Molecular Mechanisms of Target Recognition in an Innate Immune System: Interactions Among Factor H, C3b, and Target in the Alternative Pathway of Human Complement

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Molecular Mechanisms of Target Recognition in an Innate Immune System: Interactions Among Factor H, C3b, and Target in the Alternative Pathway of Human Complement

Michael K. Pangburn,* Kerry L. W. Pangburn,* Vesa Koistinen,† Seppo Meri,† and Ajay K. Sharma†

In the alternative pathway of complement (APC) factor H is the primary control factor involved in discrimination between potential pathogens. The APC deposits C3b on possible Ags, and the interaction with factor H determines whether the initial C3b activates the APC. Factor H is composed of a linear array of 20 homologous short consensus repeats (SCR) domains with many functional sites. Three of these sites are involved in binding C3b and regulating complement activation; others bind to sialic acid and/or heparin and are responsible for host recognition. Using site-directed mutations we have examined the contributions of each of these sites to target discrimination and to functional activities of factor H. Decay acceleration by SCR1–4 of C3/C5 convertases bound to nonactivators was strongly dependent on SCR domains 11–15 and 16–20. Loss of these regions caused a 97% loss of activity, with SCR16–20 being the most critical (>90% loss). On APC activators the pattern of site usage was different and unique on each. On yeast, deletion of the 10 C-terminal domains (SCR11–20) had no effect on specific activity. On rabbit erythrocytes, this deletion caused loss of 75% of the specific activity. An examination of binding affinity to C3b on the four cell types demonstrated that factor H exhibits a unique pattern of SCR involvement on each cell. The results reveal a complex molecular mechanism of discrimination between microbes and host in this ancient innate defense system and help explain the different rates and intensities of APC activation on different biological particles.

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alternative pathway is carbohydrate specific. Once C3b amplification is allowed to begin due to the lack of membrane regulators or weak factor H binding, the efficiency of C3b attachment to the surface becomes important. We have shown that amplification is dependent on the specificity of nascent C3b for different carbohydrates (6). C3b attachment to polysaccharides exhibiting low reactivity with the thiostear acid is inefficient, and this inefficiency affects each round of amplification. The end result is that activation of the APC (i.e., amplification) can be weak or strong depending on the specificity of metastable C3b for the particular carbohydrate structures on that particle (6, 12, 19, 26, 36).

Factor H is composed of 20 homologous domains, each containing ~60 aa (37). Numerous functional sites have been identified along this structure (38–45). Three of these sites have been shown to interact with unique sites on the C3b molecule (39, 44, 46, 47). The C3b binding site at the N-terminus possesses decay-accelerating activity for the alternative pathway C3/C5 convertase and also serves as a cofactor site for factor I, a serine protease that inactivates C3b. At least three polyanion binding sites are located along the factor H molecule. Interactions between factor H and surface polyanions have been shown to be important to discrimination between activators and nonactivators of the alternative pathway of complement (7, 8, 11, 12, 14, 15, 17, 18, 23, 24, 27, 29, 43, 45, 48–50). This paper presents an analysis of the influence that different regions of factor H have on decay-accelerating activity and affinity for C3b. We have examined these interactions with C3b bound to four different cell types (two activators and two nonactivators). The results indicate that different functions of factor H rely to varying degrees on multiple sites along the factor H molecule and that the reliance on individual sites varies depending on features on the target surface recognized by this protein.

Materials and Methods

Materials

C3 (51, 52), factor B (53), factor D (54), and factor H (55) were purified from normal human plasma as described previously. Complement component C3 was repurified on a Mono S column (Pharmacia, Piscataway, NJ) (52). Cobra venom factor and cobra venom factor,Bb were prepared as previously described (56). All proteins were stored at −75°C in VBS. The concentrations of factor H and all other proteins were determined at 280 nm using E1%/1000 of 12.4, Zymosan, Trizma base, glycine, guanidine hydrochloride, and polyethylene glycol were purchased from Sigma (St. Louis, MO). Linearized Baculogold baculovirus DNA was purchased from Pharmingen (San Diego, CA). The Bac-To-Bac baculovirus expression system, Escherichia coli strain DH10Bac, Grace’s insect medium, yeastolate extract, lactalbumin hydrolysate, sodium bicarbonate, and gentamicin were purchased from Life Technologies (Gaithersburg, MD). E. coli strains NM 522 and Topp 10 (Spodoptera frugiperda (Sf9) and High Five cells were purchased from Invitrogen (San Diego, CA). FCS was purchased from Atlanta Biologicals (Norcross, GA). Restriction enzymes, T4 DNA ligase, and calf intestinal alkaline phosphatase were purchased from New England Biolabs (Beverly, MA). GeneAmp PCR reagents were purchased from Perkin-Elmer (Norwalk, CT). Buffers used were veronal-buffered saline (VBS), 5 mM veronal, 145 mM NaCl, and 0.02% NaN3, pH 7.3; VBE; VBS containing 10 mM EDTA; GVB, VBS containing 0.1% gelatin; GVBE, GVB containing 10 mM EDTA; GVB, half-physiological ionic strength buffer prepared by diluting GVB 2-fold with 5% dextrose in water; and PBS, 10 mM sodium phosphate and 140 mM NaCl, pH 7.4.

