore 3000 instrument by immobilizing 800–2000 RU of human C3b to three independent flow cells of a CM5 chip, using amine coupling. The rH19–20 mutants or wild-type rH19–20 were injected at 0–12 μ M over the flow cells. The maximum equilibrium binding, at each concentration, was plotted using the Grafit 5 program, which determined the apparent K_d by fitting the data to a single-site saturation curve, where the capacity was fixed (as a constant) to that of the average ($n = 12$) wild-type rH19–20 capacity. The SDs correspond to the K_d values obtained when samples were injected over the three different

dp4, strong anion-exchange chromatography (AS-17 Ionpac; Dionex) was subsequently performed to isolate only fully sulfated product (Δ UA2S-GlcNS6S-IdoA2S-GlcNS6S). Because of the heterogeneity associated with the length of the hexasaccharide (dp6), this purification step was omitted in dp6 preparations, resulting in variable sulfation patterns. Fluorophore-labeled species were produced by attachment of 2-aminoacridone to the oligosaccharide reducing end (50), and GMSA were performed, as described previously (51).

Analysis of C3b binding by surface plasmon resonance (SPR)

A BIAcore 3000 instrument was used to perform SPR. Human C3b (800–2000 response units (RU)) was amine coupled to a CM5 sensor chip (GE Healthcare). In addition, a streptavidin (SA) sensor chip (GE Healthcare) was coated with biotinylated C3b, generated as described recently (52). For each type of sensor chip, three flow cells (Fc2, Fc3, and Fc4) were independently coupled with C3b. Then, 20- μ l injections of the rH19–20 mutants and wild-type rH19–20 were performed at 0–12 μ M over the flow cells at 5 μ l/min, at 22°C. The running and sample buffer consisted of PBS with 0.005% Tween. The maximum equilibrium binding of the proteins to C3b was measured by subtracting RU obtained for the reference cell (Fc1) from the C3b-coupled flow cell RU. The equilibrium binding values for each concentration were plotted using the Grafit 5 program (Erithacus), which determined the apparent K_d by fitting the data to a single-site saturation curve. Because the wild-type rH19–20 interacts with C3b with a 1:1 stoichiometry, the K_d values of the mutant proteins were calculated by setting the capacity (as a constant) to that of the average ($n = 12$) capacity of wild-type rH19–20, for each C3b-coupled sensor chip.

Preparation of C3b-coated cells and zymosan particles

Deposition of C3b on sheep erythrocytes (E_s), and zymosan particles (Sigma-Aldrich) was accomplished using purified C3, factor B and factor D as described previously (53). The number of bound C3b molecules was determined to be 125,000 per E_s cell or 100,000 per zymosan particle by measuring binding of radiolabeled factor Bb (53).

Binding assays with C3b-coated cells and zymosan

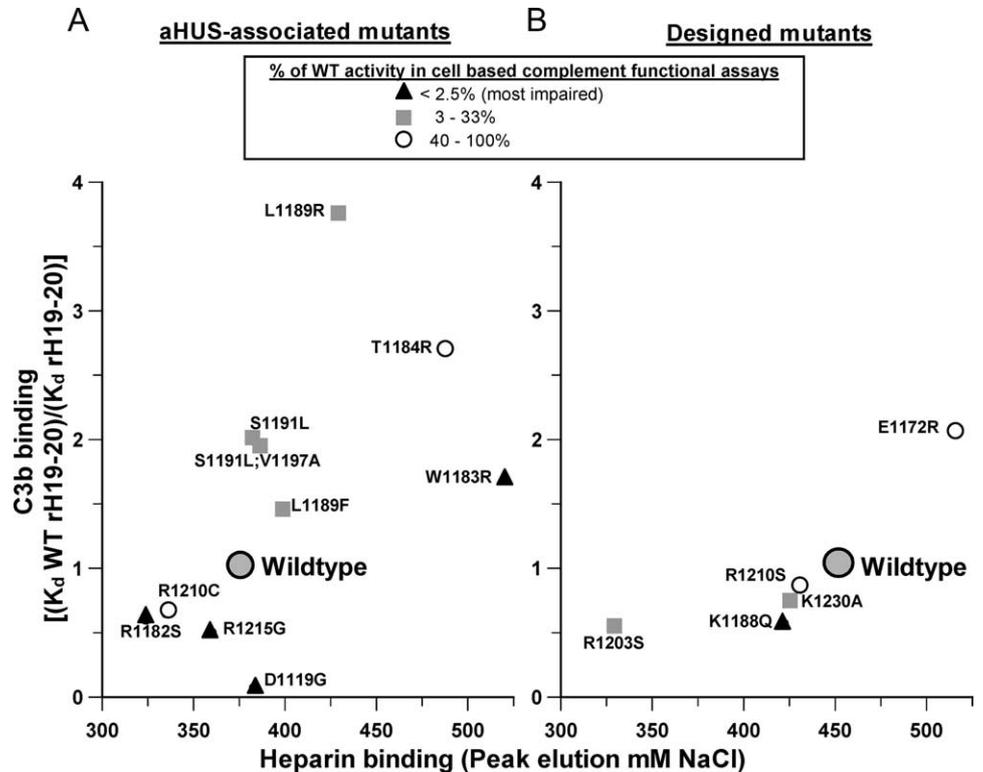
Sheep erythrocytes (E_s C3b) bearing a total of 1 μ g of C3b were incubated with \sim 20 ng of radiiodinated human fH and 0 to 8 μ M nonlabeled rH19–20 (wild-type or mutants) in 100 μ l of half-ionic strength buffer (DGVB). Alternatively, the cells were incubated with \sim 20 ng of 125 I-rH19–20 and the nonlabeled proteins. After 20 min at 22°C, the bound and free radiolabeled proteins were separated as described previously (34). The competition experiments with C3b-coated zymosan (ZymC3b) were conducted as described above, except only the IC₅₀ dose of the unlabeled wild-type rH19–20 (1 μ M) was compared with a 1 μ M dose of various rH19–20 mutants.

