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## Cutting Edge: Localization of the Host Recognition Functions of Complement Factor H at the Carboxyl-Terminal: Implications for Hemolytic Uremic Syndrome<sup>1</sup>

Michael K. Pangburn<sup>2</sup>

**Incidents of hemolytic uremic syndrome (HUS) include a subset of patients that exhibit mutations in C factor H. These mutations cluster in the C-terminal domains of factor H where previous reports have identified polyanion and C3b-binding sites. In this study, we show that recombinant human factor H with deletions at the C-terminal end of the protein loses the ability to control the spontaneous activation of the alternative C pathway on host-like surfaces. For the pathology of HUS, the findings imply that mutations that disrupt the normal functions of the C-terminal domains prevent host polyanion recognition. The resulting uncontrolled activation of complement on susceptible host tissues appears to be the initiating event behind the acute renal failure of familial HUS patients. *The Journal of Immunology*, 2002, 169: 4702–4706.**

A typical hemolytic uremic syndrome (HUS)<sup>3</sup> is inherited 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 C factor H (1–10). HUS is characterized by hemolytic anemia, thrombocytopenia, and acute renal failure. Homozygous patients typically present in the first few months of life and exhibit a very high mortality. Heterozygous individuals may intermittently exhibit symptoms of varying severity throughout life (4–7). Familial HUS is not associated with diarrhea, whereas the noninherited form of HUS, typically associated with epidemics of *Escherichia coli* infections, is accompanied by severe diarrhea but has low mortality and is non-recurring (6). Mutational analyses have demonstrated point mutations and frame shifts introducing stop codons, some of which result in reduced plasma factor H concentrations (8–10). The majority of the mutations associated with HUS clustered at the C-terminal end, specifically domain 20, of C factor H (8–11).

Factor H plays a key role in the homeostasis of the C system. In its absence, spontaneous activation of the alternative pathway of complement occurs which leads to consumption of C components C3 and factor B. In a deficient line of pigs, homozygous individuals die soon after birth from C-mediated acute renal failure (12), and factor H-deficient mice develop membranoproliferative glomerulonephritis which was shown to be alternative pathway-dependent (13). By recognizing polyanionic markers, primarily sialic acid, and sulfated polysaccharides such as heparin on host cells and tissues, factor H provides the alternative pathway of C with the ability to discriminate between self and potential pathogens (11, 14–18). Factor H is normally present in plasma at high concentrations ( $\geq 50$  mg/dl). Its structure is that of a string of 20 beads (domains) with different domains performing different functions. The domains of factor H are each composed of  $\sim 60$  aa with three to eight aa spacers between the domains (19, 20). The N-terminal four C control protein domains (CCPs) regulate alternative pathway activation, while at least three polyanion binding regions and two additional C3b-binding sites are present in the C-terminal 16 domains (11, 17, 21–29).

In the present report, a series of recombinant factor H proteins were examined each lacking one or more of the known polyanion and C3b-binding regions, but possessing the N-terminal C control region. These proteins were examined for their ability to discriminate between the standard activating cells used in C research (lacking polyanionic markers) and nonactivating cells possessing surface polyanions similar to human polyanionic markers. The results support the clinical and genetic observations in HUS patients, indicating that mutations affecting the C-terminal domains of factor H result in increased C activation on normal host tissues despite the presence of polyanionic markers.