Preparation and purification of recombinant proteins

Site-specific deletions in human factor H cDNA (57) were created by overlap extension PCR (58) as previously described (44). The constructs were restriction digested with XbaI and ligated with XbaI-treated, dephosphorylated pBacPAK 8/9 (Clontech, Palo Alto, CA) or pFastBac1 (Life Technologies). The recombinant plasmid was transformed into competent Topp 10 or DH10Bac cells and checked for correct orientation by restriction mapping. All PCR-derived regions of DNA were sequenced entirely to verify that the expected mutation had been made and to determine that no unwanted alterations in amino acid sequence had been introduced. Sf9 cells were either transfected with different constructs in pBacPAK 8/9 plus Baculogold baculovirus DNA or with constructs in pFastBac1 using the Bac-To-Bac baculovirus expression system (Life Technologies) according to the manufacturers’ procedures. Recombinant viruses developed using Baculogold DNA were further cloned by end-point dilution. The Sf9 cells were maintained in complete insect medium containing 10% FCS and 50 µg/ml gentamicin at 27°C. Cells in suspension culture were maintained in Spinner flasks at 80 rpm on a magnetic stirrer. Media were collected after 4 days and analyzed by Western blotting for synthesis of recombinant proteins. Polyeethylene glycol was added to media to a final concentration of 15%, and the mixture was stirred at 4°C for 30 min. The precipitated proteins were collected by centrifugation, redissolved in VBE, and applied to an anti-factor H-Sepharose immunoadsorbant column. Recombinant factor H was eluted with 6 M guanidine, dialyzed against VBS, and concentrated by ultrafiltration.

Radiolabeling

Human factor H and recombinant H proteins (20 µg in 20–140 µl) were labeled with 50 µCi of 125I for 30 min at 0°C in a glass tube coated with Iodogen (Pierce, Rockford, IL). After the incubation 250 µg of potassium iodide and 500 µg of OVA in 50 µl of buffer were added. The free 125I was removed by centrifugal desalting through G25 pre-equilibrated with GVB (59). Specific activities of the labeled proteins ranged from 0.2–2.0 µCi/µg. Specific activities for 125I-labeled factor B ranged from 0.6–1.4 µCi/µg.

Preparation of C3b-coated cells

Deposition of C3b on zymosan (Zym) and erythrocytes (sheep, E, rabbit, E; and human, E,) was accomplished using purified C3 and factors B and D as previously described (11, 60, 61), with the substitution of nickel for magnesium and nephritic factor (62) to stabilize the C3 convertase on the surface of the cells. The number of C3b molecules bound was determined to be between 41,000 and 170,000/particle by radiolabeled Bb binding (61).

C3/C5 convertase decay acceleration assays

Decay-accelerating activity expressed by factor H and the rH proteins was measured by determining their ability to accelerate the natural release of 125I-labeled Bb from cell-bound C3b,Bb. The C3b,Bb complexes were formed by incubating 2.3–4.6 × 107 ZymC3b, E,C3b, E,B or E,), with 0.9 µg (0.5 µCi) of [125I]factor B and 0.6 µg of factor D in 75 µl of GVB containing 1 mM NCI, at 22°C for 3 min. E,C3b were preincubated with sufficient rabbit anti-DAF purified IgG (47 µg) to inactivate all the intrinsic DAF activity of the cells (63). Anti-DAF-treated E,C3b exhibited the same Bb decay rate as cells lacking DAF. Formation of the C3 convertase was stopped by the addition of 145 µl of GVEB. The cells (20 µl) were added immediately to reaction mixtures containing varying amounts of human H, II, or a rH mutant (0–10 µg) in 80 µl of GVB. After 10 min at 22°C the cells were sedimented rapidly (1 min, 10,000 g) through 250 µl of 20% sucrose in GVB in a microfuge tube. The bottoms of the tubes were cut off, and the radioactivity in the cell pellet and the supernatant was measured to determine the percentage of Bb remaining bound. The specific activity of factor H-derived proteins in this assay was determined from the concentration required to release 50% of the [125I]Bb compared with that remaining bound in the absence of factor H.

Binding assays

C3b-bearing cells (ZymC3b, E,C3b, E,C3b, and E,) had between 41,000 and 170,000 C3b/cell. Varying amounts of cells were incubated for 15 min at 22°C with 10–20 ng of radiolabeled humH, rH, or mutant rH proteins in half-ionic strength buffer (DGVB) in a total volume of 100 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 (11). Maximum functional radiolabeled protein was determined for each preparation using saturating amounts of C3b (up to 300 × 1011 C3b/assay). The percentage bound is reported minus nonspecific background binding to the appropriate cell lacking C3b. This was, in all cases, <2% of the total counts per minute.

