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Critical Role of the C-Terminal Domains of Factor H in Regulating Complement Activation at Cell Surfaces

Viviana P. Ferreira,* Andrew P. Herbert,† Henry G. Hocking,† Paul N. Barlow,† and Michael K. Pangburn2*

The plasma protein factor H primarily controls the activation of the alternative pathway of complement. The C-terminal of factor H is known to be involved in protection of host cells from complement attack. In the present study, we show that domains 19–20 alone are capable of discriminating between host-like and complement-activating cells. Furthermore, although factor H possesses three binding sites for C3b, binding to cell-bound C3b can be almost completely inhibited by the single site located in domains 19–20. All of the regulatory activities of factor H are expressed by the N-terminal four domains, but these activities toward cell-bound C3b are inhibited by isolated recombinant domains 19–20 (rH 19–20). Direct competition with the N-terminal site is unlikely to explain this because regulation of fluid phase C3b is unaffected by domains 19–20. Finally, we show that addition of isolated rH 19–20 to normal human serum leads to aggressive complement-mediated lysis of normally nonactivating sheep erythrocytes and moderate lysis of human erythrocytes, which possess membrane-bound regulators of complement. Taken together, the results highlight the importance of the cell surface protective functions exhibited by factor H compared with other complement regulatory proteins. The results may also explain why atypical hemolytic uremic syndrome patients with mutations affecting domains 19–20 can maintain complement homeostasis in plasma while their complement system attacks erythrocytes, platelets, endothelial cells, and kidney tissue. The Journal of Immunology, 2006, 177: 6308–6316.

A typical hemolytic uremic syndrome (aHUS)3 is inherited either as an autosomal dominant or autosomal recessive trait and the recessive form has been firmly associated with mutations clustering at the C-terminal end of complement factor H (1–11). aHUS is characterized by hemolytic anemia, thrombocytopenia, and acute renal failure. Homozygous patients typically present the disease in the first few months of life and exhibit a very high mortality. Heterozygous individuals may intermittently exhibit symptoms of varying severity throughout life (2, 8–10) depending on mutations in other complement regulators (12, 13). Mutational analyses demonstrated that the majority of the mutations associated with aHUS clustered at or indirectly affected the C-terminal end, specifically domain 20, of complement factor H (1, 6, 14–17).

Factor H plays a key role in the homeostasis of the complement system by acting as a cofactor for factor I-mediated cleavage of C3b and by accelerating the decay of the alternative pathway C3/C5 convertase C3bBb complexes. In the absence of factor H, 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 plasma, factor H also binds to human endothelial cells and basement membranes (18), protecting them from complement-mediated attack (19). Moreover, some cancer cell surfaces (20, 21) and pathogenic microorganisms (22–27) have developed immune evasion strategies whereby they bind factor H, thus disguising themselves as self cells and escaping elimination by the alternative pathway of complement. In a factor H-deficient line of pigs, homozygous individuals die soon after birth from complement-mediated acute renal failure (28), and factor H-deficient mice develop membranoproliferative glomerulonephritis, which was shown to be alternative pathway dependent (19).

The concentration of factor H in plasma is ~500 μg/ml. The 20 homologous domains of factor H are each composed of ~60 aa with 3–8 aa spacers between the domains (29, 30). In the electron microscope factor H appears to have the structure of flexible “beads on a string” (31, 32). The N-terminal four complement control protein domains (CCP) regulate alternative pathway activation while at least three polyanion binding regions and two additional C3b binding sites are present in the 16 C-terminal domains (15, 22, 25, 33–40). The three binding sites for C3b have been mapped to the CCP 1–4, 8–15, and 19–20 (40–42). The site on CCP 19–20 interacts with C3b, iC3b, and C3d (35, 42, 43). The three-dimensional structure of domains 19–20 has been elucidated by two groups, one using nuclear magnetic resonance spectra (16) and the other, x-ray crystallography (17). Structure-function analysis have determined that the C3b-binding site located in the N-terminal four CCP domains possesses decay-accelerating activity for the AP C3/C5 convertase and also serves as a cofactor site for factor I, a serine protease that inactivates C3b (33, 35, 36, 44). Inactivation of C3b requires that C3b be in complex with the N-terminal domains of factor H (45).

