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An Engineered Construct Combining Complement Regulatory and Surface-Recognition Domains Represents a Minimal-Size Functional Factor H

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Complement is an essential humoral component of innate immunity; however, its inappropriate activation leads to pathology. Polymorphisms, mutations, and autoantibodies affecting factor H (FH), a major regulator of the alternative complement pathway, are associated with various diseases, including age-related macular degeneration, atypical hemolytic uremic syndrome, and C3 glomerulopathies. Restoring FH function could be a treatment option for such pathologies. In this article, we report on an engineered FH construct that directly combines the two major functional regions of FH: the N-terminal complement regulatory domains and the C-terminal surface-recognition domains. This minimal-size FH (mini-FH) binds C3b and has complement regulatory functions similar to those of the full-length protein. In addition, we demonstrate that mini-FH binds to the FH ligands C-reactive protein, pentraxin 3, and malondialdehyde epitopes. Mini-FH was functionally active when bound to the extracellular matrix and endothelial cells in vitro, and it inhibited C3 deposition on the cells. Furthermore, mini-FH efficiently inhibited complement-mediated lysis of host-like cells caused by a disease-associated FH mutation or by anti-FH autoantibodies. Therefore, mini-FH could potentially be used as a complement inhibitor targeting host surfaces, as well as to replace compromised FH in diseases associated with FH dysfunction. The Journal of Immunology, 2013, 191: 912–921.

The complement system is a major humoral component of innate immunity, participating in the protection against infections, removal of immune complexes, waste disposal, and regulation of the adaptive immune response (1). It is activated via three major pathways: the classical, lectin, and alternative pathways. Complement activation is targeted and limited both in time and space. The activation of this plasma enzyme system is controlled by several fluid-phase and cell-bound complement regulators that intervene at various points of the activation cascade. The alternative pathway is spontaneously and constantly activated in plasma at a low rate. In addition, all three pathways lead to activation of C3 via C3 convertase enzymes, and the generated C3b molecules can form additional alternative pathway C3 convertases and, thus, amplify complement activation.

Therefore, proper regulation of the alternative pathway is essential (2). Misdirected or systemic, overwhelming activation leads to complement-associated diseases, such as age-related macular degeneration (AMD), atypical hemolytic uremic syndrome (aHUS), and C3 glomerulopathies, including C3 glomerulonephritis and dense deposit disease (DDD) (1–5). A number of complement inhibitors are being developed with the hope of applying them in the treatment of such diseases (6).

Factor H (FH) is the major regulator of the alternative pathway in plasma, and it plays an important complement inhibitory role when attached to host cell surfaces and basement membranes (7, 8). FH is a 150-kDa glycoprotein with a plasma concentration ∼250 μg/ml (9). It regulates the alternative pathway C3 convertase (C3bBb) through three mechanisms. First, it competes with factor B for

R.S. performed cloning and protein expression. M.A.-D., P.S.-C., M.A.-D.-D., and M.J. performed hemolysis assays. L.T.R. and M.J. performed the endothelial cell assays. P.S.-C. and M.J. wrote the manuscript with the help of the other investigators. Portions of this work were presented at the 24th International Complement Workshop, October 10–15, 2012, Chania, Greece (Immunobiology 217: 1137).

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Abbreviations used in this article: aHUS, atypical hemolytic uremic syndrome; AMD, age-related macular degeneration; CCP, complement control protein domain; CCR1, factor H–related protein 1; CRP, C-reactive protein; DDD, dense deposit disease; DPBS, Dulbecco’s PBS; ECM, extracellular matrix; FH, factor H; MAA-BSA, malondialdehyde-acetaldehyde adduct of BSA; MDA, malondialdehyde; mini-FH, minimal-size functional factor H; PNH, paroxysmal nocturnal hemoglobinuria; PTX3, pentraxin 3; RU, resonance unit.

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C3b binding, thus inhibiting convertase formation. Second, it enhances the decay of existing convertases by displacing the C3b-bound Bb fragment. Third, it acts as a cofactor for the protease factor I in the cleavage and irreversible inactivation of C3b. By these mechanisms, FH downregulates the alternative complement pathway (7). Mutations, polymorphisms, and autoantibodies affecting FH, which are associated with AMD, aHUS, DDD, and other C3 glomerulopathies, influence the ability of FH to interact with C3b and/or with host cells or extracellular ligands, thus causing alterations in the activity of this important complement regulator (10–12).

