The MFHR1 Fusion Protein Is a Novel Synthetic Multitarget Complement Inhibitor with Therapeutic Potential

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ABSTRACT

The complement system is essential for host defense, but uncontrolled complement system activation leads to severe, mostly renal pathologies, such as atypical hemolytic uremic syndrome or C3 glomerulopathy. Here, we investigated a novel combinational approach to modulate complement activation by targeting C3 and the terminal pathway simultaneously. The synthetic fusion protein MFHR1 links the regulatory domains of complement factor H (FH) with the C5 convertase/C5b-9 inhibitory fragment of the FH-related protein 1. In vitro, MFHR1 showed cofactor and decay acceleration activity and inhibited C5 convertase activation and C5b-9 assembly, which prevented C3b deposition and reduced C3a/C5a and C5b-9 generation. Furthermore, this fusion protein showed the ability to escape deregulation by FH-related proteins and form multimeric complexes with increased inhibitory activity. In addition to substantially inhibiting alternative and classic pathway activation, MFHR1 blocked hemolysis mediated by serum from a patient with aHUS expressing truncated FH. In FH2/2 mice, MFHR1 administration augmented serum C3 levels, reduced abnormal glomerular C3 deposition, and ameliorated C3 glomerulopathy. Taking the unique design of MFHR1 into account, we suggest that the combination of proximal and terminal cascade inhibition together with the ability to form multimeric complexes explain the strong inhibitory capacity of MFHR1, which offers a novel basis for complement therapeutics.

Complement activity is strictly controlled by a panel of soluble and membrane-bound regulators that intervene at several levels of the cascade. With a serum concentration of 500 µg/ml, plasma factor...
H (FH) is the major regulator of the alternative pathway (AP). FH restricts “tick-over” C3 activation and the C3 amplification loop in the fluid phase as well as on host surfaces by acting as cofactor to promote factor I (FI)–mediated proteolysis of C3b into iC3b and preventing the formation or accelerating the decay of C3 convertases. FHR-1 as well as FHR-2, -3, -4, and -5 (summarized as FHR family) are closely related to FH, and complement regulatory as well as host/opsonin recognition features are attributed to most of them. FH regulates terminal pathway activation by preventing C5 cleavage through the C5 convertase and thereby, inhibiting C5a release, membrane attack complex (MAC) formation, and subsequent cytolysis. In addition and independent of C5 convertase regulation, FHR-1 inhibits assembly of C5b6(7) complexes and MAC formation. FHR-1, FHR-2, and FHR-5 contain a conserved dimerization interface that enables the formation of homo- and heterodimers (e.g., FHR-1:FHR-1; FHR-2:FHR-1, respectively), and these complexes can compete with FH for C3b or host cell surface binding and thereby, reduce FH surface levels, leading to local complement activation. The plasma concentration of FHR-1 is 70–100 μg/ml. However, given the regulatory as well as deregulatory properties of FHRs, it has been postulated that FHRs have evolved to act in concert with FH, but their exact role in complement regulation as well as disease has not yet been precisely elucidated.

Mutations in activators or inhibitors of the complement cascade (e.g., FH, FI, factor B, C3, CD46) or autoantibodies (e.g., anti-FH) cause uncontrolled complement activation and MAC formation on host surfaces (e.g., on endothelial cells), leading to the development of atypical hemolytic uremic syndrome (aHUS). In C3 glomerulopathies (C3Gs), fluid-phase activation is deregulated by mutations in complement regulators, FH/CFHR genomic rearrangements, or autoantibodies (e.g., C3NeF), resulting in glomerular accumulation or deposition of C3 cleavage fragments, which seem to be the major pathogenic factors. In addition to MAC-induced damage, opsonin deposits (e.g., C3b) and anaphylatoxins (C3a and C5a) promote disease progression by triggering inflammation, proliferation, or infiltration of immune cells or podocyte damage. The same factors are also thought to play a critical role in the pathomechanism of IgA nephropathy, membranous nephropathy, diabetic kidney disease, ANCA vasculitis, graft rejection, and ischemia-reperfusion injury.

Targeting the complement system pharmacologically is highly desired, and terminal pathway inhibition (i.e., C5 by the humanized antibody eculizumab) has already been successfully transferred into clinical practice and showed remarkable improved patient survival and quality of life.

However, despite the clinical success of eculizumab, incomplete responses by solely blocking C5 (serum concentration 55–113 μg/ml) have been reported for aHUS (e.g., when residual C5 activity derogates the drug’s therapeutic benefit). In C3G, eculizumab led to a partial response only in some patients, which might be explained by preserved C3 convertase activity. In addition, incomplete responses or even therapeutic failures have been described to occur in other complement-driven pathologies (e.g., paroxysmal nocturnal hemoglobinuria).