Factor H has at least three binding sites for heparin and other polyanions, located in CCP 7, CCP 20, and in the CCP 9–15 region (22, 34, 38–40, 42, 46). The C-terminal site has also been
shown to bind sialic acids (25, 34). Interactions with polyanions are important because the 10-fold higher affinity of factor H for C3b on host cells and other nonactivators (47, 48) requires the presence of sialic acid clusters or other polyanions on the surface. The polyanion binding site in domain 7 has been strongly associated with a disease of the retina, age-related macular degeneration, suggesting the possibility of tissue specific polyanion recognition by individual sites of factor H (49–52). While all these sites, as well as others, may be relevant in the recognition of nonactivator structures, the role of the C terminus of native factor H is the best characterized. It has been shown to recognize polyanionic markers, primarily sialic acid and sulfated polysaccharides such as heparin on host cells and tissues, and to mediate the binding of factor H to human endothelial cells (4, 18).

In the present report, the C-terminal domains 19–20 of factor H were examined for their ability to discriminate between C3b bound to complement-activating cells lacking polyanionic markers and C3b bound to nonactivating cells rich in surface polyanions. The results show that a small recombinant protein composed of only domains 19–20 of factor H (rH 19–20) is capable of discriminating between host-like cells and complement-activating cells. We also examined the ability of rH 19–20 to block control of the complement activation process on nonactivating cells (sheep and human erythrocytes). It selectively inhibited the control activities of factor H on these cells without affecting the fluid phase complement control functions of the full-length regulator. The presence of the rH 19–20 fragment in normal human serum led to aggressive complement-mediated lysis of normally nonactivating sheep erythrocytes as well as moderate lysis of normal human erythrocytes, which have the membrane-bound complement regulators CD35, CD55, and CD59. Our results highlight the importance of the interaction between the C-terminal end of factor H and C3b on surfaces bearing polyanions and may explain why aHUS patients with mutations in these C-terminal domains can maintain complement homeostasis in plasma while their complement system attacks normal cells and tissues, with the kidney being most susceptible.

Materials and Methods

Reagents

Buffers used were sodium PBS, 10 mM sodium phosphate, 140 mM NaCl, 0.02% NaN₃ (pH 7.4); veronal-buffered saline (VBS), 5 mM veronal, 145 mM NaCl, 0.02% NaN₃ (pH 7.5); gelatin-VBS (GVBS), VBS containing 0.1% gelatin; GVB-EDTA (GVBE), GVBS containing 10 mM EDTA; dextrose-GVB (DGVB), half physiological strength buffer prepared by diluting GVB 2-fold with 5% dextrose in water; and MgEGTA, 0.1 M MgCl₂, 0.1 M EGTA (pH 7.3).

Purified proteins

Complement proteins factor H (45), C3 (53, 54), factor B (55), factor D (56), and factor I (45) were all purified from normal human plasma as described in the references cited. A fragment consisting of residues 1107–1231 of factor H (domains 19–20, i.e., rH 19–20) was cloned, expressed, and purified by us as described previously (16). Briefly, Pichia pastoris strain KM71H was transformed with the expression vector pPICZα (Invitrogen Life Technologies), and expression of the protein was directed to the secretory pathway by placing the coding sequence upstream from the Saccharomyces cerevisiae α-factor secretion sequence. Recombinant protein was purified to homogeneity from culture supernatant using cation exchange chromatography (16). Samples of this protein were analyzed by mass spectrometry, and the results were consistent with the predicted molecular mass. The solution structure of this protein was also determined by nuclear magnetic resonance (16). A fragment consisting of factor H domains 1–3 was cloned, expressed, and purified as above. Fragments consisting of factor H domains 1–7, domains 1–6, and domains 6–15 were cloned into an insect cell expression system and were expressed and purified as described previously (43). All proteins were stored at −75°C in VBS. The concentrations of factor H and rH 19–20 were determined at 280 nm using E280/1 cm of 12.4.

Radiolabeling of proteins

Factor H, factor B, and rH 19–20 (50–100 μg) were radiolabeled with 500 μCi of 125I for 30 min at 0°C in a glass tube coated withlodogen (Pierce). After incubation, the free 125I was removed by centrifugal desalting through a G25 column pre-equilibrated with GVB (57). Specific activities for 125I-labeled proteins ranged from 4 to 7 μCi/μg.

Preparation of C3b-coated cells

Deposition of C3b on sheep and rabbit erythrocytes (E₃g, and Eₕ, respectively) was accomplished using purified C₃, factor B, and factor D as previously described (58) with the substitution of nickel for magnesium and nephritic factor (59) to stabilize the C3 convertases on the surface of the cells. The number of bound C3b molecules was determined to be between 40,000 and 170,000 per cell by radiolabeled factor Bb binding (58).