Results

Deletion mutants of recombinant factor H

Studies of the functional roles of different sites on factor H employed proteins containing five domain and 10 domain deletions (Fig. 1). The strategy used (44) 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 (44), and stored frozen at −75°C before analysis.

Decay acceleration of C3/C5 convertase: cell surface-dependent roles of different domains

The cell surface to which a C3/C5 convertase is bound is known to determine the effectiveness of factor H decay activity (11, 15, 48). As a consequence, any examination of the roles of different sites along the molecule must include particles representative of activators and nonactivators of the human alternative pathway of complement. We have chosen two representatives from each group. Nonactivators were represented by a host cell (human erythrocytes, EH) and by sheep erythrocytes (ES), the traditional nonactivator for complement studies. Activators were represented by a rabbit erythrocyte, ER, and by a traditional complement activator similar cell type, an erythrocyte, but one that is a strong activator for complement studies. Activators were represented by a rabbit erythrocyte, ER, and by a traditional complement activator similar cell type, an erythrocyte, but one that is a strong activator for complement studies. 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Specific activities of factor H mutants for decay-accelerating C3/C5 convertase. Decay acceleration assays measured the abilities of human factor H, rH, and rH mutants to release [125I]Bb from cell-bound C3b,Bb. The C3b,Bb complexes were formed by incubating 2–5 × 107 C3b-coated cells with 0.9 μg (0.5 μCi) of factor D and 0.6 μg of factor D in 74 μl of GVB containing 1 mM NiCl2. Assays with EH C3b contained sufficient anti-DAF to inhibit all intrinsic decay-accelerating activity (63). The decay rate of the anti-DAF-treated EH C3b,Bb was indistinguishable from that of EH C3b,Bb, indicating C3b,Bb enzyme was measured by determining the radiolabel remaining bound after incubation for various times in the presence and the absence of factor H, as shown in Fig. 2. The specific activities of human factor H and the different recombinant factor H mutants were determined by measuring the percentage of [125I]Bb remaining bound after 10 min at 22°C compared with that remaining bound in the absence of any factor H. Plots of the concentration dependence of decay acceleration (Fig. 3) were used to calculate the molar concentration needed to decay dissociate 50% of the C3/C5 convertases during the 10-min incubation. Comparison of the molar specific activities of eight species of factor H on four different cell types is shown in Table I. The values were normalized to the molar specific activity of wild-type human factor H on E6 C3b,Bb to aid comparison. In these assays only 48 fmol of factor H (480 pM) was required to decay accelerate 50% of the C3/C5 convertase on ES C3b. The full-length rH and human factor H exhibited almost identical specific activities on each cell type (Table I). All recombinant proteins that lacked the first four domains of the protein (rHΔ1–5 and rHΔ1–10) also lacked decay-accelerating activity. Trace levels of factor H activity were observed in sham purifications from fresh medium containing FCS and account for the trace (<0.02%) background activity in Fig. 3. Activities of the other deletion mutants were very similar on the two nonactivators, EH and EH. The intrinsic decay-accelerating activity present on human erythrocytes due to DAF (CD55) was inactivated by including sufficient purified rabbit anti-DAF to inactivate all the DAF (63). The decay rate of the anti-DAF-treated EH C3b,Bb was indistinguishable from that of EH C3b,Bb.
As expected from previous studies (11, 15, 48) the decay-accelerating activity of factor H was lower on the two activator surfaces. Compared with the nonactivating cells, E₅C₃b,Bb required 8-fold higher concentrations of factor H to decay 50% of the C3/C5 convertases in 10 min, and Zym required 65-fold more factor H (Fig. 3 and Table I). Deletion of the second C3b binding site, localized to the SCR6–10 region in our previous study (44), showed little effect on decay acceleration, except on E₅. Removal of the polyanion binding site located in the 11–15 domain region of factor H (40) showed little or no effect on decay acceleration with the two activators, as expected. On the sialic acid-bearing E₅ and E₅H, however, there was a 6- to 10-fold drop in specific activity with the loss of this region. Similarly, removal of the sialic acid/polyanion binding site and the third C3b binding site located at the C-terminal of factor H (rHΔ16–20) had almost no effect on the activators, while resulting in an 11- to 16-fold drop in activity on nonactivators (Table I). Deletion of the entire C-terminal half of the molecule (rHΔ11–20) removed both the polyanion binding sites and the C3b binding site and resulted in the loss of 97% of the decay-accelerating activity on E₅ and E₅H, indicating that the sites in this region of the protein are important for full expression of decay-accelerating function on host cells and other sialic acid-bearing cells. On Zym, loss of this region had no effect. In fact, the C-terminal 15 domains of factor H appear to play no role in decay acceleration when the C3/C5 convertase resides on zymosan. Thus, the high efficiency of convertase decay on host cells and other nonactivators is not the result of interactions at the decay-accelerating site itself, but is regulated by interactions with domains of factor H that are perhaps >800 Å away (64–66).