Hemolysis assays

Lysis of human erythrocytes (E_H) (35, 51) was measured by preincubating 5×10^6 E_H with 7 μ g/ml anti-CD59 mAb (clone MEM43; Abcam) for 20 min at 4°C, and then mixing, on ice, with GVB, normal human serum (NHS; 40% final), and 0.1 M MgEGTA (5 mM final concentration) in the presence of 1 μ M wild-type or mutant rH19–20. This concentration of wild-type rH19–20 is enough to achieve \sim 50% hemolysis (IC₅₀) of

C3b-coated flow cells. *B*, Same as *A*, except biotinylated C3b was used to coat a SA sensor chip. The nonsignificant (n.s.) differences between the ability of the wild-type rH19–20 vs each of the mutants to bind to C3b ($p > 0.05$) are indicated. *C*, Representative equilibrium C3b-biotin binding curves for rH19–20 wild type as well as a high binding (L1189R) and a low binding (R1215G) mutant. *D*, Analysis of the ability of rH19–20 mutants to compete with radiolabeled wild-type rH19–20 for binding to C3b on zymosan particles. Zymosan particles bearing 1 μ g of C3b were incubated with \sim 20 ng of 125 I-rH19–20 (wild type) in combination with the IC₅₀ dose of unlabeled wild-type rH19–20 (1.5 μ M) or with 1.5 μ M of the rH19–20 mutants in 100 μ l of DGVB. After 20 min at 22°C, the bound and free radiolabel were separated by centrifugation of cells through 20% sucrose in DGVB. The average ($n = 4$) percentage of 125 I-rH19–20 remaining bound to the zymosan-C3b (ZymC3b) and SDs are shown for each protein. The differences between the ability of the wild type vs each of the mutants to bind to ZymC3b were all significant ($p < 0.05$). The results shown are from one of two representative experiments.

FIGURE 4. Summary figure of relative affinities of rH19–20 wild-type (WT) and mutant proteins for C3b and heparin, and of complement functional cell-based assays. The salt concentration (mM NaCl) at which the rH19–20 WT or mutant proteins eluted from the heparin affinity column are graphed on the *x*-axis. The relative affinity of the mutants vs the WT protein for C3b is graphed on the *y*-axis using the formula: relative affinity = $[(K_d \text{ of WT rH19-20})/(K_d \text{ of each rH19-20 mutant})]$. The average of the K_d values obtained on the C3b-coated CM5 and SA sensor chips were used. The higher values represent higher affinity for each ligand, whereas lower values represent the opposite. The symbols that represent each mutant depend on their percentage of remaining WT activity in the cell-based assay that measured the ability of the mutants to inhibit fH binding to C3b-coated host-like cells (Fig. 5A). These values are in agreement with the results obtained in the E_H lysis assay (Fig. 6).



anti-CD59-treated cells, because of inhibition of fH-mediated protection. The mix was immediately transferred to a 37°C water bath and incubated for 20 min. To determine the extent of hemolysis induced by the mutants vs the wild-type rH19–20, 200 μ l of cold GYBE was added, the samples were centrifuged, and the OD of the supernatant was determined at 414 nm. The percent lysis was determined by subtracting the $A_{414 \text{ nm}}$ of the background lysis control and dividing by the maximum lysis in water.

Statistical analysis

Conventional Student's *t* tests (two-tailed) were used to assess the statistical significance of the difference between wild-type rH19–20 and the mutants in their ability to bind to C3b (BIAcore), to C3b on zymosan, and to lyse E_H in the presence of NHS, and *p* values <0.05 were considered statistically significant.

Results

A panel of mutant recombinant forms of fH domains 19–20 (rH19–20) was expressed in *P. pastoris*

A total of 16 mutations of rH19–20 were expressed in *P. pastoris*, as summarized in Fig. 1A. Ten of these correspond to aHUS-linked sequence variants (38). As previously noted (41), these, to a large extent, congregate on one face of module 20 (Fig. 1A). A further six mutants, which have not been identified in aHUS patients but that cover a wider surface region of the disease-linked domain 20, were designed to help delineate binding sites for C3b and heparin. On the basis of previous studies (40, 41), the residues chosen for these “designer” mutants are hypothesized to lie within C3b or polyanion binding sites. All the mutants were homogeneous as judged by SDS-PAGE under both oxidizing and reducing conditions (data not shown). The expected m.w. of all the mutants was confirmed by electrospray ionization mass spectrometry (data not shown).

The aHUS-linked mutations do not perturb structure

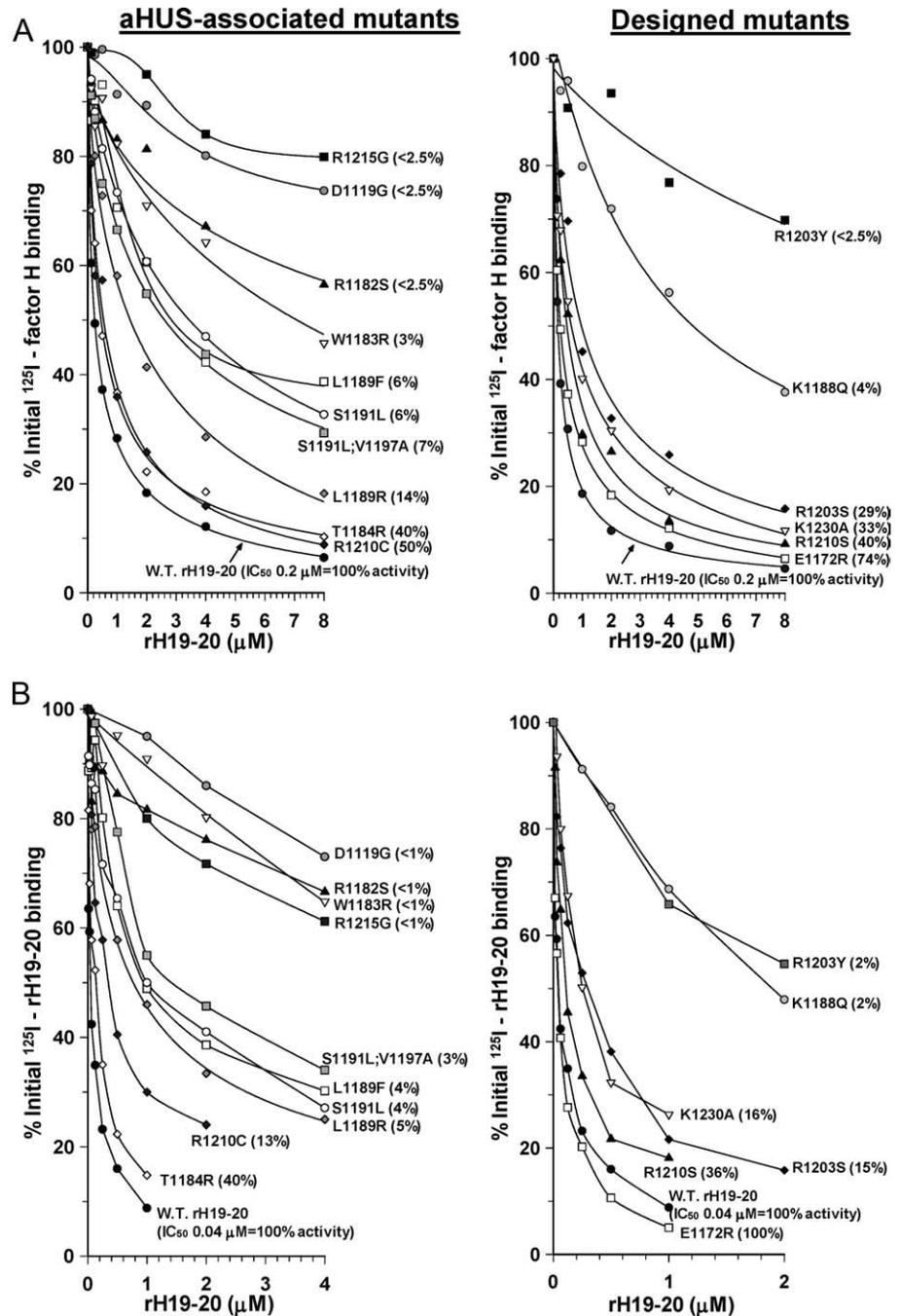
Samples of wild-type sequence rH19–20, and of the mutant forms, were isotopically enriched with ^{15}N . Each sample was used to collect a 2D ^{15}N , ^1H -HSQC spectrum. Nearly every amino acid