Binding assays

C3b-bearing cells (E₃gC₃b and EₕC₃b) had between 100,000 and 120,000 molecules of C3b per cell. Varying numbers of cells were incubated for 20 min at 22°C with 10–20 ng of radiolabeled human factor H or rH 19–20 in half ionic strength buffer (DGVB) in a total volume of 10 μl. Bound and free radiolabeled proteins were separated by layering 80 μl of the mixture on top of 20% sucrose in DGVB, centrifuging for 2 min at 10,000 × g, and cutting the tube to separate the pellet and supernatant (48). Background binding was measured using E₃g and Eₕg cells without C3b on their surface. For binding inhibition assays, cells bearing 1 μg C3b were incubated with ~20 ng of radioiodinated human factor H and 0–20.7 μM (0–300 μM/ml) of nonlabeled rH 19–20, or 0–0.75 μM of rH 1–3, rH 6–15, rH 1–6, and rH 19–20, in 100 μl of DGVB. Alternatively, the cells were incubated with ~20 ng 125I-rH 19–20 and 0–1.35 μM nonlabeled rH 19–20 or rH 1–7. After 20 min at 22°C, the samples were processed as described above. The percentage of factor H bound under these conditions, in the absence of rH 19–20, was ~40%.

C3/C5 convertase decay acceleration assays

Decay accelerating activity expressed by factor H was measured by determining its ability to accelerate the natural release of 125I-labeled Bb from cell bound C3bBb. The C3bBb complexes were formed by incubating 4.2 × 10⁴ E₃gC₃b with 0.8 μg (~1 μCi) of 125I-factor B and 0.5 μg of factor D in 75 μl of GVB containing 1 mM NiCl₂ at 22°C for 5 min. Formation of the C3 convertase was stopped by the addition of 145 μl of GVBE. The cells (10 μl) were added immediately to reaction mixture containing varying amounts of factor H or to 0.67 nM factor H (concentration required to release 50% of the 125I-Bb) in the presence of various concentrations of rH 19–20 (0–27.6 μM), in 40 μl of PBS. After 10 min at 22°C the cells were sedimented rapidly (2 min, 10,000 × g) through 250 μl of 20% sucrose in GVBE in a Microfuge tube. The bottoms of the tubes were cut off, and the radioactivity in the cell pellet and the supernatant were measured to determine the percent Bb remaining bound.

Factor H cofactor activity for factor I: cell surface cofactor activity

Cofactor activity assays measured the ability of human factor H to behave as a cofactor for factor I in the digestion of cell-bound 125I-C₃b to C₃bi, in the presence of various concentrations of rH 19–20. The 125I-C₃b-coated cells were formed by incubating 100 μl of GVB containing 5 × 10⁸ E₃gC₃b, 10 μg of factor B, 50 ng of factor D, 0.25 mM NiCl₂, for 5 min at 22°C. EDTA was added to a concentration of 16 mM, immediately followed by 30 μg (350 μCi) of 125I-C₃, and incubation for 30 min at 37°C. The 125I-C₃b-loaded cells were washed thoroughly in GVB and 10 μl of cells (~20,000 cpm) were mixed with 14 ng of factor I and various concentrations of factor H in GVB, for 10 min at 37°C. Reactions were placed on ice, 200 μl of GVB with 5 μg of trypsin was added to each reaction, and the samples were incubated for 5 min at 37°C. Only C₃b converted to iC₃b by factors H and I was cleaved by trypsin to release 125I-C₃ into the supernatant. The cells were sedimented by centrifugation (2 min, 10,000 × g), and the distribution of the radioactivity between the cell pellet and the supernatant was determined. Inhibition of factor H cofactor activity by rH 19–20 was measured using an amount of factor H that caused 50% C₃b conversion to iC₃b (50% maximal C₃c release) and varying concentrations of rH 19–20.
Factor H cofactor activity for factor I: fluid phase cofactor activity

This assay measured the ability of human factor H to behave as a cofactor for factor I in the digestion of fluid phase C3b to iC3b, in the presence of various concentrations of rH 19–20. This assay was also used to verify the cofactor activity of the rH 1–7. Five micrograms of C3b (1.8 μM) was incubated with 2 μg (1.4 μM) of factor I and 5 ng (2.1 nM) of factor H, or 2.7 ng (3.3 nM) of rH 1–7, in the presence of up to 17.9 μM (260 μg/ml) rH 19–20, in 16 μl of total volume. C3b cleavage was analyzed by 10% SDS-PAGE. Reactions with or without factor H, rH 1–7, or rH 19–20 were included as controls.