**Binding of factor H: cell surface-dependent contributions of different domains**

Our previous study measured binding of rH deletion mutants to E₅C₃b (44). The present study expands the examination of binding to investigate the influence of the cell surface to which C₃b is attached. Each of the purified recombinant proteins was radiolabeled, and binding was measured to the various C₃b-loaded cells. Because the rH proteins were of limited supply, the C₃b concentration, instead of factor H, was varied to generate binding curves (Fig. 4). In Fig. 4, A and B, it can be seen that a maximum of 79% of the radiolabeled human H and 60% of the radiolabeled rH were capable of binding to E₅C₃b. The maximum binding of rHΔ11–15 was ~11%. In all other functional assays except binding assays, which were performed after radiolabeling, the proteins appeared to be fully active. Radiolabeled proteins had reduced activity, and rebinding experiments on the unbound fractions indicated that the unbound material was not capable of binding. Thus, it was necessary to determine the amount of functional protein in each preparation. Increasing concentrations of cell-bound C₃b (as much as 10 times the highest levels shown in Fig. 4) were used to obtain maximum binding levels for each radiolabeled protein. The maximum binding observed with any cell type was taken as the amount of functional protein present.

The binding of factor H to surface-bound C₃b is well known to be extremely heterogeneous, producing highly nonlinear Scatchard plots (13, 67, 68). Because of this heterogeneous binding and the limitations imposed by the need to use cell-bound ligands, it was impossible to determine affinities as Kₘ values. Nevertheless, because binding was dependent only on C₃b/assay and was not affected by the number of cells/assay or the C₃b/cell, the affinity of factor H for C₃b on different surfaces could be taken to be proportional to the concentration of C₃b needed to bind similar amounts of the radiolabeled protein. Therefore, the concentration of C₃b needed to bind 20% of the functional protein was used for comparing the relative affinities of the proteins. Twenty percent was chosen because the concentration of C₃b needed to bind 50% of many of the weaker binding rH mutants was unattainable. In several cases even the 20% level could not be attained, and it was necessary to estimate this value by extrapolation. All binding isotherms were repeated and confirmed at least twice with at least two different preparations of each recombinant protein.

The binding behavior of full-length factor H was similar to the on the two nonactivators and on the two activators (Fig. 4, A and B). Like factor H, rH showed lower affinities for C₃b on the activators compared with C₃b on the nonactivators. The difference is identical with the published 10-fold affinity difference in human H on these surfaces (12). Because rH bound to every cell type with a 5-fold higher affinity than human H, the binding of all mutant rH molecules will be compared with that of rH. Loss of SCR1–5 containing the first C₃b binding site (Fig. 4C) reduced the affinity for C₃b on all cells, but a significant difference was found in the affinities on the two activators. Loss of the second C₃b binding site and the first sialic acid/heparin binding site by deletion of SCR6–10 (Fig. 4D) caused a greater reduction in affinity than loss of the first C₃b site on all four cells. However, the affinity loss on Zym was considerably greater than that on any other cell (compare

**Table I. Decay acceleration of C₃b,Bb residing on different cells: relative⁶ molar concentrations of human H and rH mutants required for 50% decay⁵**

<table>
<thead>
<tr>
<th>Protein</th>
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⁶ Values given are relative to the 48 femtomoles (480 pM) humH per 100 μl assay found to cause the decay of 50% of the radiolabeled Bb from E₅C₃b,Bb in 10 min at 22°C as described in Figs. 2 and 3.

⁵ All values are averages from two or more inhibition curves (see Fig. 3) except for assays with rHΔ1–5, rHΔ1–10, and all assays on E₅b that were from single inhibition curves.

⁷ Decay on human erythrocytes was measured in the presence of sufficient anti-DAF to inactivate all cell-bound decay accelerating activity.
Fig. 4, C and D). This observation suggests that the SCR6–10 region plays a greater role in the interaction between factor H and ZymC3b than on the other cells. Removal of the SCR11–15 region containing the second sialic acid/heparin binding site caused a minor (2- to 3-fold) drop in the affinity for C3b on the nonactivators ES and EH, but no change in binding to the activators (Fig. 4E). Finally, removal of the third C3b binding site and the third sialic acid/heparin site (rHΔ16–20) resulted in the most unexpected pattern. While loss of this site dramatically reduced the affinity for C3b on EH, it had no effect on the other activator Zym, and while it caused a 5-fold effect on binding with EH, loss of this region caused the largest single-site effect on binding to C3b on ES seen in this study. This switch in activator/nonactivator discrimination can be clearly seen by comparing the patterns of the two activators in Fig. 4F (filled symbols) with patterns in the five other panels of Fig. 4.