### Materials and Methods

#### Materials

Factor H (30) was purified from normal human plasma as described previously. All proteins were stored at  $-75^{\circ}\text{C}$  in Veronal-buffered saline (VBS). The concentration of factor H and all recombinant factor H (rH) proteins was determined at 280 nm using  $E_{1\text{ cm}}^{1\%}$  of 12.4. Buffers used were: PBS, 10 mM sodium phosphate, 140 mM NaCl, 0.02%  $\text{NaN}_3$  (pH 7.4); VBS, 5 mM veronal, 145 mM NaCl, 0.02%  $\text{NaN}_3$  (pH 7.3), VBS containing 0.1% gelatin (GVB); GVB containing 10 mM EDTA, and MgEDTA, 0.1 M  $\text{MgCl}_2$ , 0.1 M EGTA (pH 7.3). Factor H-depleted serum (H-dpl serum) was prepared from normal human serum by immunoadsorption of factor H on anti-H-agarose in the presence of EDTA. The flowthrough from the immunoadsorbant column was concentrated to the original serum concentration by ultrafiltration using 10,000 molecular weight cutoff membranes. Dialysis against and storage in PBS containing 0.1 mM EDTA prevented spontaneous activation of C in the absence of factor H.

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<sup>3</sup> Abbreviations used in this paper: HUS, hemolytic uremic syndrome; CCP, C control protein domain; VBS, Veronal-buffered saline; H-dpl serum, factor H-depleted serum; rH, recombinant factor H.

### Preparation and purification of recombinant proteins

Site-specific deletions in human factor H cDNA were created by overlap extension PCR as previously described (24). The constructs were inserted into pBacPAK 8/9 (Clontech Laboratories, Palo Alto, CA) or pFastBac1 (Life Technologies, Gaithersburg, MD). *Spodoptera frugiperda* cells were transfected with the constructs and the cells were maintained in complete insect media containing 10% FCS. Recombinant proteins were purified from media supernatants by immunoabsorption on anti-factor H-Sepharose. Recombinant factor H was eluted with 6 M guanidine, dialyzed against VBS, and concentrated by ultrafiltration (24).

### Hemolytic assays

Lysis of sheep and rabbit erythrocytes was measured by mixing on ice 2.5  $\mu$ l 0.1 M MgEGTA, various amounts of H-dpl serum containing factor H or rH, and sufficient GVB to bring the mix to 40  $\mu$ l. The H-dpl serum containing factor H or rH was also 20% of normal. Cells (10  $\mu$ l containing  $1 \times 10^7$  cells) were added, the mix was immediately transferred to a 37°C water bath and incubated for 20 min. To determine the extent of hemolysis, 250  $\mu$ l cold GVB containing 10 mM EDTA was added, the samples were centrifuged, and the OD of supernatant was determined at 414 nm. The percent lysis was determined by subtracting the  $A_{414}$  in the absence of serum and dividing by the maximum possible  $A_{414}$  determined by water lysis of erythrocytes.

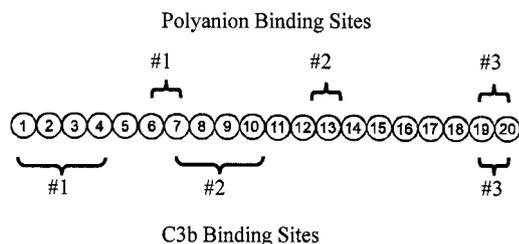
## Results

### Domain deletion mutants of factor H

Studies of the functional roles of different sites on factor H (Fig. 1) used proteins containing 5 and 10 domain deletions and designated rH $\Delta$ 1–5, rH $\Delta$ 6–10, rH $\Delta$ 11–16, rH $\Delta$ 16–20, and rH $\Delta$ 11–20. The strategy used to make these deletions (24) resulted in exact deletion of whole domains starting from the first Cys of each domain and ending with the residue before the first Cys of the next expressed domain. This strategy removes the domains as well as the entire interdomain linker on the C-terminal side of the deleted domains. The proteins were produced in a baculovirus expression system, purified by immunoaffinity chromatography (24), and stored frozen at –75°C before analysis.