Hemolytic assays

Lysis of ES or human erythrocytes (Eh) was measured by mixing, on ice, GVB, normal human serum (NHS, 40% final), and 0.1 M MEGTA (5 mM final concentration) in the presence of 0–24 μM (0–360 μg/ml) rH 19–20. Cells (5 × 10⁶) were added and the mix (24 μl total) was immediately transferred to a 37°C water bath and incubated for 20 min. To rule out any effect that anti-Eh Abs in the NHS could have on the lytic assays, control experiments were performed in which 400 μl NHS was preadsorbed with 2 × 10⁷ Eqi for 1 h at 4°C, before the lytic assays. Eqi (5 × 10⁶) were also preincubated with 7 μg/ml anti-CD59 mAb (clone MEM43; Abcam) or 10 μg/ml anti-CD55 mAb previously prepared by us (60) for 20 min at 4°C, then mixed with NHS and rH 19–20, as above. rH 6–15 was included as a control in these assays. To determine the extent of hemolysis, 100 μl of cold GVBE was added, the samples were centrifuged, and the OD of supernatant was determined at 414 nm. The percent lysis was determined by subtracting the A₄₁₄ in the absence of serum, and dividing by the maximum possible A₄₁₄ determined by water lysis of the erythrocytes.

Detection of C3b deposition

C8-depleted sera was prepared by immunoadsorption of serum on anti-C8 Sepharose. Deposition of C3b on Eh was measured by mixing, on ice, GVB, C8-depleted serum (40% final), and 0.1 M MEGTA (5 mM final concentration) in the presence of 12 μM rH 19–20. Eqi (5 × 10⁶) preincubated with or without anti-CD55 polyclonal Ab, as described above, were added and the mixture transferred to a 37°C water bath for 20 min. Cells were washed with GVB, and C3b deposition was detected with FITC-conjugated goat F(ab')₂ anti-human C3 (Cappel). Cells were analyzed by flow cytometry using BD Biosciences FACSscan equipped with Cell Quest Pro software, and a minimum of 10,000 events were acquired.

Results

Discrimination between activators and nonactivators by CCP domains 19–20 alone

Factor H confers on the human alternative pathway of complement the ability to discriminate between activating cells that possess minimal surface polyanions (e.g., Es, bacteria, fungi, etc.) and nonactivating host and host-like cells (e.g., Eh). Eh and most human cells and tissues possess high levels of sialic acid-containing polysaccharides as well as other polyanions. Work done in the late 1970s demonstrated that removal of sialic acid leads to activation of the human alternative pathway (48, 61). These early studies also showed that factor H bound 5- to 10-fold better to C3b than to cells bearing polyanions than to cells without polyanions as shown in Fig. 1A. Fig. 1A demonstrates that rH 19–20, without the aid of the rest of factor H, exhibits a quantitatively similar ability to distinguish between complement-activating and nonactivating cells. Both factor H and rH 19–20 bind with 7-fold higher affinity to C3b-bearing cells that possess high levels of sialic acid-containing polysaccharides (Es/C3b) than to cells (Eh/C3b) possessing minimal surface sialic acid. This can be seen in Fig. 1 as the 7-fold difference in C3b concentrations required to bind similar amounts of factor H.

Domains 19–20 inhibit binding of full-length factor H to cell-bound C3b

Fig. 2A shows that rH 19–20 inhibits, in a dose-dependent manner, the binding of full-length factor H to C3b-coated host-like cells (Eh). Inhibition by the two-domain fragment exhibited an IC₅₀ of 0.3 μM. Fig. 2B shows that the recombinant subfragments of factor H that contain other C3b or heparin binding domains (rH 1–6 and rH 6–15) partially inhibit the binding of intact factor H, although not to the same extent as rH 19–20. rH 1–3, which does not contain the full N-terminal C3b-binding site, has no effect at the concentrations tested. Unlabeled rH 19–20 was effective (Fig. 3) against itself, exhibiting an IC₅₀ of 0.2 μM. In contrast, no inhibition of rH 19–20 binding was seen using rH 1–7 up to 1.5 μM, the maximum concentration attainable with this purified recombinant protein. When tested in a fluid phase cofactor assay and compared with equimolar concentrations of factor H, rH 1–7 was at least 80% active (results not shown).
Decay acceleration assays measured the ability of factor H to increase the rate of dissociation of the two subunits of the alternative pathway C3/C5 convertase (C3b,Bb). The dose dependence of this factor H activity is shown in Fig. 4A. The release of radio-labeled Bb from surface-bound C3b was measured and a concentration of factor H was chosen that decayed half the enzymes remaining after a 10-min incubation. This concentration of factor H was then used for decay acceleration assays in the presence of various concentrations of rH 19–20 (Fig. 4B). At 28 μM rH 19–20, the accelerated decay due to factor H was strongly inhibited and controls showed that rH 19–20 alone did not significantly increase or decrease decay. The IC_{50} of rH 19–20 for decay acceleration was 0.7 μM.