Although the binding curves in Fig. 4 allow comparisons of relative affinities on different types of cells, the presentation of the data have been normalized to the maximal activity of individual radiolabeled preparations, increasing concentrations of cell-bound C3b (as much as 10 times the highest levels shown) were used. The maximum binding levels for humH, rH, rHΔ1–5, rHΔ6–10, rHΔ11–15, rHΔ16–20, rHΔ1–10, and rHΔ11–20 were 79, 60, 45, 43, 11, 34, 37, and 28%, respectively.

In Table II the relative differences in apparent affinity are presented. All the values for binding affinity are reported relative to the concentration of C3b on ES C3b needed to bind 20% of the full-length rH protein. In these experiments this required sufficient ES C3b cells to provide 220 pM C3b. For ES C3b a 10-fold higher concentration of C3b was required to bind 20% of the protein, indicating the 10-fold reduction in affinity for C3b bound to this cell. Table II includes data from 28 binding curves, including those for the half-molecules rHΔ1–10 and rHΔ11–20, which are not shown in Fig. 4 or 5 (data in Table II are summarized from two or more independently measured binding curves for each cell/rH combination). Not shown in Table II are the comparable values for normal human factor H, which are 5, 4, 57, and 55 for ES, EH, and Zym, respectively, relative to 1 for the rH and ES C3b pair. The origin of this 5-fold difference has been attributed to charge differences due to the lack of sialic acids on the insect cell-derived rH and is illustrated by comparing Fig. 4, A and B.

The data in Table II allow a quantitative comparison of the relative affinities of the different deletion mutants for clusters of C3b on four different cells. Two patterns are apparent from this table. First, major differences in the roles of various regions of factor H were found between activators and nonactivators of the APC. Second, there were also significant differences between the members of these groups. The interaction between factor H and C3b on ES and EH appears to use the SCR1–5 region equally and differ only slightly, if at all, in their reliance on SCR6–10. However, binding to C3b on the two nonactivators differs considerably in the use of the C-terminal domains 16–20, where the third C3b site and a sialic acid/polyanion binding sites are located. In fact, on ES a 50-fold reduction in affinity occurred with the loss of SCR domains 16–20. In contrast, on EH the loss of this region had only

**FIGURE 4.** Comparison of binding of human factor H, rH and four rH deletion mutants to C3b on two cell types that do not activate the human alternative pathway (ES C3b (□) and EH C3b (■)) and two cell types that activate the pathway (ZymC3b (●) and ES C3b (□)). Various concentrations of cells bearing the indicated amounts of C3b were incubated with 10 ng of each radiodinated protein in 100 μl of DGVB. After 15 min at 22°C, the bound and free radiolabel were separated by centrifugation of cells through 20% sucrose. Background binding to the appropriate cell lacking C3b at the appropriate concentration was subtracted from each assay, but was <2% in all cases. To determine the amount of functional protein in the radiolabeled preparations, increasing concentrations of cell-bound C3b (as much as 10 times the highest levels shown) were used. The maximum binding levels for humH, rH, rHΔ1–5, rHΔ6–10, rHΔ11–15, rHΔ16–20, rHΔ1–10, and rHΔ11–20 were 79, 60, 45, 43, 11, 34, 37, and 28%, respectively.
a 5-fold effect, while loss of SCR domains 6–10, containing the second C3b site and the first sialic acid/polyanion binding site, had the greatest effect on binding.

Table II. Relative reduction in binding affinity of factor H deletion mutants for C3b on different activators and nonactivators of the alternative pathway

<table>
<thead>
<tr>
<th>Protein</th>
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<td>rHΔ16–20</td>
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*Numbers given are numbers of cell-bound C3b/100 μL assay needed to bind 20% of the radiolabeled rH relative to the 1.3 × 10<sup>7</sup> C3b/100 μL on E<sub>0</sub>C3b (220 pM C3b) needed to bind 20% of rH. All C3b concentrations were normalized to this concentration, which was set to 1. See text and Fig. 4 for a description of the binding assays.

Comparison of the two activators demonstrates that a distinct pattern of functional site usage governs the interactions between factor H and C3b on these surfaces compared with the nonactivators (Table II). The data show that activators relied more on the C3b binding site in the SCR6–10 region than on any other site. This was most significant with ZymC3b where there was an almost 10-fold greater reliance on the second C3b binding site in the SCR6–10 region than on the first C3b binding site in SCR1–5 where both decay-accelerating and factor I cofactor activity reside. Reliance on these two sites (SCR1–5 and SCR6–10) differed by <2-fold on E<sub>R</sub> and on the two nonactivators. The biggest difference in site usage between the two activators was found in the importance of the C-terminal regions. Deletion of any or all of the last 10 domains had no effect on rH binding to C3b on Zym (compare the 11-fold reduction in affinity found for rH (Table II) with the 10-, 12-, and 12-fold reductions found for rHΔ11–15, rHΔ16–20, and rHΔ11–20, respectively). In contrast, E<sub>R</sub>C3b exhibited a decrease from 10- to 90-fold in affinity for rH lacking the C-terminal SCR16–20 region. Deletion of the entire last 10 domains showed less of an effect on E<sub>R</sub>, but data in Table II suggest that deletion of two five-domain regions did not always have as great an effect as would be expected from the loss of multiple binding sites.