Newer therapeutic strategies are focusing on the intervention of C3 activation by using small peptides (e.g., Compstatin and Amyndas), antibodies (e.g., targeting factor D, factor B, or C3), or naturally occurring physiologic regulators or their functional domains for designed therapeutic inhibitors. Especially in FH deficiencies, replacement of FH has been proposed as a viable treatment option to replace dysfunctional protein and restore AP regulation, and the latest technological advancements now allow the recombinant production of glycosylation-optimized FH with full bioactivity. Also, minimized factor H (mini-FH) variants, soluble CR1, or combinational approaches, like TT30, inhibit C3 activation.

Regarding the complexity of the complement system, which if not properly regulated, leads to accumulation of various potential pathogenic intermediates, a combinational or “multitarget” strategy to modulate complement activation could potentially improve therapeutic efficiency while diminishing unwanted side effects, and it might also be more efficient in controlling complex disease systems and less prone to drug resistance.

We describe here a novel fusion protein MFHR1, which combines the C3 regulatory and cell surface/opsonin targeting domains of FH with the terminal pathway regulatory domains derived from FHR-1 displaying strong complement inhibitory activity in vitro. MFHR1 modulates complement by acting on C3 activation and amplification as well as the C5/C5b-9 level. Our data show that targeting complement activation on multiple levels simultaneously offers a basis for an optimized complement inhibition, which was also shown by the ability of MFHR1 to prevent AP deregulation in clinically relevant models of aHUS and C3G.

**RESULTS**

**Structure, Expression, and Characterization of MFHR1**

MFHR1 was designed to combine the terminal pathway regulatory and dimerization properties of FHR-1 with the C3 regulatory and surface binding properties of FH. We directly linked the two N-terminal C5/C5b6 binding domains FHR-1 to the regulatory (FH1-4) and surface recognition domains (FH19-20) of FH (Figure 1A). MFHR1 was expressed in insect cells and purified from the supernatant, yielding approximately 5 mg protein per liter culture. The purity of MFHR1 was confirmed by Coomassie and silver staining (Figure 1B). MFHR1 migrated at its predicted molecular mass of 59 kD. The presence of FHR-1-2, FH1-4, and FH20 was confirmed by immune detection using specific antibodies and antisera (Figure 1C).
MFHR1 Inhibits AP and CP Activation

Global complement inhibitory potency of MFHR1 was analyzed by complement ELISA measuring C5b-9 formation after LPS-induced AP activation in human serum (HS) with the addition of MFHR1 or controls. MFHR1 inhibited C5b-9 formation with an IC50 of 3.2 nM and exceeded the inhibitory activities of eculizumab (5.5-fold; IC50=17.5 nM), human factor H (hFH; 52-fold; IC50=167 nM), and mini-FH (3.9-fold; IC50=12.4 nM), whereas FHR-1 showed only AP inhibition at very high concentrations (3.16 mM) (Figure 2A, Table 1). In addition, MFHR1 inhibited classical pathway (CP) activation after IgM-induced CP activation in HS and C5b-9 formation at an IC50=15.9 nM (Figure 2B, Table 1).

MFHR1 Inhibits Complement Activation on the C3 Axis

To investigate whether MFHR1 maintained the functional properties of FH, we first measured the ability of MFHR1 to bind to C3b, the key opsonin of the AP, using an ELISA-based binding assay. MFHR1 specifically and in a dose-dependent manner bound C3b at levels comparable with those of hFH (Supplemental Figure 1A). In addition, MFHR1 bound to human umbilical vein endothelial cells, which were used as a model of host cells37 (Supplemental Figure 1B). As analyzed in a fluid-phase cofactor assay after SDS-PAGE (Supplemental Figure 2) and quantitative analysis of α′-chain cleavage by densitometry (Figure 3A), MFHR1, hFH, and FH1-4 mediated a dose-dependent cleavage of α′-chain fragments. hFH with an IC50 of 4 nM showed an 8.75-fold higher cofactor activity compared with MFHR1 (IC50=35 nM) that was in a similar range to FH1-4 (IC50=26 nM), whereas FHR-1 showed no activity (Figure 3A, Table 2). In addition, MFHR1 and hFH showed cofactor activity when bound to human umbilical vein endothelial cells (Supplemental Figure 2B). We then compared the decay acceleration activity of MFHR1 and hFH by their ability to displace factor B from preformed C3 convertases (C3bBb), where MFHR1 showed nearly identical activity to hFH (Figure 3B). After AP activation, C3b depositions initiate and further amplify the AP and the generation of proinflammatory anaphylatoxins (e.g., C3a).