### Control of C lysis by deletion mutants of factor H

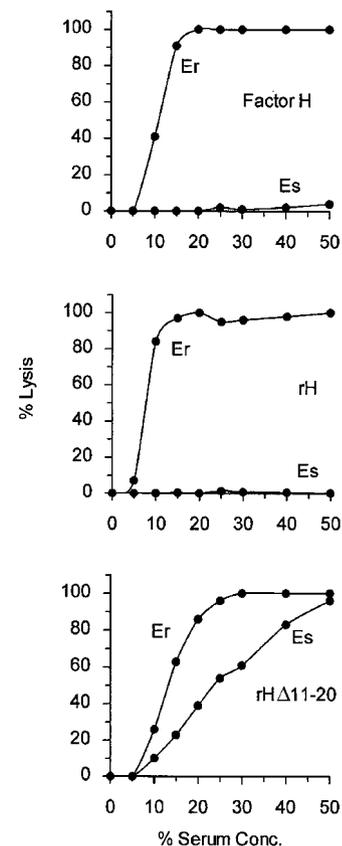
The human alternative pathway of C spontaneously activates on and lyses rabbit erythrocytes which possess minimal surface polyanions. Sheep erythrocytes, in contrast, possess high levels of sialic acid-containing polysaccharides, as do most human cells and tissues. Sheep erythrocytes are used as the standard nonactivator for human C analysis. The ability of deletion mutants of factor H to recognize surface polyanionic markers and prevent activation of the alternative pathway was examined by replacing normal human



**FIGURE 1.** Schematic representation of factor H indicating the locations of the polyanion and C3b-binding sites. Factor H is composed of 20 homologous domains each possessing a similar core structure (45). Approximate locations of polyanion binding sites 1, 2, and 3 (24–29) are indicated at the *top*, and of the three C3b-binding sites are indicated at the *bottom* (21–24).

factor H with various purified recombinant factor H proteins lacking regions known to contain polyanion binding sites. This reconstituted serum was then examined for its ability to lyse rabbit erythrocytes (Fig. 2) which verified that a functional alternative pathway had been reestablished in the H-dpl serum. Similar assays using sheep erythrocytes examined the ability of the various deletion mutants of factor H to recognize the presence of polyanions on the cell surface and block activation and lysis of those cells. Human erythrocytes could not be used because they possess the membrane-bound regulators decay-accelerating factor and C receptor 1 which block C activation, and CD59 which blocks lysis even if the C system does activate on that surface (31).

Reconstitution of H-depleted serum with purified human factor H resulted in normal C target discrimination and normal alternative pathway activation (Fig. 2, *top panel*). Identical results were observed when the baculovirus-expressed recombinant factor H was used. In both cases, rabbit erythrocytes were lysed while sheep erythrocytes did not activate the alternative pathway (Fig. 2, *middle panel*). However, when depleted serum was reconstituted with the recombinant protein possessing the N-terminal C regulatory domains, but lacking the two C-terminal polyanionic recognition



**FIGURE 2.** Lysis of C-activating cells (Er) and host-like polyanion-bearing cells (Es) in factor H-depleted human serum replenished with normal human factor H, with recombinant factor H, or with a deletion mutant of recombinant factor H lacking the C-terminal 10 domains. Human serum depleted of factor H was reconstituted with normal molar concentrations of human factor H (*top panel*), full-length recombinant factor H made in insect cells (*middle panel*), or a deletion mutant factor H lacking the C-terminal 10 domains, rH $\Delta$ 11–20 (*bottom panel*). Various concentrations of the reconstituted sera were incubated with Er or Es cells for 20 min at 37°C. The assays contained 2.5 mM MgEGTA to inhibit classical and lectin pathway activation and restrict activation to the calcium-independent alternative pathway of C. Lysis was subsequently measured by hemoglobin release after centrifugation to remove unlysed cells.