Direct interactions between factor H and cell surface polyanions were not detected in the present study. Although binding assays were performed at half the normal ionic strength, binding to particles lacking C3b was <2% for all cells. This background binding has been subtracted from the data reported here. An additional concern regarding ionic interactions was that the lack of sialic acids on rH, made in insect cells, might alter the binding to cells bearing high surface polyanion concentrations (E<sub>R</sub> and E<sub>H</sub>) compared with cells with little surface charge (Zym). A comparison of Fig. 4, A and B, reveals that this is apparently not the case. Although humH bound 5-fold more poorly to E<sub>0</sub>C3b than rH, this 5-fold difference was observed with every cell type from the highly sialic acid-loaded E<sub>R</sub> to the nearly neutral Zym, which lacks sialic acid. These results suggest that the primary interaction responsible for the difference in affinity of humH and rH is due to charge interactions between C3b and factor H.

Cross-comparison of the roles of different sites for different activities on different cells

Because the binding of full-length rH to each cell type showed large and variable differences, it is difficult to compare the roles that each region of factor H plays in decay acceleration and binding. Table III allows such a comparison. Table III shows the data from both portions of this study normalized to the activity of full-length rH on each particular cell type. This analysis permits, for example, one to quickly see that decay acceleration by rH on Zym was unchanged by deletion of any region of the molecule except the decay-accelerating site in SCR1–5 (i.e., the relative specific activity remained 1 for all deletions, except where activity was lost due to removal of domains 1–5). This presentation of the data also allows easy comparison of the relative importance of a region to either activity on all four cell types. For example, rHΔ6–10 can be seen to be most important to decay of C3/C5 convertase activity on E<sub>R</sub> (4-fold reduction in activity with loss of this region), but for binding this region was far more important. The greatest effect was observed on E<sub>H</sub> and Zym, where 19- and 32-fold reductions in binding affinity were found. This apparent contradiction between binding and decay-accelerating activity is also made clear by Table III and will be discussed below. Finally, a careful study of Table III allows a comparison of the roles of all the regions of factor H.
in each activity on all cell types. What is apparent is that the functions of factor H on every surface were dependent upon a unique set of interactions with the different domains of factor H.

Discussion

Discrimination between potential pathogens and the host is a fundamental function of any system of innate immunity. The molecular mechanisms by which the molecules of these systems recognize and elicit responses or prevent (i.e., to the host) responses has recently been recognized to be fundamental to understanding the adaptive immune systems of higher organisms (1–4). Complement appears to have evolved in nonvertebrates before the arrival of rearranging genes (2, 3, 69). Many lectin-based innate systems bind carbohydrates and signal cellular responses directly through cell receptors for the lectin. In the case of the complement-activating mannose-binding lectin, lectin binding initiates a cascade that subsequently elicits chemotaxis and phagocytosis. The alternative pathway of complement uses a more generalized recognition system. Idealistically, it recognizes the host and attacks everything else. In reality micro-organisms display a spectrum of activation rates and activation intensities (33, 70, 71), and many have evolved effective evasion mechanisms. Many evasion strategies involve host mimicry. Factor H with its multiple binding sites is the key recognition protein in this system. Understanding the mechanisms of its action and its specificities may allow 1) better design of implantable or extracorporeal biomaterials to make them more host-like (72); 2) better control of complement-mediated tissue damage in xenotransplantation (73); 3) the design of vaccines that promote APC activation to elicit maximal responses from the adaptive immune system (1); 4) development of antibiotics that prevent mimicry of the host, which allows escape from complement (22, 27, 30); and 5) development of means to control inadvertent or misdirected complement activation in the host (5).

The unique structure of factor H allows it to simultaneously use multiple recognition sites (Fig. 1). These domains appear to be arranged as a flexible chain of beads on a string (64–66, 74), and such domains can interact with multiple ligands that are not contiguous on target surfaces. The discriminatory ability of such an arrangement is illustrated in the data presented in this paper. Tables I, II, and III demonstrate that factor H interacts uniquely with each of these four potential biological targets by using a unique set of sites for each cell and each activity.