Factor I cofactor assays measured the ability of the N terminus of factor H to bind to C3b and promote cleavage and inactivation of C3b by the serine protease factor I. The product of this reaction is iC3b, a protein extremely sensitive to trypsin, whereas C3b is relatively resistant. Cells coated with radiolabeled C3b were incubated with increasing concentrations of factor H in the presence of excess factor I for 10 min, then the reactions were diluted in GVB containing trypsin. Fig. 5A shows the dependence of C3c release on the concentration of factor H. A concentration of factor H was chosen that caused 50% C3c release, and the effect of increasing concentrations of rH 19–20 on this reaction was determined (Fig. 5B). The IC_{50} of rH 19–20 for factor I cofactor activity of factor H was 1.9 μM.

Measurement of factor H-mediated fluid phase complement control activities in the presence of rH 19–20

To determine whether the inhibitory effects of rH 19–20 on factor H functions were limited to cell surface bound C3b, we measured the effect of rH 19–20 on the factor I-mediated cleavage of soluble C3b (Fig. 6). In this system, as shown for surface-bound C3b
above, soluble C3b forms a complex with factor I and factor H that results in cleavage of the C3b α-chain to form the iC3b α-chain fragments of 68 and 43 kDa (data not shown). The presence of rH 19–20 (0.179 μM) did not inhibit factor H/factor I-mediated cleavage (Fig. 6, lanes 3–5). Lanes 1 and 2 were negative controls for the cleavage of C3b in the absence of factor H or without rH 19–20, which can be seen at the bottom of lane 2 at a molecular mass of 14,000 Da. Identical results (data not shown) were obtained in experiments using H 1–7 as the cofactor molecule, in the presence of the same concentrations of rH 19–20.

Complement homeostasis requires factor H for control of spontaneous fluid phase activation of the alternative pathway amplification feedback system (62). The absence of factor H, or inhibition of its function, will result in activation. Because of the results shown in Figs. 2–5, we measured the effect of rH 19–20 on the fluid phase stability of the alternative pathway. Incubation of 40% normal human serum at 37°C with 3.5 μM rH 19–20 (over three times the average ICso concentration), for up to 1 h, resulted in <10% loss of alternative pathway function. This compares with complete consumption of both C3 and factor B in <60 s in the absence of factor H function. The results indicate that fluid phase control of the amplification cascade by factor H is largely unaffected by rH 19–20. This result and that shown in Fig. 6 suggest that the inhibitory effects of rH 19–20 are largely confined to cell surfaces.

rH 19–20 promotes complement activation on the surface of host-like cells

The human alternative pathway of complement spontaneously activates on and lyases Ehrlichia, which possess minimal surface polyanions. Ehrlichia, in contrast, possess high levels of sialic acid-containing polysaccharides as do most human cells and tissues. Sheep erythrocytes are used as the standard nonactivator for human complement analysis and as shown in Fig. 1B factor H binds more efficiently to Ehrlichia than to sheep erythrocytes. Because the fragment rH 19–20 behaves similarly (Fig. 1A) and because it does not inhibit factor H-mediated homeostasis in the fluid phase, we examined whether cell-specific binding of rH 19–20 might inhibit the ability of factor H to control complement activation on what are normally nonactivating surfaces.

Fig. 7A shows that incubation of Ehrlichia or E. coli in the presence of 40% NHS and MgEGTA resulted in no lysis in the absence of rH 19–20 (see the origin of Fig. 7A). Thus, in the absence of rH 19–20, spontaneous activation on Ehrlichia and E. coli was controlled, indicating normal host cell recognition. However, in the presence of increasing concentration of rH 19–20, lysis of both cell types was observed. Lysis of Ehrlichia was aggressive, with an IC50 of 0.9 μM rH 19–20, whereas lysis of E. coli only reached 20% hemolysis with 27-fold higher concentrations of inhibitor (Fig. 7A). NHS was also preadsorbed before use in these lytic assays with Ehrlichia to eliminate any possible anti-Ehrlichia Abs that could affect complement activation. Adsorbed and nonadsorbed NHS yielded identical results. It is noteworthy that significant lysis of E. coli occurred upon loss of protection by factor H, even though E. coli possess the membrane bound regulators CD35, CD55, and CD59 (63, 64). This result suggests that factor H plays a substantial role in red cell survival in blood.