FH is composed of 20 complement control protein domains (CCPs; also known as short consensus repeat domains). The N-terminal domains 1–4 of FH are necessary and sufficient for the cofactor and decay-accelerating activities of the protein (13, 14). The crystal structure of FH domains 1–4 with C3b and that of factor I revealed the molecular basis for the cofactor and decay-accelerating activities of FH, indicating that FH facilitates C3b cleavage by providing a binding platform for factor I and stabilizing the domain structure of C3b, while promoting destabilization of the C3 convertase through competition and electrostatic repulsion (15, 16). The C-terminal domains 19–20 play a critical role in host cell recognition by FH, targeting the complement regulatory activity of FH to the surfaces of host cells and tissues (17, 18). Recent reports on the structure of the FH domains 19–20 in complex with C3d provided structural insights into this host-protection mechanism (19, 20). Collectively, these data suggest a folded-back structure of FH when bound to C3b on a surface, with CCP4 and CCP19 coming into proximity of each other (15, 19–21).

FH-based therapeutics could be of advantage over certain current treatments, such as plasma infusion or eculizumab, which inhibits complement at a later step (5). Purified full-length FH showed efficacy in controlling the C3 convertase in a mouse model of FH deficiency (22) and in vitro C3-deposition and cell-protection assays in the presence of aHUS-associated anti-FH autoantibodies or mutations in FH or C3 (23–25).Domains 19–20, which naturally evolved to dock FH on host cells, could be exploited in drug design to direct the regulatory activity of FH, or other complement inhibitor, to host surfaces (26). Therefore, we speculated that a minimal-size functional FH (mini-FH) could be created by directly fusing the complement regulatory domains and the host cell–recognition domains of FH. This notion gathered further support from structural data that was published recently (19, 20). We generated such a construct that is limited to the two major functional regions (i.e., domains 1–4 and 19–20) and assessed the functional activity of this recombinant protein as a potential therapy.

Materials and Methods

The study was performed in accordance with the Declaration of Helsinki and was approved by the Research Ethics Committee of the Medical Faculty of the Friedrich Schiller University (control number 2269-04/08), the University Hospital ‘La Paz,’ and Comité de Protection des Personnes du Faculty of the Friedrich Schiller University (control number 2269-04/08), The study was performed in accordance with the Declaration of Helsinki.

Proteins, sera, and Abs

Mini-FH was generated by gene synthesis (GenScript, Piscataway, NJ). Nucleotides coding for CCPs 1–4 and 19–20 from the FH cDNA sequence (accession number Y00716) were codon optimized, synthesized, cloned, inserted into the pBSV-SHis Baculovirus expression vector, expressed in Spodoptera frugiperda (SpI) cells, and purified by nickel-affinity chromatography, as described (27). Recombinant FH-related protein 1 (CFHR1) and the FH fragments consisting of CCPs 1–4 (FH1–4) and CCPs 15–20 (FH15–20) were generated and produced as described (28).

FH was purified from normal human serum (29) or obtained from Merck Chemicals (Schwalbach, Germany) or from Complement Technologies (Tyler, TX). C2-depleted serum, C3, C3b, C3d, factor I, factor B, factor D, properdin, goat anti-human FH Ab, and goat anti-human factor B Ab were purchased from Merck, and FH-depleted serum, C3b, and C3d were from Complement Technologies. HRP-conjugated goat anti-human C3 was from MP Biomedical (Salon, OH). Rabbit anti-human C3d Ab was obtained from Dako (Hamburg, Germany). HRP-conjugated swine anti-rabbit Iggs and rabbit anti-goat Igs were from Dako. The mAb C18 was from Alexis Biochemicals (Lörrach, Germany). The mAb OX24 was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-C3c Ab was from Quidel (Teco Medicals, Paris, France).

Sequence analyses

Nucleotide and protein sequences were analyzed using the Bioinformatics Resource Portal (expasy.org) of the Swiss Institute of Bioinformatics (http://www.expasy.org) with the Translate, Compute pI/Mw, and ProtParam tools.

Microtiter plate-binding assays

To analyze C3b and C3d binding, 100 nM mini-FH or FH, diluted in Dulbecco’s PBS (DPBS; Lonza, Wuppertal, Germany), was immobilized in microtiter plates. Nonspecific binding sites were blocked with DPBS containing 3% BSA (Sigma-Aldrich, Taufkirchen, Germany). Serial dilutions of C3b and C3d in DPBS were added for 1 h at 22°C, and binding was detected using HRP-conjugated anti-C3 (for C3b) or anti-C3d Ab, followed by the respective secondary Ab. TMB PLUS substrate (Kem-Entec Diagnostics, Taarssur, Denmark) was used to visualize Ab binding, and the absorbance was read at 450 nm.

Binding of mini-FH and FH to wells coated with MaxGel (diluted 1:30), gelatin (25 μg/ml), pentraxin 3 (PTX3; 10 μg/ml; ∼30 nM), and C-reactive protein (CRP; 10 μg/ml; ∼87 nM) was performed as for C3b and C3d but using DPBS containing Ca2+ and Mg2+ (Lonza).