Expression of decay-accelerating activity for the APC C3/C5 convertase required 65-fold higher concentrations of factor H if the enzyme was on a Zym particle rather than on a nonactivator such as a host cell (Table I). No other sites appeared to interact with the Zym surface, because the specific activity of rH was unaffected by removal of any region other than the decay-accelerating site itself (Table III). Similarly, on the second activator, E$_r$, the C-terminal domains of the molecule contributed only minimally to the activity of the decay-accelerating site. On nonactivators, however, regions other than the decay-accelerating site (SCR1–5) of factor H enhanced activity greatly, especially the C-terminal half of the molecule. In fact, loss of SCR domains 11–20 yields a protein (rH11–20) that has the same specific activity as full-length rH on Zym, but has lost all of the interactions that enhance decay, and this mutant recognizes little difference between activators and nonactivators (see last line of Table I). The enhanced decay activity on nonactivators is most likely the result of interactions with polymers on these surfaces for the following reasons. In terms of alternative pathway activation the major distinguishing feature between these cells is the presence of high densities of sialic acid on E$_s$ and E$_r$. The surface density of sialic acids on E$_s$ is ~10% that on E$_r$, and Zym has no sialic acid (12). Furthermore, we and others have shown that removal of sialic acid from E$_r$ and sialic acid-bearing complement-resistant bacteria reduces the activities of factor H on these surfaces and causes the cells to become activators of the human APC (7, 11, 12, 15, 24, 48). Interactions of multiple C3b molecules on the surface with the C3b binding site at the C-terminal of factor H cannot account for the observed results, because the C3b densities on the activators and nonactivators used in this study were similar (for example, in one set of assays the number of C3b per cell was between 76,000 and 93,000 on the four cell types and produced the same results). Thus, the evidence suggests that the SCR1–15 and SCR16–20 regions of factor H interact with surface sialic acids to enhance decay-accelerating activity on nonactivators of the APC.

If the involvement of individual regions of factor H in decay acceleration is compared with the involvement of regions controlling binding, an apparent contradiction becomes evident. For example, in Table III the removal of domains 6–10 had no effect on decay acceleration activity on Zym; however, this same deletion (rH11–20) that has the same specific activity as full-length rH on Zym, but has lost all of the interactions that enhance decay, and this mutant recognizes little difference between activators and nonactivators (see last line of Table I). The enhanced decay activity on nonactivators is most likely the result of interactions with polymers on these surfaces for the following reasons. In terms of alternative pathway activation the major distinguishing feature between these cells is the presence of high densities of sialic acid on E$_s$ and E$_r$. The surface density of sialic acids on E$_s$ is ~10% that on E$_r$, and Zym has no sialic acid (12). Furthermore, we and others have shown that removal of sialic acid from E$_r$ and sialic acid-bearing complement-resistant bacteria reduces the activities of factor H on these surfaces and causes the cells to become activators of the human APC (7, 11, 12, 15, 24, 48). Interactions of multiple C3b molecules on the surface with the C3b binding site at the C-terminal of factor H cannot account for the observed results, because the C3b densities on the activators and nonactivators used in this study were similar (for example, in one set of assays the number of C3b per cell was between 76,000 and 93,000 on the four cell types and produced the same results). Thus, the evidence suggests that the SCR1–15 and SCR16–20 regions of factor H interact with surface sialic acids to enhance decay-accelerating activity on nonactivators of the APC.

If the involvement of individual regions of factor H in decay acceleration is compared with the involvement of regions controlling binding, an apparent contradiction becomes evident. For example, in Table III the removal of domains 6–10 had no effect on decay acceleration activity on Zym; however, this same deletion reduced binding on Zym to 3% of that of full-length H. Scientific principles tell us that no biological event can occur without an interaction, thus binding must accompany decay acceleration. If the results are correct, then the binding occurring in these two events must be different. We believe that the different patterns arise from the kinetic differences between the two assays. In the assays for decay acceleration we measured the rate of destruction of the binding site. That is, C3b,Bb is the ligand for this binding event, and no signal occurs until it is destroyed by interaction with factor

### Table III. Relative reduction in decay activity and affinity of factor H deletion mutants compared to the activity of full-length rH on the same cell type

<table>
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<tr>
<th>Protein</th>
<th>Decay Acceleration</th>
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Numbers given are from Tables I and II and have been normalized to the values for full-length rH on the indicated cell type. The numbers presented represent the fold reduction in activity from rH and allow a comparison of the relative roles of each deleted region to binding or decay accelerating activity on each cell type. Symbols (∞) under decay acceleration indicate complete loss of specific decay accelerating activity, which accompanied removal of SCR domains 1–5.
H. Extrapolations from earlier work (14) (M. K. Pangburn, unpublished observations) suggest that the half-life of the C3 convertase (C3b,Bb) in plasma is very short (in the millisecond range) due to decay by factor H. The binding assays performed here (Fig. 4 and Tables I and III) measured a much more stable interaction. In fact, only if the binding was stable during the 1- to 2-min spin through 20% sucrose would a signal have been observed in these assays. Thus, during decay acceleration the N-terminal end of this 800-Å-long flexible protein must act like the end of a whip that splits the C3b,Bb complex with only brief contact. On surfaces bearing sialic acids, ionic interactions of the opposite end of the protein with surface charges aid in making this contact. They may also hold the protein on the surface, allowing the active end to decay many nearby C3/C5 convertases in an ~800-Å radius, thus contributing to the enhanced effectiveness of decay on host and host-like surfaces. Zym and most bacteria lack these sialic acid sites. The results of binding assays suggest that a longer term interaction also occurs that involves many other sites in factor H. In light of these considerations, the evidence suggests that each different function of this molecule involves a unique set of binding sites and that their relative importance to each activity depends on the markers on the surface to which C3b is bound.