To compare the regulatory efficacy of factor H, CD55 and CD59 in protecting the surface of Ehrlichia, each were inhibited individually and in combination (Fig. 7B). No lysis was observed in the absence of any inhibitor (EH at zero input of rH 19–20). No lysis was observed with saturating doses of anti-CD55 Ab, 10% lysis was observed with saturating doses of anti-CD59 Ab, and 16% of the cells lysed upon inhibition of factor H by 12 μM rH 19–20. Loss of the protection by factor H appeared to be at least as important to red cell survival as the other regulators on these cells. Interestingly, while the effect of completely inhibiting either of the two cell-bound complement regulatory proteins (CRP) caused little or no lysis in this assay (origin of Fig. 7B), inhibition of either cell-bound regulator combined with the loss of factor H cell surface control resulted in aggressive lysis with an IC50 of 0.9 μM when rH 6–15 was used in this system, at the IC50 concentration of rH 19–20 (0.7–1 μM), it yielded no measurable effect (data not shown).

Fig. 7C shows that deposition of C3b is readily detectable when inhibiting factor H complement control on the surface of human erythrocytes with rH 19–20. The effect of inhibiting CD55, which unlike CD59 participates in the regulation of C3b, is only detectable when factor H is also inhibited.

Discussion

The results presented in this study lead to three conclusions. First, the two C-terminal domains of factor H (rH 19–20) are able to distinguish between activators and nonactivators, without the aid of the rest of the N-terminal 18 domains of the factor H molecule. Second, rH 19–20, by efficiently competing with factor H for binding to cell-bound C3b, is able to inhibit regulatory activities of the N-terminal site directed toward cell-bound C3b, while not affecting control of fluid-phase C3b. Third, factor H through recognition of host cell surfaces by its domains 19–20, is a critical component of the complex system of control factors used by host cells and tissues to protect their surfaces from complement-mediated attack. This last conclusion derives from the observation that addition of rH 19–20 to normal human serum leads to aggressive complement-mediated lysis of nonactivating sheep erythrocytes, and even to partial lysis of human erythrocytes, which have membrane-bound regulators of complement. When either CD55 or CD59 was blocked on Ehrlichia, aggressive lysis was prevented by factor H. This finding demonstrates the importance of the factor H contribution to regulatory redundancy at host cell surfaces.

Discrimination between potential pathogens and the host is a fundamental function of any system of innate immunity. Early studies showed that regulation of alternative pathway activation on
targets was quantitatively different from that on host cells (61).
This was later shown to be due to the presence of sialic acids or other polyanions on nonactivating cells (47, 48) and this was subsequently shown to be due to polyanion recognition by factor H (37). Clinical evidence has appeared in recent years indicating that alleles and mutations that affect polyanion recognition domains in factor H result in pathology involving complement activation. Inherited HUS focused attention on the C-terminal domains bearing C3d and sialic acid/polyanion binding sites (1–11). Moreover, age-related macular degeneration suggested a similar role might exist for the polyanion binding site in CCP 7 (49–52). Our results extend the existing understanding of the mechanism by which factor H discriminates between activator and nonactivator surfaces. Fig. 1 shows that rH 19–20 and full-length factor H each bind 7-fold better to cells bearing C3b and polyanions than to cells bearing C3b but lacking polyanions. A maximum difference of 10-fold for this effect has been reported for full-length factor H (37, 48). This observation suggests that the two C-terminal domains may account for most of the discriminating ability of factor H, at least for cells where sialic acid is thought to play the primary protective role (E0 and E6). It is important to note that this interaction is C3b as well as sialic acid dependent. It is difficult to show any binding of factor H to most cells lacking C3b, although recently endothelial cells were shown to bind factor H directly via the C-terminal domains at reduced ionic strength (18). The nature of the receptor on endothelial cells has not been identified but has been proposed to be glycosaminoglycans (18). In our model the presence of C3b on the cell surface is essential for detectable factor H binding under physiological conditions, as shown by the absence of binding to E6 alone (Fig. 1B).