Therefore, we analyzed the ability of MFHR1 to block surface deposition of the opsonin C3b after LPS activation in HS in the AP ELISA. MFHR1 strongly inhibited C3b deposits with an IC50 of 12.4 nM that exceeded the inhibitory activity of hFH (IC50=513 nM) and mini-FH (IC50=40.4 nM) (Figure 3C, Table 1). The IC50 values of hFH and mini-FH were comparable with observations reported in a previous study.29 As expected, eculizumab and FHR-1 had no inhibitory activity, because these proteins are known to regulate complement activity only in later stages of the cascade (Figure 3C). Because anaphylatoxins have proinflammatory effects and play an...
important role in disease progression, we investigated the inhibitory potency of MFHR1 on C3a generation in activated sera. Here again, MFHR1 and mini-FH exhibited the highest efficiency for preventing C3a generation followed by hFH (Figure 3D). Eculizumab did not result in any reduction of C3a levels, and FHR-1 at 0.316 and 1 μM increased C3a levels, presumably as a consequence of FHR-1–mediated deregulation in this serum-based assay.

MFHR1 Binds C5 and Regulates AP Activation at the Level of C5 and C5b-9 Formation

To test whether MFHR1 has the ability to interact with C5, microtiter plates were coated with MFHR1, FHR-1, or hFH, respectively (Supplemental Figure 3). After incubation of the wells with purified C5, anti-C5 antibody strongly and dose-dependently detected C5 bound to FHR-1 and MFHR1, but only a weak C5 signal was detected in hFH- or BSA-coated wells (Figure 4A). In a next step, inhibitory effects of MFHR1 on the C5 convertase were investigated. MFHR1 inhibited C5a formation in HS after activation of the AP slightly stronger than mini-FH and eculizumab and considerably stronger than hFH (Figure 4B). Again and consistent with our results from the C3a ELISA, FHR-1 increased C5a generation, probably as a consequence of FHR-1–mediated deregulation in this serum–based assay. To investigate the influence of MFHR1 on C5 cleavage in a serum-free approach, an experimental cobra venom factor (CVF) convertase (CVFBB) was generated and added to C5 preincubated with or without test proteins, sheep erythrocytes (sEs), and C6–C9, and the lytic activity of the samples was measured. Eculizumab38 as well as FHR-14 are known to prevent convertase–mediated C5 cleavage by binding to C5. MFHR1 significantly inhibited CVF-induced lysis at comparable levels to eculizumab and FHR-1, whereas hFH and BSA had no significant effect (Figure 4C). We then assayed whether MFHR1, like FHR-1, has the ability to inhibit the C5 convertase–independent step of C5b-9 formation. We incubated C5b6 with test proteins before addition to sE and C7–C9. C5b-9 formation was measured by the rate of lysis (Figure 4D). MFHR1 and FHR-1 significantly reduced C5b-9 formation, although FHR-1 tended to be slightly less effective, whereas neither eculizumab nor hFH and BSA inhibited C5b-9 at significant levels compared with controls without regulators added (Figure 4D). These data show that MFHR1 is a complement inhibitor combining C3 and terminal pathway regulatory properties acting on the C3/C5 and C5b-9 axes simultaneously.

Multimer Formation Potentiates AP Inhibitory Activity of MFHR1

In the preparative size exclusion chromatography, the elution profile of MFHR1 from the purified protein stocks migrated as a broad peak, whereas hFH purified from human plasma was found to migrate in a single and narrow peak fraction at approximately 300 kD (Figure 5A, middle panel). Collected fractions of MFHR1 were analyzed via SDS-PAGE and silver stained (Figure 5B). The six fractions contained decreasing concentrations of MFHR1 were analyzed approximately 300 kD (Figure 5A, middle panel). Collected fractions of MFHR1 were analyzed via SDS-PAGE and silver stained (Figure 5B). The six fractions contained decreasing concentrations approximately 300 kD (Figure 5A, middle panel). Collected fractions of MFHR1 were analyzed via SDS-PAGE and silver stained. The six fractions contained decreasing concentrations of MFHR1 were analyzed via SDS-PAGE and silver stained. The six fractions contained decreasing concentrations approximately 300 kD (Figure 5A, middle panel). Collected fractions of MFHR1 were analyzed via SDS-PAGE and silver stained. The six fractions contained decreasing concentrations of MFHR1 were analyzed via SDS-PAGE and silver stained.
in fractions II–VI (Figure 5C). These data suggest that MFHR1 migrates predominantly in a multimeric state in the fluid phase. Preparations containing multimeric MFHR1 complexes have higher inhibitory activity than monomeric MFHR1 fractions.