residue within wild-type rH19–20 yielded a distinct peak in such a spectrum, as previously reported (41) and as expected for a properly folded polypeptide. For 15 of 16 mutants, the ^{15}N , ^1H -HSQC spectra (data not shown) were also consistent with folded proteins; the exception was the designed mutant R1203Y, which was judged to be unfolded by ^1H NMR. Furthermore, a comparison of HSQC spectra showed good conservation of chemical shifts with variations confined to the vicinity of the mutated residues. Moreover, line shapes and spectral quality were consistent with monomeric protein for all proteins tested. These observations suggest that none of the aHUS-linked mutations resulted in major structural changes.

The aHUS-linked mutants bind with varying affinity to heparin

Previous work suggests that host GAGs form at least part of the target recognized by the C-terminal anchoring region of fH (25, 28, 31, 35, 37, 41). Thus, it was of interest to assess the extent to which our mutations affect GAG binding. Heparin is commonly used as an experimental model compound for GAGs that are found on cell surfaces such as heparan sulfate and dermatan sulfate. The wild-type and mutant forms of rH19–20 were loaded onto a heparin-affinity column and eluted using a salt gradient (Fig. 2, A and B). Some aHUS-linked mutants bound to the resin more tightly, others bound more weakly and the remainder eluted similarly to wild-type rH19–20. For example, the strongest interaction was found with W1183R (eluting at 520 mM NaCl), whereas the weakest was R1182S (eluting at 325 mM NaCl). The wild-type rH19–20 eluted at 370 mM NaCl. In general, mutations that resulted in gain of positive charge increased affinity (aHUS mutants W1183R, T1184R, and L1189R; designed mutant E1172R), whereas mutations causing a loss of positive charge decreased affinity (aHUS mutants R1182S, R1210C, and R1215G; designed mutants K1188Q, R1203A, R1210S, and K1230A). To investigate further, GMSA were performed. In these assays, the migration of a fluorescently tagged defined-length GAG fragment through an agarose gel was monitored in the presence of wild-type rH19–20

FIGURE 5. The rH19–20 mutants are impaired in their ability to bind to C3b-coated host-like erythrocyte surfaces. **A**, Binding of radiolabeled fH to C3b on E_sC3b cells in the presence of unlabeled wild-type or mutant rH19–20. Cells bearing 34×10^{11} C3b (1 μ g) were incubated with ~ 20 ng of radioiodinated human fH and 0–8 μ M of nonlabeled wild-type or mutant rH19–20, in 100 μ l of DGVB. After 20 min at 22°C, the bound and free radiolabel were separated by centrifugation of cells through 20% sucrose in DGVB. The results are graphed relative to the initial binding observed with labeled fH in the absence of the rH19–20 proteins. Each experiment was conducted with five proteins at a time, using wild-type rH19–20 as an internal control in each set of assays. The average ($n = 5$) wild-type rH19–20 IC₅₀ is shown and was 0.20 ± 0.04 . The percentage of inhibitory activity remaining for each protein is shown in parenthesis and was calculated using the following formula: $(IC_{50} \text{ wild-type rH19-20} / IC_{50} \text{ mutant rH19-20}) \times 100$. **B**, Same as above, except the C3b-coated cells were incubated with radioiodinated rH19–20 instead. The average wild-type ($n = 3$) rH19–20 IC₅₀ was 0.04 ± 0.01 . The *left* and *right panels* in **A** and **B** show the data obtained with the aHUS-associated mutants or the designed mutants, respectively.



or the mutants. The wild-type protein binds and hence retards the mobility of both a chemically pure fully sulfated heparin-derived tetrasaccharide (dp4; data not shown), as well as heparin-derived hexasaccharides (dp6) that contain heterogeneous sulfation patterns (Fig. 2C). The experimental conditions were optimized so that the wild type showed some free dp6 migrating toward the anode. Smaller amounts of observed free dp6 for some mutants indicated stronger binding, whereas larger amounts corresponded to weaker binders. The ranking of affinities of rH19–20 mutants (aHUS-associated and designed mutants) for GAGs, as inferred from the GMSA (Fig. 2C), was very similar to the ranking derived from heparin affinity chromatography (Fig. 2, A and B). In summary, there is no uniform link between the affinity for heparin of the aHUS-associated mutants and aHUS pathology. Thus, some of the aHUS-associated mutants (W1183R, T1184R, L1189R,

L1189F, S1191L, and S1191L;V1197A) were either unaffected or had increased affinity for heparin.

The aHUS-linked mutants bind with varying affinity to C3b

We measured the affinity of each mutant protein for C3b using SPR. C3b was immobilized on a CM5 sensor chip by amine coupling. Fig. 3A summarizes the value of K_d obtained for each mutant. Four of the aHUS mutants (D1119G, R1182S, R1210C, and R1215G) bound with lower affinity to immobilized C3b, compared with the wild type. The other aHUS mutants were either unaffected in their ability to bind (V1197A in the context of the S1191L;V1197A double mutant) or had a slightly (L1189F) or significantly (W1183R, T1184R, L1189R, and S1191L) increased affinity for C3b. It is noteworthy that most of the aHUS mutants that clearly bind more strongly to C3b (i.e.,

W1183R, T1184R, and L1189R) correspond to those with a higher affinity for heparin, while most of those with low affinity for C3b (i.e., R1182S, R1210C, R1215G) correspond to those exhibiting weaker heparin binding, as summarized in Fig. 4. Interestingly, the aHUS mutant S1191L, which bound with greater affinity to C3b (Figs. 3 and 4), maintained a normal ability to bind heparin (Figs. 2 and 4).