Studies analyzing the functions of deletion mutants of factor H support the conclusions drawn from the present studies. These studies (62) showed that deletion of the C-terminal 10 domains caused a 40-fold reduction in binding to E6C3b. Similarly, a protein lacking the last 5 domains resulted in a 50-fold reduction in binding. Both results suggested that the C-terminal domains play a dominant role in binding to cells bearing C3b and polyanions. When the effect on decay acceleration of the complement C3/C5 convertase was measured (62) recombinant proteins lacking the C-terminal 5 domains exhibited an 11-fold reduction in functional activity. Mutants lacking the entire C-terminal half of the protein exhibited a 30-fold reduction in decay accelerating activity on sheep E6C3b,Bb. Significantly, systematic removal of clusters of domains spanning the entire factor H molecule showed that no other region, aside from the N terminus, affected factor H function on E6C3b,Bb as much as removal of the C-terminal domains.

In this study, the use of rH 19–20 as a site-specific competitor allowed the dissection of the protective functions of factor H. rH 19–20 was able to enhance complement-mediated lysis of normally complement-resistant cells (Fig. 7) and to act against control of the surface-bound C3 convertase (Figs. 4 and 5), without affecting fluid-phase functions of factor H (Fig. 6). This facilitated the study of the cell surface-specific regulatory functions of factor H, within the context of a fully functional fluid phase complement system. rH 19–20 was able to compete with full length factor H for binding to cell-bound C3b more efficiently than the other C3b/polyanion binding sites of factor H (Fig. 2B). Although rh 1–6 showed significant inhibition of factor H binding, it is the active site and thus cannot be used as a functional inhibitor. The use of domain-specific inhibitory Abs for this purpose has the disadvantage that the Abs bind directly to factor H and have variable dose-dependent effects on function, probably due to dimerization or steric hindrance of the 150-kDa factor H by the 150-kDa Abs (41, 46, 65, 66).

FIGURE 7. rH 19–20 inhibits the factor H-mediated control of alternative pathway complement lysis of host-like polyanion-bearing cells (EH and EH). A total of 40% NHS was incubated with E6 or E6 in the presence of 0–24.1 μM (0–350 μg/ml) rH 19–20, for 20 min at 37°C. The assays contained 5 mM MgEGTA to inhibit classical and lectin pathway activation and restrict activation to the calcium-independent alternative pathway of complement. Lysis was subsequently measured by hemoglobin release (A412) after centrifugation to remove unlysed cells. Same as above, except that the EH were preincubated for 20 min on ice with saturating concentrations of neutralizing anti-CD59 or -CD55 Abs, before the addition of NHS and rH 19–20. EH were preincubated for 20 min on ice with or without saturating concentrations of neutralizing anti-CD55 Abs. C8 depleted sera, in the presence or absence of 12 μM rH 19–20, was added and incubated for 20 min at 37°C. C3b deposition on EH was detected with FITC-conjugated goat anti-human C3 Ab, by FACS analysis. Deposition of C3b on EH is expressed as mean fluorescence intensity (MFI). A minimum of 10,000 events per sample were counted. Results are representative of three separate experiments, and the data show the mean of duplicate observations.
The data shown in Figs. 4–7 indicate that there is a fundamental difference between the control of soluble C3b and that of cell-bound C3b. Nilsson et al. (67) describe antigenic differences between bound and soluble C3b. Conformational neo-epitopes were suggested to be present on surface-bound C3b, which are not present on soluble C3b, although these epitopes have not been characterized. This difference may also be due to multisite attachment of factor H to C3b clusters and to bifunctional binding by domains 19–20 to polyanions and C3b. Notably, one interpretation of the recently determined structure of rH 19–20 suggested binding sites for C3b and polyanions on opposite faces of CCP 20 (16). According to such a model, the C-terminal site of factor H would bind poorly to C3b on cells lacking the second cell surface ligand, i.e., a polyanion. However, domain 20 would bind bifunctionally between C3b and the polyanion, providing that the surface bears a sufficient density of polyanions. This would raise the apparent affinity of factor H for the C3b on these cells and would increase regulation of complement activation on these surfaces. The degree of enhancement of factor H binding by polyanions appears to be limited to 5- to 10-fold (37, 48) raising the question of how this modest difference could be sufficient to account for the difference between activation or no activation of this system. One rationale for the adequacy of this mechanism is the following: because the alternative pathway is dependent on a feedback loop and because all three reactions in that loop are inhibited by factor H, a 10-fold increase in the affinity of H for the activation site could be as effective as a 1000-fold increase in factor H function.