**MFHR1 Is Resistant to Competition by FHR-1 and FHR-5**

FHR-1 and FHR-5 act as competitive agonists for FH binding to C3b and thereby, act as modulators of complement activation (i.e., antagonists of FH-directed complement regulation). We speculated that dimeric or multimeric MFHR1 preparations might influence its susceptibility to FHR competition. Therefore, we performed C3b binding ELISAs, where equimolar amounts of MFHR1 or hFH were incubated with increasing amounts of FHR-1 or FHR-5 on C3b-coated microtiter plates. MFHR1 binding to C3b was completely resistant to competition by FHR-1 or FHR-5 (up to 100-fold molar excess), whereas hFH binding to C3b was affected by an already 3.1-fold molar excess of either FHR-1 or FHR-5 (Figure 6A). To determine whether MFHR1-mediated inhibition of complement activation on cell surfaces is also resistant to competition by FHRs, we incubated sE with FH-depleted HS, leading to subsequent AP complement activation and hemolysis. MFHR1 or hFH were added together with increasing amounts of FHR-5. Here again, MFHR1 was completely resistant to FHR-5–mediated competition up to 100-fold molar excess, whereas hFH-mediated protection of sE was inhibited after the addition of tenfold molar excess of FHR-5 (Figure 6B).

**MFHR1 Controls Complement Activation In Vitro and Shows Therapeutic Benefit in C3G In Vivo**

We then explored the capacity of MFHR1 to suppress complement activation under pathophysiological conditions. Serum derived from a patient with aHUS carrying a C-terminal FH deletion caused complement overactivation and lysis of sE.

The addition of MFHR1 protected sE from complement-mediated lysis more effectively than eculizumab (Figure 7A). We also investigated the efficacy of MFHR1 in an animal model of C3G, namely FH knockout mice. FH−/− mice display abnormal glomerular C3 accumulation and low serum C3/C5 levels. Administration of a single dose of MFHR1 increased serum C3 levels at all analyzed time points, reaching a peak of approximately 26% of wild-type levels after 12 hours, whereas hFH increased serum C3 to comparable levels to MFHR1 after 12 hours but led to a further increase, reaching approximately 53% of wild-type levels after 24 hours (Figure 7B). Serum C5 was detectable 24 hours after injection of MFHR1 or hFH, whereas it was not present in PBS-injected mice (Figure 7C). In addition, glomerular C3 staining was significantly reduced at a comparable degree in mice injected with MFHR1 or hFH, although no changes in hematoxylin- and eosin–stained samples were detected (Figure 7, D and E). Injected proteins MFHR1 and hFH were detected in the glomeruli of treated mice (Figure 7E, Supplemental Figure 4). These data show that MFHR1 has the ability to reverse an inherent complement defect in vivo or in vitro.

**DISCUSSION**

Hereditary or acquired complement defects may lead to constitutive overactivation of the complement system downstream of the defective effector protein. Therapeutic complement inhibition focusing on C5 inhibition as a single target has improved disease progression and survival, especially in patients with aHUS and patients with paroxysmal nocturnal hemoglobinuria, but it only provides partial complement control and misses full efficiency in C3G and other diseases associated with complement dysregulation.

Because the complement cascade produces active effector proteins on several levels, a multitargeted strategy could be beneficial for better control of a complex disease—causing imbalance between complement activation and regulation.

We, therefore, combined relevant complement regulatory domains of FHR-1 and FH to design a complement inhibitor that operates on different levels of the cascade (Supplemental Figure 5). This new fusion protein, MFHR1, strongly inhibited complement activation as shown by distinct reductions of AP- and CP-mediated C5b–9 complex formations and considerably exceeded the AP inhibitory activity of hFH (>50-fold), mini-FH (>3.5-fold), and the C5inhibitor eculizumab (greater than fivefold). It also prevented the release of the potent immune modulators C3a and C5a in HS and completely reversed complement overactivation after in vitro
supplementation to serum of a patient with aHUS, showing the effectiveness of MFHR1 in a disease model and proving the concept of our multitarget approach. Furthermore, MFHR1 partially inhibited serum AP activation and reduced pathologic C3 depositions in FH−/− mice at a comparable degree to recombinant FH, mini-FH, soluble CR1, and TT30, despite their individual mechanisms of action. In comparison with these recombinant proteins, plasma-derived hFH outperforms the in vivo efficacy, which might be explained by aspects of serum t1/2 and glycosylation. MFHR1 retained the C3 regulatory activity of FH as well as its host cell binding features. However, cofactor activity of MFHR1 was similar to FH−/− fragment but lower compared with hFH. This is in line with earlier studies and confirmed that full-length FH provides better structural rigidities than FH−/− for optimally stabilizing the conformational state of C3b to be cleaved by FI. MFHR1 bound to the effector molecule C5 inhibited experimental C5 convertase and C5b-9-mediated lysis of sE under serum-free conditions, presumably by inhibiting C5 cleavage and binding to C5b6 complexes on cell surfaces as described for FHR-1. These results confirm that the fusion of FHR-1 to FH−/− and FH19-20 is tolerated well without steric hindrances, because full C5/C5b-9 and C3 regulatory functional properties were both maintained by MFHR1.