Real-time binding assay

The interaction of FH and mini-FH with C3b and C3d was analyzed in real time using a ProteOn XPR36 surface plasmon resonance system (Bio-Rad). C3b and C3d were immobilized by a standard amine coupling technology to a GLC biosensor chip. Different concentrations of FH or mini-FH were injected simultaneously through the six channels of the microfluidic system at 30 μl/min flow rate and allowed to interact with each of the three immobilized proteins for 300 s. The dissociation was also followed for 300 s. The running buffer was 10 mM HEPES, 140 mM NaCl, and 0.005% Tween-20 (pH 7.2). The surface was regenerated with an 18-s pulse of 25 mM NaOH. The experiment was repeated three times on two different chips. A bivalent analyte-binding model was used to calculate the apparent kinetic parameters because it was the closest to the real situation, where FH has two binding sites for C3b in domains 1–4 and 19–20. The 1:1 Langmuir model was used for C3d binding, because only domains 19–20 participate in the interaction. Binding curves were double referenced by subtracting the signal from the interspots and the signal from a buffer injection, as recommended by the manufacturer.

Malondialdehyde-acetaldehyde adduct of BSA—binding assay for FH, mini-FH, and FH1–4

Malondialdehyde (MDA) salt sodium (MDA•Na) was synthesized from 1,3,3-tetramethoxypropane (Sigma-Aldrich), as previously described (30), and then used in the modification of BSA (fatty acid BSA; Aldrich), with a protocol modified after Thiele et al. (30) and Ishii et al. (31). Briefly, BSA (2 mg/ml) was incubated overnight in 0.1 M phosphate (pH 7.2) with MDA•Na (50 mM) and acetaldehyde (20 mM) in rotation at 37°C until vividly yellow. The modification product was named MDA-acetaldehyde adduct of BSA (MAA-BSA), because both MDA and acetaldehyde were used in the modification. Unreacted aldehydes were removed by changing the MAA-BSA into PBS with a concentrator (Amicon Ultra-15 Ultracel 30k). The modification of the lysines of BSA by MDA was analyzed by the increase in mobility under native PAGE (fluorescence detected by Fluoroskan, ex/em 355/460 nm).

Microtiter plate wells were coated with 15 μg/ml MAA-BSA at 4°C for 17 h. The wells were blocked with 0.5% BSA/PBS for 1 h, washed with 0.05% Tween-20 in PBS, and incubated with dilution series of purified FH (33) and then with anti-human mini-FH, or FH1–4 prepared in 0.1% BSA/PBS for 1 h at 22°C. The wells were washed with 0.05% Tween-20/PBS and then incubated with mAb 90X (33) (4 μg/ml in 0.1% BSA/PBS) for 1 h at room temperature. After thorough washing, HRP-conjugated anti-mouse Ab
MINI-FACTOR H AS A COMPLEMENT INHIBITOR

Jackson ImmunoResearch; 0.7 µg/ml in 0.1% BSA/PBS was added for 1 h at 22 °C. After thorough washing, ortho-phenylenediamine (Dako) was added, according to the manufacturer’s instructions, and the bound HRP-conjugated Ab was quantitated by reading absorbance at 492 nm. For data analysis, the A492-nm value for BSA (wells that were blocked only) for each FH, mini-FH, or FH-I-4 dilution was subtracted from the respective A492-nm value for MAA-BSA.

C3 convertase–decay assay

Solid-phase alternative pathway C3 convertase was assembled on immobilized C3b in microtiter plate wells, as described previously (34). Increasing concentrations of mini-FH or FH were added for 30 min at 37 °C, and the remaining intact convertase was determined using a goat anti-factor B antisera. TMB PLUS substrate was used to visualize Ab binding, and the absorbance was read at 450 nm.

Cofactor assays

Cofactor activity of mini-FH and FH was measured in fluid-phase assays by incubating C3b (220 nM) and factor I (440 nM) with 10 nM FH or mini-FH for 1 h at 37 °C in a final volume of 20 µl. The reactions were stopped by adding reducing SDS–sample buffer. Samples were loaded onto 11% SDS-PAGE gels, separated by electrophoresis, and subjected to Western blot. C3 fragments were revealed using HRP-conjugated goat anti-human C3 and an ECL detection kit (Applichem, Darmstadt, Germany).

To assay cofactor activity on surfaces, 50 µl 1 µM mini-FH or FH was added to 90 µl of microtiter plate wells, as described previously (34). In these experiments, the concentration of FH or mini-FH was in 50 µl at 10 nM. After washing, the cells were incubated with 220 nM C3b and 440 nM factor I, and 30 µl of the wells were incubated for 1 h at 37 °C. Reducing SDS–sample buffer was added to block only for the wells and incubated for 1 h at 37 °C. The reactions were incubated at 37 °C for 30 min, and the absorbance was read at 450 nm.