A second type of sensor chip, preimmobilized with SA (SA chip), was used to immobilize C3b molecules that had been biotinylated at the cysteine-SH within the thioester site. This mode of immobilization emulates more closely the natural orientation of C3b on surfaces (52). Fig. 3C shows representative equilibrium binding single-site saturation curves for rH19–20 wild type as well as examples of a high-affinity (L1189R) and a low-affinity (R1215G) mutant binding to C3b. The K_d values obtained with the SA-C3b sensor chip (Fig. 3B) were very similar to those obtained on the CM5 chip (Fig. 3A). These findings indicate that the mode of C3b coupling did not bias the measured K_d values.

To further investigate the unexpected increase in affinity for C3b of some mutants as observed by SPR, C3b-coated zymosan particles (ZymC3b; which are devoid of polyanions) were incubated with radiolabeled wild-type rH19–20 in combination with unlabeled wild type or the mutants with the highest and lowest affinity for C3b (by SPR). Fig. 3D shows that aHUS mutants W1183R, T1184R, and L1189R as well as designed mutant E1172R, competed with radiolabeled rH19–20 more efficiently for binding to surface-bound native C3b than the unlabeled wild-type rH19–20, whereas aHUS mutants R1182S and D1119G were less efficient. The differences between the ability of the wild type vs each of the mutants to bind to ZymC3b were all significant ($p < 0.05$). These results agree with the SPR data, confirming that some disease-linked mutants do indeed bind with higher affinity to C3b. Taken together, the C3b-binding data show that there is no correlation between affinity for C3b and the association of the mutations with aHUS.

Delineating C3b and heparin binding sites

Considering our panel of 16 rH19–20 mutations as a whole, 14 neither introduced a cysteine (the exception being R1210C) nor resulted in structural disruption (the exception being R1203Y). Of these 14, 9 had a positive or negative effect, compared with wild-type rH19–20, on both heparin and C3b binding, whereas one (V1197A, within the context of S1191L;V1197A double mutant) had no significant effect (Fig. 4). Two other mutations (R1210S and K1230A) decreased heparin binding but did not alter C3b binding. Mutant D1119G significantly decreased C3b binding but had no effect on heparin binding. On the other hand, S1191L increased C3b binding while having no effect on heparin binding. Since these mutations resulted in only localized structural changes, it seems valid to summarize their various functional consequences on the 3D structure of wild-type rH19–20 (Fig. 1B) and to infer that the wild-type residue is directly involved in ligand binding if its substitution alters affinity. On this basis, Fig. 1B illustrates that there is an overlapping binding patch on one face of module 20 for heparin and C3b. Two of the mutants that had minor or negligible effect on heparin binding (L1189F and V1197A, respectively) both involve conservative substitutions of buried side chains.

The rH19–20 aHUS-associated mutants are impaired in their ability to bind to C3b-coated host-like erythrocyte surfaces

A clear message of the results described above is that while 10 mutants included in this study have been linked with occurrence of aHUS (48), not all of them are negatively affected in their ability to bind to C3b and polyanion ligands (summarized in Fig. 4). We

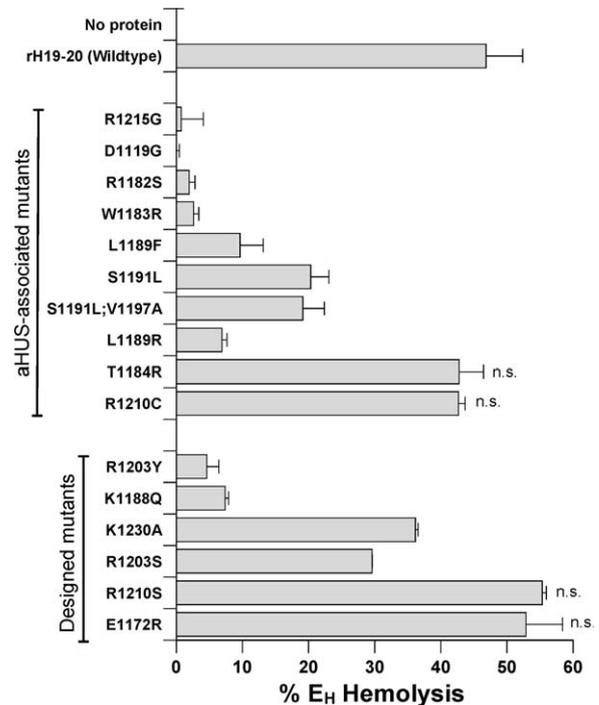


FIGURE 6. Ability of rH19–20 mutants to inhibit fH-mediated protection of human erythrocytes from complement-mediated lysis. Lysis of human erythrocytes (E_H) was measured by preincubating anti-CD59-treated E_H (5×10^6) with $1 \mu\text{M}$ wild-type or mutant rH19–20, in the presence of 40% NHS. This concentration of wild-type rH19–20 is enough to achieve $\sim 50\%$ hemolysis (IC_{50}) of anti-CD59-treated cells, due to inhibition (by rH19–20) of fH-mediated protection. The mixture was incubated for 20 min, at 37°C . To determine the extent of hemolysis induced by the mutants vs the wild-type rH19–20, $200 \mu\text{l}$ of cold GVBE was added, the samples were centrifuged, and the OD of the supernatant was determined at 414 nm . The percentage of lysis is graphed by subtracting the $A_{414 \text{ nm}}$ of the background lysis control, as well as the percentage of lysis induced by blocking CD59 alone ($\sim 20\%$), and dividing this value by the maximum lysis obtained with water. The nonsignificant (n.s.) differences between the ability of the wild-type rH19–20 vs each of the mutants to induce lysis of E_H ($p > 0.05$) are indicated. The results are representative of two separate experiments shown as means and SDs of triplicate observations.

therefore tested whether these mutations interfere with the ability of fH to bind, simultaneously, to C3b and host-like polyanions in a more physiological setting using a competitive binding assay, as described previously (35). In this assay, rH19–20 competes with radiolabeled full-length fH, inhibiting its binding to C3b and host-like polyanions on sheep erythrocytes (E_S), which carry high levels of sialic acid-containing polysaccharides (33, 34) (as do most human cells and tissues). We showed previously that such competition impairs fH complement regulatory functions and increases complement activation on host surfaces (35). Thus, the C-terminal module pair is important for anchoring fH to self-surfaces following C3b deposition. Data in Fig. 5A confirm that wild-type sequence rH19–20 inhibits, in a dose-dependent manner, the binding of full-length fH to C3b-coated host-like cells, with an IC_{50} of $0.2 \mu\text{M}$. The aHUS-linked mutant forms of rH19–20, on the other hand, were significantly impaired in their ability to compete, displaying from $< 2.5\%$ (R1215G, D1119G, and R1182S) to $40\text{--}50\%$ (T1184R and R1210C) of the inhibitory activity of the wild-type rH19–20 (Fig. 5A). Only the designed mutant (E1172R) retained $> 70\%$ activity in this assay. The presence of the V1197A mutation within the double-mutant S1191L;V1197A did not further impair its ability to compete vs S1191L alone.