The strong inhibitory potency of MFHR1 to inhibit serum AP-mediated C3b deposition on LPS-coated surfaces might partly be explained by more accessible C3b/C3dg binding sites in FH19-20 targeting complement regulation to sites of ongoing AP activation as was shown for mini-FH. But it is likely dependent on the formation of multimers. Like native FH-1, FHR-2, and FHR-5, MFHR1 contains a dimerization interface localized within its N-terminal FH-1-derived SCR1-2

Figure 3. MFHR1 displays cofactor and decay acceleration activity and blocks C3b surface deposition and C3a release (A) IC50 fitting curves for inhibition of α'-chain cleavage by MFHR1, FH−/−, FHR-19-20, or hFH. C3b and F1 were incubated with increasing concentrations of recombinant proteins or hFH (5-250 nM) for 30 minutes at 37°C. For quantification, intact C3b α'-chain densitometry was expressed relative to β-chain in each sample. Control without cofactor was set to 100% intact C3b α'-chain. Data represent mean values ±SD from n=5 experiments for MFHR1 and hFH or n=2 for FH−/− and FHR-19-20. The cofactor activity of hFH was significantly different from MFHR1 (two-way ANOVA; P<0.001), whereas the activities of MFHR1 and FH−/− were similar. (B) MFHR1 dissociates C3bBb. Convertase complexes were assembled on microtiter plates in the presence of C3b, factor B (FB), and factor D (FD), and MFHR1 or hFH was added and incubated at 37°C. Intact C3bBb and dissociation of these complexes were measured by the relative amount of FB. OD at 450 nm measured for control wells without regulator (C3b+FB+FD) was set to 100% FB. Negative control was performed without adding FD (C3b+FB). Data are mean±SD from n=3 experiments. The decay acceleration activities of MFHR1 and hFH were not significantly different from each other (two-way ANOVA). (C) IC50 fitting curves for inhibition of C3b deposition. MFHR1, hFH, FHR-1, or eculizumab was added to HS, and C3b depositions were measured after AP activation by LPS. HS or heat-inactivated (hi) HS added to wells not coated with LPS (−LPS) was used as control. Data points represent mean values from n=4 assays ±SEM using sera of four individual healthy donors. Serum without inhibitors was set to 100% C3b deposition for each individual experiment. The inhibitory activity of MFHR1 was significantly different from mini-FH (P<0.001; two-way ANOVA). (D) MFHR1 blocks generation of C3a as assayed in the supernatant of AP-activated sera using C3a ELISA (Quidel); hi HS was used as negative control. Data points are mean values from n=3 assays ±SEM using sera of three individual healthy donors. Untreated HS was set to 100% relative C3a. The C3a inhibitory activity of MFHR1 was not significantly different from mini-FH (two-way ANOVA).
domain, and MFHR1 forms multimers that increase the inhibitory activity of MFHR1. This is particularly remarkable, because homo-, hetero-, or multimeric FHR complexes result in increased binding on cell surfaces or to complement activation products (i.e., C3b and C3d). Alterations in local composition and levels are believed to play a physiologic role in modulating FH regulation, especially on pathogen surfaces, but they also have a pathologic role. In some subtypes of C3G, gene rearrangements leading to duplication of the dimerization domains and abnormal FHR proteins are held responsible for a deregulation of complement activation on host surfaces and disease development. The dimerization motif in MFHR1 mediates binding to FHR-1 and FHR-2 in vitro (Supplemental Figure 6); however, complement regulation does not seem to be influenced negatively by these potential MFHR1-FHR heterodimers under physiologic conditions (Figures 2, 3, B and C, and 4B). We further show that MFHR1 is resistant to competition by FHR-1 and FHR-5. Therefore, the unique design of MFHR1 is expected to resist inhibitory influences in subgroups of patients with C3G and disease.