There is a growing body of evidence that each polyanion-binding site on factor H has unique specificities. Recently, CCP domain 7 was shown to exhibit a polyanion recognition profile different from that of CCP 19–20 (38). It is thus probable that factor H exhibits cell type-specific recognition depending on the array of polyanions that the particular cell expresses on its surface. Thus, in the case of endothelial cells (18), the presence of sulfated glycosaminoglycans would appear to be sufficient for weak initial binding of factor H, in the possible absence of C3b. Whichever the case, the conclusion is the same in the sense that the C terminus is crucial for the ability of factor H to bind to cell surfaces, be it through interaction with the C3b-polyanion interface, or through direct interaction with polyanions. Further studies are needed to comprehend how mutations in other regions affect the function of factor H and predispose to diseases, such as macular degeneration (49–52).

All of the regulatory activities of factor H are expressed by the N-terminal four domains. In this study, all the activities of full-length factor H for cell-bound C3b were inhibited by rH 19–20 (Figs. 2–5). If rH 19–20 competes for the binding site on C3b where domains 1–4 bind, then the nearly complete inhibition shown in Fig. 2A is easily explained. However, direct competition with the N-terminal site is unlikely to explain this because rH 19–20 does not compete with factor H (Fig. 6) or with H 1–7 (data not shown) for control of fluid-phase C3b. In addition, rH 1–7 does not compete with rH 19–20 for the binding to membrane bound C3b (Fig. 3). This is in agreement with previous findings that these sites are distinct (42). However, these results do not agree with the model proposed recently (68) in which the N-terminal site is blocked by intramolecular folding in solution and is freed upon binding of the C-terminal site to C3b, C3d, or polyanions. According to such a model, rH 19–20 would prevent exposure of the N-terminal site of full-length factor H by competing for binding of the C terminus to ligands, which should block fluid-phase cofactor activity of factor H, but no evidence of inhibition was found in our study (Fig. 6). Therefore, although both studies support an important role of the C-terminal region, our results do not support the presence of a cryptic functional site at the N terminus, which is revealed upon engagement of the C terminus with ligands. Furthermore, this model is not supported by the ability of aHUS patients, with mutations affecting the C-terminal domains, to control fluid phase complement activation (7, 68).

HUS (shiga-toxin-related, familial, and sporadic) and various other diseases, presents with thrombotic microangiopathy which defines a lesion of vessel wall thickening (mainly arterioles and capillaries), intraluminal platelet thrombosis, and partial or complete obstruction of the vessel lumina, secondary to endothelial cell damage (69). The enhanced shear stress in the narrowed microcirculation causes erythrocytes to be traumatized, which can result in hemolysis. Our data shows that factor H is important in maintaining erythrocyte homeostasis, presenting an additional factor that may contribute to the damage of erythrocytes and other cell types in factor H- and factor I-related aHUS. Deposition of C3b (Fig. 7C) as well as up to 20% lysis of human erythrocytes (Fig. 7, A and B) was observed when factor H-mediated cell surface control was inhibited by rH 19–20, even when the function of the membrane-bound regulatory proteins was intact and when shear stress was absent. Interestingly, cryoprecipitation, a pathophysiological mechanism of RBC death, has recently been shown in vitro to be linked to aHUS, to be induced by complement activation, and may be factor H dependent (70). Studies defining the relative contribution of each of these mechanisms to erythrocyte destruction in aHUS are warranted. Likewise, erythrocytes from individuals with aHUS caused by mutations in CD46, which is not present on erythrocytes, may be more resistant to complement-mediated lysis, but will still be affected by enhanced shear stress encountered in the microvasculature due to endothelial cell damage.

Host cells are normally protected from autologous complement-mediated lysis due to the complex, often redundant array of CRP that control activation on the surface of the cell. These CRP can be membrane-bound (CD59, CD55, CD46, CD35), as well as fluid phase (C4BP and factors H and I) (12, 64). Human erythrocytes as well as nucleated human cells are known to be protected by CD59 and CD55 and the present data suggest a significant role for factor H. Use of rH 19–20 as a cell-specific inhibitor overcomes the primary difficulty in analyzing the role of factor H. As shown in Fig. 7 human cells activate the alternative pathway and are lysed (20% in 20 min) in the presence of the factor H inhibitor rH 19–20. Completely blocking CD55 with polyclonal Abs produced no lysis and minimal C3b deposition unless rH 19–20 was present. Inhibiting CD59 with excess mAbs resulted in levels of lysis similar to those produced by inhibition of factor H by rH 19–20. These results suggest that control of complement activation on human erythrocytes by fluid phase factor H is at least as important to red cell homeostasis as CD55 or CD59. The results also show (Fig. 7B) that on E14, inhibition of any two of these three regulators results in aggressive activation of the alternative pathway and lysis of the cells indicating that each of the three regulators performs a critical function in protecting host cells from the cytotoxic effects of autologous activation of complement.

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Disclosures

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References