We also determined the effect of the mutations on the ability of rH19–20 to compete with radiolabeled wild-type rH19–20, i.e., without the potential interference of the other C3b/polyanion-binding sites or higher-order architecture of full-length fH, and very similar results were obtained (Fig. 5B). Thus, the four aHUS mutants that were most affected (D1119G, R1182S, W1183R, and R1215G) and the two least affected mutants (T1184R and R1210C) in the competition assay with full-length ¹²⁵I-fH, ranked comparably in the competition assay with ¹²⁵I-rH19–20, and can be compared in summary Fig. 4.

Ability of rH19–20 aHUS-associated mutants to inhibit fH-mediated protection of human erythrocytes from complement-mediated lysis

Experiments were performed in which complement-mediated lysis of human erythrocytes was measured in the presence of NHS and rH19–20 mutants (Fig. 6). Wild-type rH19–20 competes with fH, inhibiting fH-mediated protective action more effectively than any of the aHUS-linked mutants, apart from R1210C and T1184R, which are comparably effective. This is largely consistent with the cell-surface binding results (Fig. 5) and provides further evidence that anchoring of fH to self-surfaces via its C terminus is essential for its protective function.

Thus, the competition data strongly indicate that a common feature of aHUS-linked fH mutants is the reduced ability to engage, in a functionally effective way, with the fH target (C3b/C3d), when in the context of polyanion-rich self-surfaces. Intriguingly, this behavior on physiological cell surfaces does not correlate with the increased affinity that some of the aHUS mutants displayed for C3b (Fig. 3) and/or for the GAG-model compound heparin (Fig. 2), when these interactions were analyzed individually.

Discussion

If a mutation were to impair the ability of fH to regulate complement at self-surfaces it would have particularly significant consequences for tissue and cell surfaces thought to rely on fH for protection from complement-mediated damage. An example is the glomerular basement membrane that lacks membrane-bound complement regulators but is exposed to plasma (and therefore the alternative pathway of complement) by fenestrations between the endothelial cells of the glomerular microvasculature. Thus, fH mutations clustered within the surface-anchoring C-terminal region, which have been detected in a significant proportion of aHUS patients, may be expected to directly trigger or exacerbate pathogenesis. Understanding the precise properties of disease-linked mutants of fH will therefore help to clarify the molecular basis of aHUS. For example, disease-associated mutations in the C-terminal region may disrupt its 3D protein structure. Alternatively, they could interfere with the recognition site in CCP 20 of fH for polyanionic self markers, or they may perturb the C-terminal binding site for surface-bound C3b, or they could affect both polyanion and C3b binding sites simultaneously. In the present study, we sought to distinguish between these various possibilities.

Our NMR analyses show that none of the 10 disease-linked mutations in the current study results in anything other than highly localized effects on the structures of CCP 19 or 20. Consequently, any functional effects of these mutations may reasonably be ascribed to their location within, or near to, critical binding sites on the wild-type protein. C3b- and heparin-binding data collected on these and five further, structurally intact, designer mutations in the context of rH19–20 allow the most comprehensive delineation of binding sites for these ligands to date. This exercise (summarized in Figs. 1B and 4) suggests that positively charged binding sites for heparin and C3b overlap on one face of rH19–20. The binding

surface for heparin (and by extension, for other GAGs) appears to extend toward the C terminus of fH while the C3b-binding surface extends into module 19. Changes in the electrostatic nature of the surface had the largest influences (gain of positive charge or loss of negative charge appeared to correlate with increases of both heparin and C3b binding); thus, it is possible that some of the residues concerned are involved in electrostatic steering effects rather than direct contacts with ligands. These findings are in partial agreement with previous reports (40, 41) but do not support a suggestion of simultaneously occupied binding sites on opposite faces of CCP 20 (41).

Functional analyses of some aHUS-associated mutants reveal effects consistent with an intuitively feasible model of aHUS pathogenesis; i.e., their reduced ability to bind to a polyanion-rich C3b-coated host-like surface correlates with their diminished performance in assays that measure directly affinity for C3b or for heparin. For example, although D1119G (involving loss of a negative charge) is unaffected in its ability to bind heparin, it has significantly diminished affinity for C3b (Figs. 3 and 4), and this mutant was also the weakest of all the mutants in the cell surface assays. Moreover, analysis of some of the designer mutants (i.e., those engineered for this study to help delineate C3b and heparin binding sites but not identified in any aHUS patients to date) also produced results that appear to link an affinity for C3b and/or heparin to a capacity for blocking fH binding to a C3b-coated erythrocyte surface. Thus, the designer mutant R1210S binds C3b with wild-type affinity and is only slightly diminished in affinity for heparin while maintaining near-wild-type activity in the cell surface assay. The case of the aHUS-linked R1210C is complicated by the likelihood that it forms disulfide bonds with itself or other proteins (54) (recombinant R1210C was found to be glutathionylated in our study). In all, seven mutations (aHUS-linked mutants R1215G and R1182S and designer mutants K1188Q, R1203S, R1210S, and K1230A) display both weaker heparin and C3b binding (Figs. 2 and 3), and, as expected, their performance is significantly impaired in the cell surface assays (Fig. 5). It should be noted that the reduced heparin binding obtained with R1182S contrasts with the unaffected heparin binding previously observed with a mutant, R1182A, which has not been found in aHUS patients (40).