### Table 2. Calculated IC50 values for cofactor activity

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC50 Values, nM</th>
<th>R²</th>
<th>95% Confidence Interval</th>
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<tbody>
<tr>
<td>hFH</td>
<td>4</td>
<td>0.83</td>
<td>2 to 7</td>
</tr>
<tr>
<td>MFHR1</td>
<td>35</td>
<td>0.85</td>
<td>17 to 71</td>
</tr>
<tr>
<td>FH1-4</td>
<td>26</td>
<td>0.89</td>
<td>14 to 51</td>
</tr>
<tr>
<td>FHR-1-2</td>
<td>No activity</td>
<td>—</td>
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Figure 4. MFHR1 binds C5 and regulates terminal pathway activation by inhibition of C5 cleavage and MAC formation. (A) MFHR1 binds to C5 as determined by ELISA. Equimolar amounts of MFHR1, FHR-1, hFH, or BSA were immobilized to Nunc plates and incubated with increasing concentrations of C5. Binding was detected using monoclonal C5 antibodies and HRP-labeled secondary antibodies. Data are mean±SD from n=3 experiments. **Statistical significance versus C5 bound to hFH (one-way ANOVA with Bonferroni test; P<0.001). (B) MFHR1 blocks generation of C5a as measured in the supernatant of AP-activated sera using C5a ELISA (Quidel). Heat-inactivated (hi) HS was used as negative control. Data points are mean values from n=3 assays ±SEM using sera of three individual healthy donors. Untreated HS was set to 100% relative C5a. The C5a inhibitory activity of MFHR1 was significantly different from mini-FH (P<0.10; two-way ANOVA), whereas the activities of MFHR1 and eculizumab were similar. (C) MFHR1 inhibits C5 cleavage and lysis of sE. CVFBb convertase was generated on sE after adding factor B (FB) and factor D (FD). Hemolysis was induced after adding C5-C9 and detected at 414 nm. Preincubation of C5b6 with MFHR1 or FHR-1 inhibited hemolysis, whereas hFH or BSA showed no inhibition. Control reactions (ctr) were performed without the addition of C5 (no C5b to generate lytic complexes) or without FB/FD (no CVFBb convertase is formed to cleave C5 into C5a and activated C5b). C5 activation control without regulators (w/o reg) was set to 100%. **Statistical significance versus w/o reg (P<0.001; one-way ANOVA with Bonferroni test). (D) MFHR1 inhibits formation of the MAC on sE. MAC formation on sE was induced by incubation with C5b6 and C7–C9 components and detected by hemolysis of cells. Preincubation of C5b6 with MFHR1 or FHR-1 inhibited hemolysis at significant levels compared with hFH, eculizumab, or BSA. MAC formation control without regulators (w/o reg) was set to 100%. Samples without C9 did not induce hemolysis. All data represent mean values ±SD of three independent experiments. **Statistical significance w/o reg (one-way ANOVA with Bonferroni test; P<0.001).
might also benefit these patients; this, however, needs to be proven. We hypothesize that MFHR1 uses the ability for dimerization/multimerization of FHRs, increasing local concentrations and ligand avidity to increase its regulatory performance.

However, comparing their individual IC50 ratios for the C3b inhibition relative to C5b-9 inhibition, hFH (IC50 C3b-ELISA/IC50 C5b-9-ELISA =3.07) and mini-FH (3.25), both primarily acting at the C3 level, showed comparable quotients. Because of its proportionately lower IC50 C5b-9 values than IC50 C3b values, the quotient for MFHR1 (3.88) was higher, reflecting a proportionately higher terminal pathway inhibitory activity. Therefore, MFHR1 acts on multiple therapeutically relevant targets in the complement cascade.

In summary, we show that regulators affecting multiple effector sites simultaneously in the complement cascade, such as C3 inhibition, inhibition of C5 cleavage, and prevention of C5b-9 formation, have advantages compared with solely C3- or C5-targeted approaches. Particularly, because MFHR1 prevents the formation of proinflammatory effectors C3a and C5a and the accumulation of C3 cleavage products, it is expected to also positively affect local inflammation and disease progression in other complement-associated diseases. In addition, resistance to competition and its ability for multimerization are unique features of MFHR1 and suggest a superior effect of MFHR1 not only in FHR-related C3Gs. In addition to the therapeutic potential of MFHR1 to regulate complement overactivation, this multitarget strategy could serve as a blueprint for developing new complement therapeutics.