sites and the C-terminal C3b-binding site (rH $\Delta$ 11–20), the system lost the ability to recognize the sheep erythrocyte as a nonactivator and these cells were lysed (Fig. 2, *bottom panel*). Fig. 3 shows three such assays using smaller five-domain deletions, each of which removes a single polyanion or C3b-binding site (24, 25, 27). Factor H lacking domains 11–15 has lost the polyanion binding site located near domain 13 (25), and this protein reconstituted full discriminatory ability to the alternative pathway (Fig. 3, *middle panel*). Deletion of the C-terminal domains 16–20 removes the polyanion binding site and the C3b-binding site located in domains 19–20 (27, 28), which resulted in loss of the discriminatory function of the system as evidenced by the lysis of sheep erythrocytes (Fig. 3, *bottom panel*). Finally, deletion of domains 6–10 (Fig. 3, *top panel*) resulted in a small degree of lysis of sheep erythrocytes suggesting that the C3b site in this region or the polyanion binding site located in domains 6 and 7 may contribute modestly to the recognition of polyanionic markers on the surface.

## Discussion

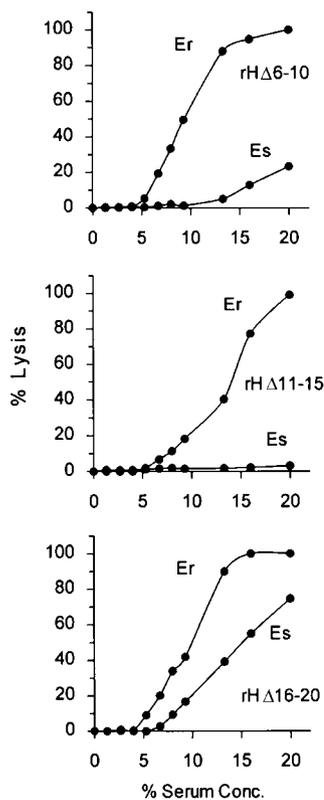
The results presented in this study lead to two conclusions. First, they indicate that the C-terminal region of factor H is critical for target/host discrimination by the alternative pathway of C. Loss of this function would be expected to result in C activation on host cells and tissues. Second, these observations define a functional defect which provides a rational link between the clinical symptoms of HUS and the structural defects indicated by genetic analysis.

The alternative pathway of C is a component of the innate immune system and two proteins of this system have been identified in sea urchins (32–34), suggesting that it arose before both adap-

tive immunity and the other C pathways. In humans, the alternative pathway of C activates continuously by depositing C3b on all surfaces in contact with plasma (35). Amplification of the initial C3b and subsequent activation of the full C system is controlled on host surfaces by regulatory proteins, some of which are membrane bound and some of which are fluid phase proteins (18, 31). Factor H is the primary fluid phase regulator and it is responsible for controlling spontaneous fluid phase activation as well as activation on host cells. Host markers that bind factor H and stop activation include polyanions such as clusters of sialic acid and sulfated proteoglycans such as heparin. Microorganisms lacking these markers are attacked by the alternative pathway. Many pathogens either bear polyanionic structures that mimic host markers or have developed cell surface receptors specific for factor H (27, 36–39).

The N-terminal four domains of factor H exhibit all of the C regulatory activities needed to prevent runaway fluid phase alternative pathway amplification (21–23). As a result, familial HUS patients with truncation or point mutations in factor H often maintain significant plasma C3 and factor B levels, but show episodic HUS and renal failure (2, 9, 10). The 16 C-terminal domains of factor H have been shown to have numerous functions which control the regulatory activity of the N-terminal domains (Fig. 1). There are two additional C3b-binding sites (18, 24) which raise the affinity of factor H for C3b clusters on the surface of activating cells. There are at least three polyanion-binding sites (24–29) distributed as indicated in Fig. 1. The results presented in Figs. 2 and 3 indicate that the functional sites at the C terminus are the most important sites for blocking alternative pathway activation on polyanion-bearing surfaces as would be found in the host. Fig. 2, *top and middle panels* show that human H and full-length rH controlled runaway fluid phase consumption or there would have been no lysis with either cell type. Furthermore, spontaneous activation on sheep erythrocytes was controlled while rabbit cells were attacked and lysed, indicating normal target recognition by recombinant factor H. However, truncations of the protein resulting in the loss of the C-terminal five domains (Figs. 2 and 3, *bottom panels*) resulted in lysis of both cell types, indicating a loss of discriminatory functions in these recombinant proteins. As the figures show, lysis of the sheep cells was less aggressive than lysis of rabbit cells. This may have been due to the presence of polyanion binding site no. 1 located in domains 6–7 (24, 29), which appeared to participate in recognition and whose removal (rH $\Delta$ 6–10) did cause some tendency for lysis of sheep cells even in the presence of the C-terminal domains (Fig. 3, *upper panel*). Although the proper experiment would be a double deletion mutant (rH $\Delta$ 6–10, 16–20), more specific mutations targeting specific crucial amino acids at each site would be more informative and these mutants are currently under development. Especially relevant will be identification of residues that specifically inactivate only C3b binding or only polyanion binding at each site. A detailed model of the polyanion binding site in domains 19–20 was analyzed by Perkins and Goodship (11). This model predicted a heparin-binding site and identified the contact residues which correlated with reported mutation sites in HUS patients (11).