Interaction of mini-FH with C3b and C3d and analysis of its functional activity in regulation of C3b deposition

Interaction of mini-FH with C3b and C3d and analysis of its functional activity in regulation of C3b deposition

Results

Generation of mini-FH

We hypothesized that a mini-FH that would retain the C3b- and host surface–binding properties of FH could be generated (Fig. 1A, 1B). To construct mini-FH, the original nucleotide sequence of exons 2–6 and 22–23 of the CFH gene, coding for domains 1–4 and 19–20, respectively, were codon optimized (Supplemental Fig. 1), synthesized, and cloned into the Baculovirus expression vector pBSV-8His (27). This construct has no artificial linker between domains 4 and 19, only the native 6 aa between the fourth cysteine in CCP4 and the first cysteine in CCP19. The translated protein has a theoretical isoelectric point of 6.32 and a molecular mass of 42,233 Da (44,891 Da with the His-tag). We hypothesized that such a mini-FH would be able to regulate surface C3b as does full-length FH (Fig. 1B). The protein was expressed in insect cells and purified by nickel-affinity chromatography; the average protein yield was ∼8.2 mg/l cell supernatant (Fig. 1C).

Interaction of mini-FH with C3b and C3d and analysis of its functional activity in regulation of C3b deposition

First, we tested the ability of the mini-FH construct to bind to C3b, the main FH ligand, which binds to both domains 1–4 and 19–20 (37, 38). Equimolar amounts of FH and mini-FH were immobilized in microtiter plate wells, and the binding of increasing concentrations of C3b was measured. C3b bound to FH and mini-FH in a similar dose-dependent manner (Fig. 2A). These results further support FH domains 1–4 and 19–20 as the main binding sites for fluid-phase C3b, although additional sites were reported (37, 38). We also tested the binding of C3d, which is bound by the domains 19–20 (37). C3d bound more strongly to mini-FH than to FH (∼4-fold) (Fig. 2B), which may indicate a better availability of the C-terminal C3d binding site in the compact mini-FH compared with FH.

To assess binding of mini-FH to surface-bound C3 fragments and to avoid the need for a detection Ab, a label-free surface plasmon resonance detection system was used, with C3b and C3d immobilized on the biosensor chip (Fig. 3). The binding of mini-FH to C3b showed a different interaction mode compared with FH. Mini-FH showed an overall 3-fold stronger binding, when the resonance unit (RU) at the same time point and the same molar concentration was compared with that of FH (Fig. 3A, Table I).

Nevertheless, mini-FH had ∼2.5-fold slower association rate but formed 1.3-fold more stable complexes compared with FH.
resulting in 2-fold weaker affinity. Mini-FH bound much more strongly to C3d compared with FH. At identical molar concentrations (25 nM), the residual amount of mini-FH in the C3d-coated chip was 80-fold higher compared with FH. The association rate of mini-FH was 8.5-fold slower than that of FH, but this was compensated for by a 22-fold slower dissociation, resulting in a 2.6-fold higher affinity (Fig. 3B, Table I).

We then tested the capacity of mini-FH to accelerate the decay of the alternative pathway C3 convertase (C3bBb), which was assembled on immobilized C3b. The spontaneous, the FH-mediated, and the mini-FH-mediated decay of the convertase was measured. Mini-FH displayed strong decay-accelerating activity, similar to that of FH (Fig. 4A). We also analyzed the activity of mini-FH as a cofactor for the factor I-mediated cleavage of C3b. Mini-FH displayed cofactor activity similar to that of FH (Fig. 4B). These data indicate that, in the functional assays in vitro, mini-FH is at least as efficient as the native, full-length protein.

Mini-FH binds to pentraxins

Other physiological FH ligands are the pentraxins CRP and PTX3, which may direct the complement-inhibitory activity of FH to sites of inflammation (39, 40). Both CRP and PTX3 bind to CCP20 of FH (40–43). Mini-FH bound to CRP and PTX3 in a dose-dependent manner (Fig. 5A). Its binding to PTX3 was similar to that of FH, whereas mini-FH showed weaker binding to CRP compared with FH. This might be explained by the presence of an additional CRP binding site in CCP7 of FH (39), which is missing in mini-FH. Accordingly, CRP-bound FH displayed stronger cofactor activity than did mini-FH, whereas the cofactor activities of FH and mini-FH, when bound to PTX3, were comparable (Fig. 5B).

Mini-FH binds to MDA epitopes

Recently, MDA epitopes formed during oxidative damage were also shown to bind domains 19–20 of FH (44). We used immobilized BSA modified with MDA and acetaldehyde (MAA-BSA) as a model of MDA epitope–containing surfaces. For the detection of the bound FH and mini-FH proteins, we used the mAb 90X, which recognizes CCP1 (33). We found that mini-FH bound to MAA-BSA, but its binding was clearly weaker than that of FH, as expected, because an additional MDA epitope binding site, within FH domain 7, is missing in mini-FH (Fig. 6A). Mini-FH showed dose-dependent binding, whereas FH1–4, used as a negative control, did not show binding to MAA-BSA (Fig. 6B).
Table I. Kinetic parameters of the interaction of FH and mini-FH with C3b and C3d