Indeed, all the aHUS-associated mutants exhibit a weaker than wild-type ability to compete with fH for erythrocyte surface binding (Fig. 5). For example, L1189R and W1183R perform very poorly in cell surface assays (Figs. 5 and 6), whereas T1184R and R1210C are the only aHUS mutants that are nearly as good at inhibiting the protective effects of fH as the wild-type rH19–20. What is striking is that several of these mutations have a higher affinity both for heparin (Figs. 2 and 4) and for C3b (Figs. 3 and 4). Such is the case for aHUS mutants W1183R, T1184R, L1189R, and the designer mutation E1172R, all of which involve an introduction of one or more positive charges. Note that in the case of the W1183R mutant, the observed increased affinity for C3b and heparin differed from what had been observed previously with another aHUS-linked mutant, W1183L (40) that had reduced binding to C3b and heparin; unlike W1183L (40), the W1183R mutant ran as a single band by SDS-PAGE (data not shown). The L1189F mutation also shows increased C3b and heparin binding, albeit to a much lesser extent than L1189R (Figs. 3 and 4). Another mutant (S1191L) with weaker than wild-type activity in the cell surface assays (Figs. 5 and 6) has enhanced affinity for C3b (Fig. 3), contrary to previously reported results (54, 55). This same mutant had no effect on heparin binding (Fig. 2), in agreement with a previous study (55). Interestingly, the presence of V1197A in the S1191L;

V1197A double mutant has no additional effect in any of the assays tested vs the S1191L mutant.

An overall summary of the heparin and C3b-binding results (where a wide range of affinities for C3b and heparin were observed), and the cell surface complement functional assays (where all aHUS-linked mutants were impaired) is illustrated in Fig. 4. Taken together, these results unexpectedly show that, measured separately, affinities for C3b and heparin do not correlate consistently with the strength of binding to a polyanion-rich erythrocyte surface (on which C3b has been deposited), nor do they correlate with the disease-risk phenotype.

In the case of heparin, it is conceivable that this material is not in fact representative of a putative host tissue-specific polyanionic ligand. The presence in such a ligand of particular patterns or densities of sulfation, e.g., might result in differential binding among the mutants that correlates better with cell-surface assays. Studies aimed at identifying physiological polyanion species, as well as elucidating their chemical nature, are warranted.

The lack of correlation between the separate ligand binding assays and the cell-based assays also suggests that a mutation may affect a third functional site in CCPs 19–20, perhaps different from those involved in C3b or polyanion binding. For example, a mutation could affect the region involved in the formation of putative dimers or tetramers (40, 56–58), which may be important for fH binding/function on the cell surface. In this regard, we have recently determined that polyanions greatly promote formation of fH dimers and tetramers and that this dimerization/tetramerization leads to an increase in fH binding and complement control activity at the cell surface (58). In addition, we have determined that this polyanion-induced self-assembly is mediated, at least in part, by C terminus to C terminus complex formation, specifically through CCP 18–20 (58).

A further possibility derives from consideration of the relative affinities of fH for each ligand in the setting of the cell surface and the order of binding events. An increased affinity between fH and polyanions, due to CCP 19–20 mutations, may result in a decrease in the ability of fH to diffuse normally over the cell surface, impairing its ability to efficiently encounter and bind C3b. Thus, such mutants would be less effective than normal CCP 19–20 that binds with the correct affinity to the combination of both ligands (polyanions and C3b/C3d). Likewise, the mutants that were found to have increased affinity for C3b may get retained/delayed in complexes with the products of decay acceleration and cofactor activity (i.e., C3b/iC3b).

Thus, we propose that aHUS-linked mutations disturb a complex relationship between affinities of fH for its individual ligands (C3b and physiological polyanions) or possibly for itself, in the context of the cell surface.

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Disclosures

M.K.P. is an officer of and has a financial interest in Complement Technology, Inc. (www.ComplementTech.com), a supplier of complement reagents.