CONCISE METHODS

Cloning, Expression, Purification, and Characterization of Recombinant Proteins

For production of the recombinant fusion protein MFHR1 (FHR-11–2xFH1–4xFH19–20), mini-FH,30 FHR-1, FHR-2, FHR-5, and truncated FHR-11–2 and FH1–4, appropriate gene fragments were cloned into a modified vector pFastBac-gp67-10His, and proteins were expressed in Spodoptera frugiperda cells following the manufacturer’s instructions (Bac-to-Bac System; Invitrogen, Life Technologies, Darmstadt, Germany). An N-terminally located decahistidine tag was included, facilitating the purification of recombinant proteins. Detailed descriptions for the cloning of pFastBac-gp67-10His and recombinant protein fragments are provided in Supplemental Material. The supernatants were adjusted with 5× binding buffer (100 mM NaH2PO4 [Merck, Darmstadt, Germany], 2.5 M NaCl [VWR, Darmstadt, Germany], and 100 mM imidazole [Roth, Steinheim, Germany], pH 7.4) and filtered using a 0.22-μm vacuum-driven bottle top filter (Stericup; Merck Millipore, Darmstadt, Germany) before purification of recombinant proteins was performed using HisTrap FF columns following the manufacturer’s instructions (GE Healthcare, Munich, Germany) on an ÄktaBasic10 using unicorn 5.0 software (GE Healthcare). Protein fractions were concentrated using a vivaspin
polyethersulfon membrane filter (Sartorius AG, Goettingen, Germany) with a 10-kD molecular mass cutoff. Buffer desalting was done by HiTrap desalting column (GE Healthcare) using NaPH buffer (50 mM NaH2PO4 and 150 mM NaCl) after volume reduction in vivaspin concentration devices. Size exclusion chromatography for qualitative distribution of MFHR1 molecular species was evaluated in comparison with the retention time of hFH (Complement Technologies) and BSA (Sigma-Aldrich, Taufkirchen, Germany) on Superdex 200 10/300 GL (GE Healthcare) columns according to the manufacturer’s instructions. Proteins were separated by SDS-PAGE and visualized by Pierce silver-staining kit (Thermo Fisher Scientific) or Coomassie R-250 (Serva, Heidelberg, Germany) staining. Immuno blotting was performed by standard methods using anti-hFH (Complement Technologies), anti-FH1–4,48 anti-FH C18,37 anti-FHR-1/2 (Hycult Biotech, Uden, The Netherlands), and anti-His antibodies (QIAGEN, Hilden, Germany). Secondary antibodies used were anti-mouse HRP (GE Healthcare), anti-goat HRP (DAKO, Eching, Germany), or anti-rabbit HRP (GE Healthcare) before detection with enhanced chemiluminescence (GE Healthcare) in the Fusion SL Chemiluminescence system (Vilber Lourmat). The protein concentration was determined by Pierce BCA Assay Kit (Thermo Fisher Scientific). Recombinant proteins were aliquoted and stored at −80°C.

**Proteins and Sera**

hFH, C3b, Fl, C5, C5b6, C7, C8, and C9 were purchased from Complement Technologies, and factor B and factor D were purchased from Merck. CVF and C6 were purchased from Quidel (San Diego, CA), and eculizumab (Soliris) was obtained from remnants of infusions. HS was obtained from healthy donors, and serum from a patient with aHUS was collected by vein punctuation, allowed to clot in serum and written informed consent was provided for sample collection and subsequent analysis. FH-deficient serum was purchased from Complement Technologies. The patient is a 15-year-old boy with aHUS that manifested in his first year. He shows a heterozygous C-terminal deletion of FH.39 He receives FFP every second week to substitute defective FH and has shown stable renal function over the last years without sign of active thrombotic microangiopathy events. Serum samples were collected before infusion of FFP.

**Inhibition of AP/CP Activity in Human Serum**

For determination of AP activity, 96-well Maxisorp plates were coated with 25 µg/ml LPS (Salmonella enteritidis; Sigma-Aldrich) diluted in DPBS (Gibco). After two washing steps with PBS containing 0.05% Tween (Sigma-Aldrich), wells were blocked with PBS/1% BSA (Thermo Scientific) at 37°C for 1 hour. Test proteins were diluted in 80 µl GVB/Mg2+/EGTA (Complement Technologies) before 25 µl HS was added. After another washing step, 100 µl of test protein serum mixture was transferred to the plate and incubated at 37°C for 1 hour. For the C3b AP ELISA, wells were washed five times before incubation with HRP-labeled C3b antibody (1:10,000 in PBS/1% BSA; MP Biomed) for 30 minutes at room temperature, whereas serum supernatants were directly transferred to −80°C for subsequent analysis of C3a and C5a levels using MicroVue kits (Quidel). After washing five times with PBS/0.05% Tween, detection of C3 deposition was performed by adding TMB substrate and H2SO4 to stop the reaction. Finally, the absorbance was read at 450 nm using an Epoch Microplate Spectrophotometer and Gen5 Software (BioTek, Bad Friedrichshall, Germany). For the C5b-9 AP ELISA, wells were