<table>
<thead>
<tr>
<th>Proteins</th>
<th>$k_1 \times 10^5$ (1/Ms)</th>
<th>$k_2 \times 10^{-2}$ (1/s)</th>
<th>$K_D \times 10^{-7}$ (M)</th>
<th>RU at 400 s at 25 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3b</td>
<td>9.58 ± 0.76</td>
<td>4.21 ± 0.85</td>
<td>4.41 ± 0.9</td>
<td>38.66 ± 2.89</td>
</tr>
<tr>
<td>FH</td>
<td>3.62 ± 0.11</td>
<td>3.21 ± 0.53</td>
<td>8.88 ± 1.45</td>
<td>122.37 ± 23</td>
</tr>
<tr>
<td>t test (n = 3)</td>
<td>p = 0.006</td>
<td>p = 0.76</td>
<td>p = 0.05</td>
<td>p = 0.003</td>
</tr>
<tr>
<td>C3b</td>
<td>5.01 ± 0.31</td>
<td>32.4 ± 4.81</td>
<td>6.45 ± 0.57</td>
<td>5.35 ± 1.95</td>
</tr>
<tr>
<td>Mini-FH</td>
<td>0.59 ± 0.3</td>
<td>1.45 ± 0.3</td>
<td>2.46 ± 0.06</td>
<td>452.06 ± 75</td>
</tr>
<tr>
<td>t test (n = 3)</td>
<td>p = 0.004</td>
<td>p = 0.004</td>
<td>p = 0.01</td>
<td>p = 0.001</td>
</tr>
</tbody>
</table>

Association and dissociation rates were determined by surface plasmon resonance analysis. The apparent $K_D$ was calculated from the rate constants.

Mini-FH is functionally active when bound to extracellular matrix or endothelial cells

Self/nonself discrimination by FH is important to protect host cells and noncellular surfaces, such as the kidney glomerular basement membrane, from complement attack. Therefore, the binding of mini-FH to extracellular matrix (ECM) and its complement regulatory activity on such surfaces were analyzed and compared with those of FH. We used the human basement membrane extract MaxGel for these studies (43). Mini-FH showed similar dose-dependent binding to MaxGel as did FH (Fig. 7A). FH and mini-FH did not show specific binding to gelatin, which was used as a negative control. ECM-bound mini-FH displayed a cofactor activity similar to that of FH (Fig. 7B).

Mini-FH also bound to HUVECs, which were used as a model of host endothelial cells (18), although, according to the flow cytometry analysis, the binding efficiency seemed lower than that of FH, because of the fewer epitopes in mini-FH recognized by the detection Ab (Fig. 8A, 8B). However, the cell-bound mini-FH displayed cofactor activity comparable to that of FH (Fig. 8C).

Mini-FH was more efficient in the protection of endothelial cells from complement attack compared with FH, when added to FH-depleted serum. Mini-FH was 2.4-fold more efficient in reaching 50% inhibition of C3 deposition (~0.22 μM for FH versus 0.09 μM for mini-FH) (Fig. 8D).

Mini-FH reverses the anomalous host cell lysis caused by FH mutations and autoantibodies

The relevance of the FH C terminus in the protection of host surfaces by FH is indicated by disease-associated mutations and autoantibodies affecting this part of the molecule (10, 11). In vitro, C-terminal fragments of FH, such as FH19–20 and FH15–20, compete with full-length FH for binding to host cells while failing to provide complement regulation due to the lack of domains 1–4. Therefore, these fragments cause impaired host cell protection by

FIGURE 4. Decay-accelerating and cofactor activity of mini-FH. (A) Decay acceleration assay. The alternative pathway C3 convertase (C3bBb) was assembled on microtiter plates. FH, mini-FH, or human serum albumin (HSA) as a control was added, and the remaining intact convertase was detected after 30 min using anti-factor B Ab. Data are mean ± SD from three experiments. The convertase decay-accelerating effects of mini-FH and FH were not statistically significantly different from each other (two-way ANOVA). (B) The cofactor activity of mini-FH in the fluid phase was measured by incubating C3b with factor I and 10 nM FH or mini-FH at 37°C for 1 h. C3b proteolysis was visualized after performing 11% SDS-PAGE and Western blotting using polyclonal anti-C3 Ab. The C3b cleavage products are indicated on the right. The blot is representative of three experiments.