Although there is ample evidence that these deletion mutants fold and function as expected (18, 24), the deletion of domains does alter the spatial distance between functional sites. Despite this change, the rH $\Delta$ 11–15 protein was fully functional even though the spatial distance between the N-terminal control site and the polyanion-binding site in CCP 19–20 was shortened by as much as 200 Å. This observation indicates that increased affinity for the cell surface may be more crucial to controlling C activation than any specific spatial relationship between the domains. This would be entirely consistent with the extended and very flexible



**FIGURE 3.** Lysis of C-activating cells (Er) and host-like polyanion-bearing cells (Es) in factor H-depleted human serum replenished with one of three recombinant mutants of factor H each lacking a 5-domain region of the 20-domain protein. The assays were performed as described in Fig. 2.

structure of CCP-containing proteins observed in electron microscopic images (40–42) and the finding that pathogen receptors that hold factor H at the cell surface bind at a variety of different sites along the factor H molecule from domain 6 to domain 20 (27, 38, 39, 43).

Even though recent genetic and functional studies of HUS patients have been very enlightening, no linear relationship is yet apparent between particular mutations and clinical presentations. At present it is only possible to conclude that mutations affecting CCP 19–20 predispose individuals to HUS (10). Complicating this picture is the fact that affected individuals have been reported with dominant and recessive pedigrees, sporadic to chronic symptoms, and C levels from normal to hypocomplementemic. Additional genetic differences most certainly predispose patients to or protect them from clinical pathology. Nevertheless, the unique features of alternative pathway activation and factor H-mediated control may shed some light on these observations. Normal levels of factor H limit both fluid phase and surface activation and maintain normal C3 and factor B levels. As factor H levels drop, surface activation becomes more aggressive (44) while fluid phase activation is controlled and C3 and B levels remain high. Thus, subnormal plasma factor H concentrations can cause C activation and HUS symptoms, possibly induced by triggering events. In this way, a heterozygous genotype can yield a dominant phenotype in one individual and a recessive phenotype in another where the levels of the normal gene product are only slightly different. As H levels drop below 30% (as might occur in a homozygous individual with a mutation which limits, but does not prevent transport), fluid phase consumption increases and blood may have a limited ability to mount a significant attack even on susceptible tissues. Depending on the particular mutation and its effect on the functions and transport of factor H (as in the case of C-terminal truncation mutants (5, 9, 10) and the deletion mutants studied in this report), one sees a situation where there is sufficient factor H control of fluid phase activation due to the N-terminal four domains, but a lack of control of surface activation as demonstrated in this study. Finally, heterozygous individuals with a normal factor H gene and homozygotes with two different mutations could present with very different phenotypes depending on the nature of the particular mutations and their affect on function and transport. Clarification of these effects will require detailed functional analyses of individual mutations in recombinant factor H and these data must be correlated with individual phenotypes of affected patients before the root causes of the pathology of familial HUS can be understood in more detail.

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