References

- Kavanagh, D., A. Richards, and J. Atkinson. 2008. Complement regulatory genes and hemolytic uremic syndromes. *Annu. Rev. Med.* 59: 293–309.
- Warwicker, P., T. H. Goodship, R. L. Donne, Y. Pirson, A. Nicholls, R. M. Ward, P. Turnpenny, and J. A. Goodship. 1998. Genetic studies into inherited and sporadic hemolytic uremic syndrome. *Kidney Int.* 53: 836–844.
- Perez-Caballero, D., C. Gonzalez-Rubio, M. E. Gallardo, M. Vera, M. Lopez-Trascasa, S. Rodriguez de Cordoba, and P. Sanchez-Corral. 2001. Clustering of missense mutations in the C-terminal region of factor H in atypical hemolytic uremic syndrome. *Am. J. Hum. Genet.* 68: 478–484.
- Richards, A., M. R. H. Buddles, R. L. Donne, B. S. Kaplan, E. Kirk, M. C. Venning, C. L. Tielemans, J. A. Goodship, and T. H. Goodship. 2001. Factor H mutations in hemolytic uremic syndrome cluster in exons 18–20, a domain important for host cell recognition. *Am. J. Hum. Genet.* 68: 485–490.
- Dragon-Durey, M. A., V. Fremeaux-Bacchi, C. Loirat, J. Blouin, P. Niaudet, G. Deschenes, P. Coppo, F. W. Herman, and L. Weiss. 2004. Heterozygous and homozygous factor H deficiencies associated with hemolytic uremic syndrome or membranoproliferative glomerulonephritis: report and genetic analysis of 16 cases. *J. Am. Soc. Nephrol.* 15: 787–795.
- Neumann, H. P., M. Salzmann, B. Bohnert-Iwan, T. Mannuelian, C. Skerka, D. Lenk, B. U. Bender, M. Cybulla, P. Riegler, A. Konigsrainer, et al. 2003. Haemolytic uraemic syndrome and mutations of the factor H gene: a registry-based study of German speaking countries. *J. Med. Genet.* 40: 676–681.
- Caprioli, J., P. Bettinaglio, P. F. Zipfel, B. Amadei, E. Daina, S. Gamba, C. Skerka, N. Marziliano, G. Remuzzi, and M. Noris. 2001. The molecular basis of familial hemolytic uremic syndrome: mutation analysis of factor H gene reveals a hot spot in short consensus repeat 20. *J. Am. Soc. Nephrol.* 12: 297–307.
- Esparza-Gordillo, J., J. E. Goicoechea de, A. Buil, B. L. Carreras, M. Lopez-Trascasa, P. Sanchez-Corral, and C. S. Rodriguez de. 2005. Predisposition to atypical hemolytic uremic syndrome involves the concurrence of different susceptibility alleles in the regulators of complement activation gene cluster in 1q32. *Hum. Mol. Genet.* 14: 703–712.
- Esparza-Gordillo, J., E. G. Jorge, C. A. Garrido, L. Carreras, M. Lopez-Trascasa, P. Sanchez-Corral, and C. de Cordoba, Sr. 2006. Insights into hemolytic uremic syndrome: Segregation of three independent predisposition factors in a large, multiple affected pedigree. *Mol. Immunol.* 43: 1769–1775.
- Caprioli, J., M. Noris, S. Brioschi, G. Pianetti, F. Castelletti, P. Bettinaglio, C. Mele, E. Bresin, L. Cassis, S. Gamba, et al. 2006. Genetics of HUS: the impact of MCP, CFH, and IF mutations on clinical presentation, response to treatment, and outcome. *Blood* 108: 1267–1279.
- Caprioli, J., F. Castelletti, S. Bucchioni, P. Bettinaglio, E. Bresin, G. Pianetti, S. Gamba, S. Brioschi, E. Daina, G. Remuzzi, and M. Noris. 2003. Complement factor H mutations and gene polymorphisms in haemolytic uraemic syndrome: the C-257T, the A2089G and the G2881T polymorphisms are strongly associated with the disease. *Hum. Mol. Genet.* 12: 3385–3395.
- Fremaux-Bacchi, V., E. J. Kemp, J. A. Goodship, M. A. Dragon-Durey, L. Strain, C. Loirat, H. W. Deng, and T. H. Goodship. 2005. The development of atypical haemolytic-uraemic syndrome is influenced by susceptibility factors in factor H and membrane cofactor protein: evidence from two independent cohorts. *J. Med. Genet.* 42: 852–856.
- Pangburn, M. K. 1986. Differences between the binding sites of the complement regulatory proteins DAF, CR1 and factor H on C3 convertases. *J. Immunol.* 136: 2216–2221.
- Harrison, R. A., and P. J. Lachmann. 1980. The physiological breakdown of the third component of human complement. *Mol. Immunol.* 17: 9–20.
- Discipio, R. G. 1992. Ultrastructures and interactions of complement factors H and I. *J. Immunol.* 149: 2592–2599.
- Moore, M. D., R. G. Discipio, N. R. Cooper, and G. R. Nemerow. 1989. Hydrodynamic, electron microscopic, and ligand-binding analysis of the Epstein-Barr virus/C3dg receptor (CR2). *J. Biol. Chem.* 264: 20576–20582.
- Alsenz, J., J. D. Lambris, T. F. Schulz, and M. P. Dierich. 1984. Localization of the complement component C3b binding site and the cofactor activity for factor I in the 38 kDa tryptic fragment of factor H. *Biochem. J.* 224: 389–398.
- Gordon, D. L., R. M. Kaufman, T. K. Blackmore, J. Kwong, and D. M. Lublin. 1995. Identification of complement regulatory domains in human factor H. *J. Immunol.* 155: 348–356.
- Kuhn, S., and P. F. Zipfel. 1996. Mapping of the domains required for decay acceleration activity of the human factor H-like protein 1 and factor H. *Eur. J. Immunol.* 26: 2383–2387.
- Kuhn, S., C. Skerka, and P. F. Zipfel. 1995. Mapping of the complement regulatory domains in the human factor H-like protein 1 and in factor H. *J. Immunol.* 155: 5663–5670.
- Jokiranta, T. S., J. Hellwage, V. Koistinen, P. F. Zipfel, and S. Meri. 2000. Each of the three binding sites on factor H interacts with a distinct site on C3b. *J. Biol. Chem.* 275: 27657–27662.
- Pangburn, M. K., M. Shreedhar, N. Alam, N. Rawal, A. Herbert, and A. Haque. 2004. Functional analysis of each of the three C3b binding sites of factor H and comparison with full length factor H [abstract]. *Mol. Immunol.* 41: 291.
- Sharma, A. K., and M. K. Pangburn. 1996. Identification of three physically and functionally distinct binding sites for C3b in human complement factor H by deletion mutagenesis. *Proc. Natl. Acad. Sci. USA* 93: 10996–11001.
- Blackmore, T. K., T. A. Sadlon, H. M. Ward, D. M. Lublin, and D. L. Gordon. 1996. Identification of a heparin binding domain in the seventh short consensus repeat of complement factor H. *J. Immunol.* 157: 5422–5427.
- Blackmore, T. K., J. Hellwage, T. A. Sadlon, N. Higgs, P. F. Zipfel, H. M. Ward, and D. L. Gordon. 1998. Identification of the second heparin-binding domain in human complement factor H. *J. Immunol.* 160: 3342–3348.
- Pangburn, M. K., M. A. L. Atkinson, and S. Meri. 1991. Localization of the heparin-binding site on complement factor H. *J. Biol. Chem.* 266: 16847–16853.
- Prodingier, W. M., J. Hellwage, M. Spruth, M. P. Dierich, and P. F. Zipfel. 1998. The C-terminus of factor H: monoclonal antibodies inhibit heparin binding and identify epitopes common to factor H and factor H-related proteins. *Biochem. J.* 331 (Pt. 1): 41–47.