FIGURE 5. Mini-FH is functionally active when bound to pentraxins. (A) PTX3 and CRP were immobilized in microtiter plate wells. Binding of mini-FH and FH was detected using an FH antiserum. Data represent mean ± SD from three experiments. FH bound more strongly to CRP than to mini-FH (p < 0.001, two-way ANOVA), whereas the binding of FH and mini-FH to PTX3 was similar. (B) The cofactor activity of PTX3- and CRP-bound mini-FH and FH, added at 1 μM, was measured after incubation with C3b and factor I. Supernatants were analyzed by 11% SDS-PAGE, followed by Western blotting using an anti-C3 Ab to detect C3b degradation fragments. A representative blot of two experiments is shown.
FIGURE 6. Mini-FH binds to MDA epitopes. (A) Binding of FH, mini-FH, and FH1–4 (0.13 μM) to MAA-BSA immobilized in microtiter plate wells. The binding was detected with the mAb 90X. Data represent mean ± SD of five values. The binding of FH to MAA-BSA was significantly stronger than that of mini-FH. p < 0.001, one-way ANOVA. (B) Dose-dependent binding of mini-FH to MAA-BSA. FH1–4 was used as a control. Binding was measured as in (A).

occupying cellular FH binding sites. When added to human plasma, FH19–20 or FH15–20 cause anomalous lysis of SRBCs (17, 35), which are used as an in vitro model of host-like cells.

We used hemolytic assays to assess the protective activity of mini-FH in a host cellular model using C2-depleted serum. Mini-FH bound to SRBCs (Fig. 9A); however, in contrast to FH15–20, it did not cause cell lysis up to the tested 2-μM concentration (∼90 µg/ml), because of the presence of the complement regulatory domains in this construct (Fig. 9B). As a control, we also used CFH1, which does not effectively bind to the cells and, therefore, does not inhibit cellular binding of FH (Fig. 9A, 9B) (35).

The mAb C18 binds to CCP20 of FH and inhibits FH binding to host cells (18, 45), resulting in improper protection of the cells from complement-mediated lysis (35, 36). Because the aHUS-associated anti-FH autoantibodies share a binding site on FH with this mAb (25, 46), it can be used to mimic the action of autoantibodies (35). We tested the capacity of mini-FH and FH to inhibit the SRBC lysis induced by mAb C18. Both proteins showed a dose-dependent lysis-inhibitory effect, but mini-FH was more effective (∼4-fold) than was FH (Fig. 9C).

Similarly, anti-FH autoantibody–positive plasma of aHUS patients causes hemolysis of SRBCs, because the autoantibodies block FH binding to the cells (25, 36). Addition of purified FH reverses this anomalous lysis very efficiently (Fig. 9D) (25, 35). Likewise, mini-FH inhibited SRBC lysis caused by anti-FH autoantibodies in patient-derived plasma (Fig. 9D). In addition, we tested whether mini-FH can prevent hemolysis caused by a mutation (W1183L) in the C-terminal recognition domain of FH (23). Again, mini-FH showed a dose-dependent inhibitory effect, stronger than that of FH (Fig. 9E). Furthermore, the anomalous SRBC lysis caused by the mAb OX24, which inhibits the N-terminal complement regulatory domains, could be reversed by the addition of mini-FH (Fig. 9F).

Recently, an overall neutralization of FH by autoantibodies in the acute phase of autoimmune aHUS was described (47). Plasma samples containing autoantibodies against multiple FH epitopes were collected at the very onset from three patients diagnosed with the autoimmune form of aHUS. The autoantibodies in these samples bound to both the N- and C-terminus of FH, as well as to the central part of FH in patient 2. In the hemolysis assays, performed as described (47), mini-FH strongly inhibited the SRBC lysis induced by the autoantibodies (Fig. 10). In this assay, mini-FH was also more effective in inhibiting hemolysis than was FH.

**Discussion**

FH is a major complement regulator whose deficiency or altered function due to mutations or autoantibodies is associated with several diseases. In some of these diseases, such as aHUS, the therapy includes plasma infusion or plasma exchange that provides functional FH (48). Purified FH could be used to replace the dysfunctional protein in the acute phase of such diseases.

FH is a large molecule with multiple cysteine bridges; therefore, its recombinant production is difficult and expensive, although production of recombinant full-length FH was recently reported (49, 50). In this study, we describe mini-FH, a compact form of FH limited to the two main functional regions, as a potent complement inhibitor. Such a construct has several advantages over human plasma-derived, purified FH. It can be expressed in large quantities with standardized quality and concentration. There are no batch-to-batch differences in protein sequence as there would be in plasma-purified FH because of common and rare polymorphisms and occasional mutations. When using recombinant material, there is also no risk for transmitting infectious agents, such as prions and viruses. Moreover, the availability of human material does not limit production. A disadvantage could be the lack of natural posttranslational modifications of FH, about which the current knowledge is unsatisfactory. However, posttranslational modifications, such as glycosylation of FH, were not shown to be functionally important, and domains 1–4 and 19–20 present in mini-FH are not glycosylated in native FH (51). Regarding

FIGURE 7. Mini-FH binds to ECM. (A) Microtiter plates were coated with the human basement membrane extract MaxGel or gelatin as control. Binding of mini-FH and FH was detected using an FH antiserum. Data represent mean ± SD from three experiments. The binding of FH and mini-FH to MAA-BSA was significantly different from each other (two-way ANOVA). (B) The cofactor activity of MaxGel-bound mini-FH and FH, added at 1 μM, was measured as described in Fig. 5B. A representative blot of two experiments is shown.
potential immunogenicity, the lack of artificial linker sequences minimizes the risk for autoimmune reaction; however, this needs to be tested further in vivo.