Binding, measured here as a long-term interaction between factor H and surface-bound C3b, may be important to several activities of factor H. First, because factors B and H are competitive, with a 70-fold affinity difference in favor of factor H on E$_k$ (11), resident factor H would effectively prevent binding of factor B. With factor H bound, formation of the C3/C5 convertase (C3b,Bb) would be prevented, and APC amplification would be stopped. If factor H arrives after C3b,Bb is formed, then the enzyme would first be decay dissociated, and reformation would be prevented by the long term association of factor H with the C3b-coated surface. Second, permanent inactivation of C3b requires cleavage of C3b by the plasma serine protease factor I. Factor H is a required cofactor for factor I, and factor H, residing for extended periods bound to C3b, would provide the site necessary for factor I to function. Once cleaved, C3b no longer supports continued complement activation. Furthermore, upon inactivation to iC3b, C3b loses affinity for factor H, and the regulator would be free to move on to another C3b. Because no factor I was present during our assays, factor H remained bound.

Binding assays using the deletion mutants (Table II) indicated that the long term interaction involves a more complex pattern of sites on factor H than are involved in decay acceleration (Table III). Furthermore, these multiple interactions appeared to discriminate more strongly among all four cell types used in this study than those regulating decay. Although decay acceleration showed little difference between E$_k$ and E$_e$ for any of the deletion mutants, binding showed large differences. Stable binding to C3b on E$_k$ was more dependent on the SCR1–10 region, while binding to E$_e$ relied less on this region and much more on SCR16–20. In fact, a 10-fold difference in affinity was caused by deletions at the C-terminus of the protein on these two cells (see rHΔ16–20, Table II). On APC activators little difference in decay activity was observed when SCR16–20 were removed (Table III). However, when binding was measured, large differences were seen, with the affinity on E$_e$ dropping 9-fold, consistent with the presence of low amounts of sialic acids on this surface. Binding to E$_k$ dropped 50-fold with loss of SCR16–20, while the affinity on Zym was unaffected by this deletion. The major site mediating binding to C3b on Zym was the second C3b site located in SCR6–10. Clearly, this was not due to interactions with the heparin/sialic acid binding site located in this region (43, 44) and was most likely due to binding to the second C3b site located in the same region. Surprisingly, binding to C3b on human erythrocytes also depended primarily on this SCR6–10 region and less on the N- and C-terminal five-domain regions. Here it was less clear whether the sialic acid or the C3b binding sites in these regions were the dominant factors. These observations are consistent with the finding that human erythrocytes stripped of most of their sialic acid (and all DAF and CR1 activity) still do not activate the alternative pathway (M. K. Pangburn, unpublished observations), whereas sheep erythrocytes stripped of most of their sialic acid do become activators (11, 15, 48).

One caveat regarding the extension of binding data to normal human factor H is that rH exhibited 5-fold higher affinity. Even though both rH and humH had nearly identical specific activities in decay assays, and rH affinity drops 10-fold on activators in exact quantitative agreement with results for humH, some caution should be exercised as to the quantitative contributions of each site in humH. If the cause of the affinity difference between rH and humH turns out to be nonuniform across the protein, then the contribution of any affected site might be exaggerated in rH.

It is not yet clear how many functional sites factor H possesses, but its discriminatory power could be impressive. If we assume that each domain of factor H provides it with a unique ligand specificity and that these sites could work together in pairs, threes, fours, etc., then factor H would by simple combinatorial math have the ability to discriminate among $>10^6$ target surfaces. Its unique flexible structure and its length of $>800$ Å allow factor H the opportunity to use its 20 domains to scan a large area on a target surface for ligands. This flexibility removes the restriction that the ligands be arranged precisely on the target surface. Perhaps even more impressive, this argument assumes only two states at each site (high affinity or no affinity). If each site possesses a spectrum of affinities for different structures found on the surfaces of biological particles, then the combinatorial potential of this single molecule rapidly exceeds that of the entire Ab repertoire of the adaptive immune system. While some diversity of factor H specificities would be necessary for this innate complement system to recognize all the different cells and tissues of the host organism, there appears to be no biological need in the host for a level of diversity equal to the adaptive system to have evolved in factor H. However, micro-organisms may have adapted to the host-imposed specificities of this innate defense system and thus many appear to express varying degrees of host-like markers. Whatever drove factor H to evolve its discriminatory ability, we have begun to show how this single protein, with a defined primary structure, controls diverse target recognition in an ancient system of innate immunity. Furthermore, the molecular mechanism used by this protein to control complement activation illustrates the principles and advantages of single molecule combinatorial chemistry.

Acknowledgments

We express our appreciation to Kim Anderson and Nicole Narlo for their excellent technical work.

References