28. Ram, S., A. K. Sharma, S. D. Simpson, S. Gulati, D. P. McQuillen, M. K. Pangburn, and P. A. Rice. 1998. A novel sialic acid binding site on factor H mediates serum resistance of sialylated *Neisseria gonorrhoeae*. *J. Exp. Med.* 187: 743–752.
29. Schmidt, C. Q., A. P. Herbert, D. Kavanagh, C. Gandy, C. J. Fenton, B. S. Blaum, M. Lyon, D. Uhrin, and P. N. Barlow. 2008. A new map of glycosaminoglycan and C3b binding sites on factor H. *J. Immunol.* 181: 2610–2619.
30. Prosser, B. E., S. Johnson, P. Roversi, A. P. Herbert, B. S. Blaum, J. Tyrrell, T. A. Jowitt, S. J. Clark, E. Tarelli, D. Uhrin, et al. 2007. Structural basis for complement factor H linked age-related macular degeneration. *J. Exp. Med.* 204: 2277–2283.
31. Jokiranta, T. S., Z. Z. Cheng, H. Seeberger, M. Jozsi, S. Heinen, M. Noris, G. Remuzzi, R. Ormsby, D. L. Gordon, S. Meri, et al. 2005. Binding of complement factor H to endothelial cells is mediated by the carboxy-terminal glycosaminoglycan binding site. *Am. J. Pathol.* 167: 1173–1181.
32. Pickering, M., H. Cook, J. Warren, A. Bygrave, J. Moss, M. J. Walport, and M. Botto. 2002. Uncontrolled C3 activation causes membranoproliferative glomerulonephritis in mice deficient in complement factor H. *Nat. Genet.* 31: 424–428.
33. Fearon, D. T. 1978. Regulation by membrane sialic acid of BIH-dependent decay-dissociation of amplification C3 convertase of the alternative complement pathway. *Proc. Natl. Acad. Sci. USA* 75: 1971–1975.
34. Pangburn, M. K., and H. J. Muller-Eberhard. 1978. Complement C3 convertase: cell surface restriction of BIH control and generation of restriction on neuraminidase treated cells. *Proc. Natl. Acad. Sci. USA* 75: 2416–2420.
35. Ferreira, V. P., A. P. Herbert, H. G. Hocking, P. N. Barlow, and M. K. Pangburn. 2006. Critical role of the C-terminal domains of factor H in regulating complement activation at cell surfaces. *J. Immunol.* 177: 6308–6316.
36. Jozsi, M., M. Oppermann, J. D. Lambiris, and P. F. Zipfel. 2007. The C-terminus of complement factor H is essential for host cell protection. *Mol. Immunol.* 44: 2697–2706.
37. Pangburn, M. K. 2002. Cutting edge: Localization of the host recognition functions of complement factor H at the carboxyl-terminal: implications for hemolytic uremic syndrome. *J. Immunol.* 169: 4702–4706.
38. 2008. FH aHUS Mutation Database Version 2.1. R. E. Saunders and S. J. Perkins of the University College of London, UK, and The European Working Party on the Genetics of HUS. www.fh-hus.org.
39. Pickering, M. C., E. G. de Jorge, R. Martinez-Barricarte, S. Recalde, A. Garcia-Layana, K. L. Rose, J. Moss, M. J. Walport, H. T. Cook, C. de Cordoba, Sr., and M. Botto. 2007. Spontaneous hemolytic uremic syndrome triggered by complement factor H lacking surface recognition domains. *J. Exp. Med.* 204: 1249–1256.
40. Jokiranta, T. S., V. P. Jaakola, M. J. Lehtinen, M. Parepalo, S. Meri, and A. Goldman. 2006. Structure of complement factor H carboxyl-terminus reveals molecular basis of atypical haemolytic uremic syndrome. *EMBO J.* 25: 1784–1794.
41. Herbert, A. P., D. Uhrin, M. Lyon, M. K. Pangburn, and P. N. Barlow. 2006. Disease-associated sequence variations congregate in a polyanion-recognition patch on human factor H revealed in 3D structure. *J. Biol. Chem.* 281: 16512–16520.
42. Lecak, J., K. Scott, C. Young, J. Hannon, and J. P. Acker. 2004. Evaluation of red blood cells stored at -80°C in excess of 10 years. *Transfusion* 44: 1306–1313.
43. Pangburn, M. K., R. D. Schreiber, and H. J. Muller-Eberhard. 1977. Human complement C3b inactivator: isolation, characterization, and demonstration of an absolute requirement for the serum protein BIH for cleavage of C3b and C4b in solution. *J. Exp. Med.* 146: 257–270.
44. Hammer, C. H., G. H. Wirtz, L. Renfer, H. D. Gresham, and B. F. Tack. 1981. Large scale isolation of functionally active components of the human complement system. *J. Biol. Chem.* 256: 3995–4006.
45. Pangburn, M. K. 1987. A fluorimetric assay for native C3. The hemolytically active form of the third component of human complement. *J. Immunol. Methods* 102: 7–14.
46. Gotze, O., and H. J. Muller-Eberhard. 1971. The C3-activator system: an alternative pathway of complement activation. *J. Exp. Med.* 134: 90s–108s.
47. Lesavre, P. H., T. E. Hugli, A. F. Esser, and H. J. Muller-Eberhard. 1979. The alternative pathway C3/C5 convertase: chemical basis of factor B activation. *J. Immunol.* 123: 529–534.
48. Kavanagh, D., T. H. Goodship, and A. Richards. 2006. Atypical haemolytic uraemic syndrome. *Br. Med. Bull.* 77–78: 5–22.
49. Expert Protein Analysis System (EXPASY) proteomics server of the Swiss Institute of Bioinformatics. 2008. ProtParam tool. www.expasy.org/tools/protparam.html.
50. Lyon, M., J. A. Deakin, D. Lietha, E. Gherardi, and J. T. Gallagher. 2004. The interactions of hepatocyte growth factor/scatter factor and its NK1 and NK2 variants with glycosaminoglycans using a modified gel mobility shift assay: elucidation of the minimal size of binding and activatory oligosaccharides. *J. Biol. Chem.* 279: 43560–43567.
51. Ferreira, V. P., and M. K. Pangburn. 2007. Factor H-mediated cell surface protection from complement is critical for the survival of PNH erythrocytes. *Blood* 110: 2190–2192.
52. Mitchell, D. A., R. Ilyas, A. W. Dodds, and R. B. Sim. 2008. Enzyme-independent, orientation-selective conjugation of whole human complement C3 to protein surfaces. *J. Immunol. Methods* 337: 49–54.
53. Rawal, N., and M. K. Pangburn. 1998. C5 convertase of the alternative pathway of complement: kinetic analysis of the free and surface-bound forms of the enzyme. *J. Biol. Chem.* 273: 16828–16835.
54. Sanchez-Corral, P., D. Perez-Caballero, O. Huarte, A. M. Simckes, E. Goicoechea, M. Lopez-Trascasa, and S. Rodriguez de Cordoba. 2002. Structural and functional characterization of factor H mutations associated with atypical hemolytic uremic syndrome. *Am. J. Hum. Genet.* 71: 1285–1295.
55. Heinen, S., P. Sanchez-Corral, M. S. Jackson, L. Strain, J. A. Goodship, E. J. Kemp, C. Skerka, T. S. Jokiranta, K. Meyers, E. Wagner, et al. 2006. De novo gene conversion in the RCA gene cluster (1q32) causes mutations in complement factor H associated with atypical hemolytic uremic syndrome. *Hum. Mutat.* 27: 292–293.
56. Nan, R., J. Gor, and S. J. Perkins. 2008. Implications of the progressive self-association of wild-type human factor H for complement regulation and disease. *J. Mol. Biol.* 375: 891–900.
57. Okemefuna, A. I., H. E. Gilbert, K. M. Griggs, R. J. Ormsby, D. L. Gordon, and S. J. Perkins. 2008. The regulatory SCR-1/5 and cell surface-binding SCR-16/20 fragments of factor H reveal partially folded-back solution structures and different self-associative properties. *J. Mol. Biol.* 375: 80–101.
58. Pangburn, M. K., N. Rawal, C. Cortes, M. N. Alam, V. P. Ferreira, and M. A. Atkinson. 2009. Polyanion-induced self-association of complement factor H. *J. Immunol.* 182: 1061–1068.