In this study, we characterized mini-FH for binding to the FH ligands C3b, C3d, CRP, PTX3, and MDA epitopes; for decay acceleration of solid-phase C3 convertase and for cofactor activity in the fluid phase and when bound to various ligands; and for binding and functional activity on host cellular surfaces (ECM, endothelial cells, and SRBCs).

Mini-FH bound to the tested FH ligands, including the main ligand C3b (Figs. 2, 3), as well as C3d, pentraxins, and MAA-BSA (Figs. 5, 6). Mini-FH is able to regulate C3b by retaining the complement regulatory functions of FH (Fig. 4). Interestingly, mini-FH bound more strongly to C3d compared with FH. This may be explained by the better availability of the C3d binding site in the compact mini-FH molecule compared with FH. The differences in the binding mode of FH and mini-FH to C3b on the sensor chip could reflect the physiological situation on the cell surface, where FH can bind to two adjacent C3b molecules (one with CCPs 1–4 and another with CCPs 19–20) because of the presence of a long stretch of 14 domains (CCPs 5–18) between the two binding sites. Such binding was suggested to be more physiologically relevant compared with the one when FH wraps around the same C3b molecule (51). In contrast, mini-FH can bind only to one C3b molecule, and this can assure better anchoring (by domains 19–20) of the regulatory domains CCPs 1–4 to the same C3b molecule. This and/or the stronger residual binding of mini-FH to C3b compared with FH could be a reason why mini-FH proved to be a more efficient inhibitor of C3 deposition on HUVECs and complement-mediated SRBC lysis in comparison with full-length FH (Figs. 8–10). Although mini-FH has only a short linker sequence between domains 4 and 19, it worked rather well in the ligand binding and functional assays. However, further optimization of the linker may improve the structural flexibility and, thus, the activity of the protein.

The pentraxins PTX3 and CRP, which activate complement, on one hand, were shown, on the other hand, to bind the efficient downregulator of complement activation FH and were suggested to target FH to the sites of inflammation (39, 40). Both pentraxins were shown to interact with FH via domains 7 and 20 (39–43). In addition, disease-associated FH variants were shown to have impaired binding to CRP or PTX3 (41, 43, 52, 53). For PTX3, the main binding site in FH was localized to CCP20 (43); mini-FH, although lacking the CCP7 domain, showed comparable PTX3 binding and complement regulatory activity when bound to PTX3 as the full-length FH (Fig. 5). The AMD-associated FH polymorphic variant 402H binds less efficiently to CRP than does the 402Y variant, suggesting an impaired function in the clearance of cellular debris and regulation of inflammation (52–54). Mini-FH could compensate for the impaired binding of FH to surface-bound CRP caused by pathogenic mutations in FH domain 20 (41). Because our results showed weaker binding and activity of mini-FH on CRP compared with that of FH, mini-FH might not fully compensate for the impaired FH function caused by mutations/polymermorphisms in domain 7 (Fig. 5).

Recently, MDA epitopes were shown to bind FH through two sites: one in domain 7 and another in domains 19–20 (44). The importance of MDA binding of FH in vivo is not clear, but it was suggested to be involved in the pathogenesis of AMD causing blindness, because the AMD-associated 402H variant of FH showed reduced binding to MDA, resulting in reduced local protection from oxidative stress (44). Because mini-FH contains only one MDA epitope binding site, compared with the two sites in full FH, it was expected to show weaker binding than FH, as observed. Thus, compared with full-length FH, mini-FH likely confers reduced protection from complement activation to surfaces containing MDA epitopes.

FH also interacts with ECM and protects from excessive complement activation on this surface (43). Mini-FH showed cofactor activity comparable to that of FH when bound to the model ECM (Fig. 7). This activity is likely relevant in renal diseases in which FH binding to the glomerular basement membrane is primarily mediated by CCPs 19–20, and impaired FH binding was suggested to play a role in disease pathogenesis (43, 55).

An important function of FH is its participation in the protection of host cells from complement attack. Our in vitro assays demonstrate that recombinant mini-FH rescues host cells from complement-mediated damage caused by FH mutations, FH deficiency, or autoantibodies associated with aHUS. Mini-FH also compensates for FH dysfunction in the N-terminal complement regulatory domains, demonstrated by assays using the mAb OX24, which mimics autoantibodies associated with DDD (56–58). Autoantibodies against the N-terminal domains of FH were also described recently in the acute phase of aHUS (47), and mini-FH
very efficiently protected SRBCs from hemolysis caused by such Abs (Fig. 10). Therefore, mini-FH could potentially be applied as a complement inhibitor in diseases in which dysregulation of the complement alternative pathway is particularly implicated, such as aHUS and C3 glomerulopathies (59, 60). The applicability may not be limited to FH-related disorders, because the addition of

FIGURE 9. Mini-FH reverses the anomalous hemolysis caused by aHUS-associated FH mutations or autoantibodies. (A) Mini-FH binds to SRBCs. SRBCs were incubated with 15% C2-depleted human serum (C2depl), with or without 2 μM mini-FH and FH-related protein 1 (CFHR1). SRBCs were washed and lysed, and the lysates were subjected to 12% SDS-PAGE and Western blotting using an FH antiserum. A representative blot of three is shown. (B) Mini-FH does not induce SRBC hemolysis in human serum. SRBCs were incubated, as described in (A), with 2 μM mini-FH, CFHR1, or the FH fragment consisting of CCPs 15–20 (FH15–20). After 30 min, hemoglobin in the supernatants was measured. In a control sample, water was added to achieve total SRBC lysis (white bar). Compared with the negative control (C2-depleted serum only), CFHR1 and mini-FH did not increase SRBC lysis, whereas FH15–20 significantly increased cell lysis (p < 0.01, one-way ANOVA). (C) SRBC lysis was induced in 15% C2-depleted serum by the mAb C18, which blocks FH binding to the cells. Mini-FH and FH were added in the indicated concentrations, and the extent of hemolysis was determined as described in (B). The difference between the inhibitory activity of FH and mini-FH was statistically significant (p < 0.001, two-way ANOVA). SRBCs were lysed by 10% serum from an aHUS patient with anti-FH autoantibodies (D) or from an aHUS patient carrying the W1183L mutation in CCP20 in heterozygosis (E). (F) SRBC lysis was induced in 10% human serum by the mAb OX24, which blocks the complement regulatory activity of FH. The effect of mini-FH and FH on the hemolysis was determined as described in (B). The difference between the inhibitory activity of FH and mini-FH was statistically significant (p = 0.0063, two-way ANOVA). In (B), (C), and (F), data represent mean ± SD from three experiments. In (D) and (E), representative data from two experiments are shown.

FIGURE 10. Mini-FH reverses the anomalous hemolysis caused by autoantibodies directed against multiple FH epitopes. Plasma samples from three patients diagnosed with autoimmune aHUS were collected at the very onset. The autoantibodies in these samples bound to the N- and C-terminal parts of FH, as well as to the central part (domains 8–11) in patient 2. Nonsensitized SRBCs were incubated with the patients’ plasma diluted 6, 10, and 15%. Increasing amounts of mini-FH or FH were added before performing the assay, as described (47). Data are representative of two experiments.
purified FH was able to control the increased complement activation on the endothelial cell surface in the presence of sera of aHUS patients with a C3 mutation (24). Nevertheless, attention should be paid because certain mutations in factor B or C3 are resistant to regulation by FH, and C3 nephritic factors may also render the C3 convertase resistant to regulation (61–65). Mini-FH could likely also be used to protect erythrocytes from complement-mediated damage in patients with paroxysmal nocturnal hemoglobinuria (PNH) (66). FH plays a role in the protection of PNH erythrocytes, which are characterized by strongly reduced numbers of the membrane-anchored complement regulatory proteins CD55 and CD59 due to a defect in the glycosylphosphatidylinositol anchor (67). A recent report showed that targeting CCPs 1–5 of FH to sites of complement activation at the surface of PNH erythrocytes by using a chimeric molecule confers protection of these erythrocytes from C3 fragment deposition and lysis (68).

Because the C-terminal domains are designed by nature to direct FH to sites of ongoing complement activation, mini-FH is expected to downregulate complement activation under various pathological conditions. Our data demonstrate that mini-FH retains the complement regulatory activity of FH, and it is likely more active on a molar basis. The therapeutic use of mini-FH requires further studies, but the results shown suggest its potential use in the treatment of kidney diseases, such as aHUS and C3 glomerulopathies.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1

gaagactgtaacgacagccgagacgcaacagcgaatctggaccggctcatggagc
E  D  C  N  E  L  P  R  R  N  T  E  I  L  T  G  S  W  S  20
gaccaacatacaccagaggaaaccaggcgcattacagcgggcaccctacagcagc
D  Q  T  Y  P  A  I  Y  K  C  R  P  G  Y  R  S  40
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L  G  N  V  I  M  V  C  R  K  G  E  W  V  A  L  N  P  L  R  60
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tgggacagggctgaattaccgccagcttgcagaagagg
W  D  G  K  L  E  Y  P  T  C  A  K  R  373

Figure S1. Nucleotide and amino acid sequence of codon-optimized mini-FH.
The 1119 nucleotide long sequence of FH exons coding for domains 1-4 and domains 19-20 were codon-optimized for expression in insect cells. The translated protein sequence is shown below the nucleotide sequence. The linker between CCP4 and CCP19 is shown in bold, the boundary is indicated by a double vertical line.