**Figure 6.** MFHR1 is resistant to deregulation by FHR-1 and FHR-5. (A) MFHR1 binding to C3b is not competed off by FHR-1 or FHR-5. MFHR1 (triangles) or hFH (squares; termed test protein) was added to C3b-coated microtiter plates alone or with increasing concentrations of FHR-1 (black line) or FHR-5 (gray line) ranging from equimolar amounts to 100-fold excess. MFHR1 or hFH bound to C3b was detected using specific antibodies. Average of n=3 assays ±SD is shown. *P<0.05; **P<0.01; ***P<0.001. (B) MFHR1-mediated protection of sE from serum-induced AP activation is not attenuated by FHR-5, whereas AP regulatory function of hFH was dose dependently deregulated by FHR-5. MFHR1 or hFH was used at concentrations that reduce FH-depleted serum-induced lysis of sE to 50%, and increasing concentrations of FHR-5 ranging from equimolar amounts to 100-fold excess were added. Hemolysis was determined at 414 nm, and data are expressed as relative hemolysis increase over samples where no FHR-5 has been added. Data represent mean values from n=3 assays ±SD. Asterisks mark treatments significantly different from hFH or MFHR1 alone (one-way ANOVA with Bonferroni test). **P<0.01.
Figure 7. MFHR1 controls complement activation in vitro and shows therapeutic benefit in C3G in vivo. (A) IC_{50} fitting curves for inhibition of aHUS-induced lysis of sE. MFHR1 added to serum derived from a patient with aHUS (C-terminal depletion in FH39) protects sE from AP-mediated lysis with higher efficiency than eculizumab. Data represent mean values ± SEM from n=4 experiments. The inhibitory activity of MFHR1 was significantly different from eculizumab (P<0.001; two-way ANOVA). (B) C3 serum levels of FH−/− mice after intraperitoneal injection of MFHR1 (n=2), hFH (n=3), or PBS (n=3). Mean values are shown with plotted individual data points. Serum C3 of wild-type mice was 1275±122 µg/ml (n=3). (C) MFHR1 or hFH treatment of FH−/− mice but not PBS treatment restores serum C5 as analyzed by Western blotting of serum after 24 hours. Serum of wild-type mice (FH+/+) was used as positive control. (D) MFHR1 and hFH reduce abnormal glomerular C3 depositions in FH−/− mice. Glomerular C3 fluorescence immunostaining intensity was determined 24 hours after administration of MFHR1, hFH, or PBS to treated FH−/− mice. Sections of untreated wild-type mice were used as negative control. Means are shown with plotted individual data points obtained from five glomeruli per section expressed as relative fluorescence units (RFUs). (E) Sections of glomeruli from MFHR1- or hFH-treated mice after 24 hours. Light microscopy images from hematoxylin and eosin (HE)-stained sections (HE 63×) and representative immunofluorescence images of glomerular C3 depositions (C3 Alexa-488 63× and 20×) and bound MFHR1 or hFH both detected with FH antibody (anti-FH1-4 Alexa-488 20×). No abnormality could be assessed by HE staining on glomeruli from FH−/− mice treated with PBS, MFHR1, or hFH or wild-type mice at an age of 2 months. Immunofluorescence microscopy shows capillary wall and mesangial deposition of C3 in FH−/− mice, whereas
washed three times before incubation with alkaline phosphatase-conjugated mAb (WIESLAB AP ELISA; Euro-Diagnostica, Malmo, Sweden), recognizing the C9 neoantigen formed during C5b-9 assembly, followed by incubation with alkaline phosphatase substrate (WIESLAB AP ELISA) solution for 30 minutes, and the absorbance was read at 405 nm. OD values for serum without regulatory proteins were set to 100% activity; heat-inactivated HS or serum without LPS was used as the negative control.

For analysis of CP activity, we used the WIESLAB CP ELISA (Euro-Diagnostica) following the manufacturer’s instructions. Briefly, MFHR1 was added to HS and incubated in wells precoated with human IgM using CP-specific buffer conditions and C5b-9 formation, and the absorbance was read at 405 nm.

Cofactor and Decay Acceleration Activity Assay
The cofactor activity was measured in a fluid-phase assay, and decay acceleration activity was measured by ELISA as previously described.28

Microtiter Binding and Competition ELISAs
For C5 ELISA, FHR-1, MFHR1, or hFH was immobilized to microtiter plates (MaxiSorb) at equimolar concentrations (133 nM). After washing with PBS/0.05% Tween and blocking with PBS/2% BSA, increasing amounts of C5 were incubated. Bound C5 was detected using anti-human C5 antibody (Complement Technologies) followed by incubation with an HRP-conjugated rabbit anti-mouse serum (DAKO). The C3b competition ELISA was adapted from the work by Goicoechea de Jorge et al. Briefly, 25 μg/ml of C3b was coated on MaxiSorb plates in a carbonate buffer (pH 9.6). After washing with PBS/0.01% Tween and blocking with PBS/2% BSA for 2 hours, 10 nM MFHR1 or hFH was incubated alone or with increasing concentrations of FHR-1 or FHR-5 (10, 31.6, 100, 316, and 1000 nM), respectively, and applied to the plate. After 2 hours at room temperature, plates were washed four times, and C3b-bound MFHR1 or hFH was detected using anti-hFH for FHR-5 competitive ELISA or anti-FH1,4 for FHR-1 competitive ELISA. After 1 hour, detection was performed using anti-goat or anti-rabbit Ig HRP. After washing, ELISAs were developed, and the OD at 450 nm was measured after stopping the reaction with 10% H2SO4 (Roth).

Hemolytic Assays
To test C5 convertase inhibition, 25 nM CVF, 25 nM factor B, and 2.5 nM factor D were diluted in PBS/5 mM MgCl2 (Sigma-Aldrich) and incubated for 1 hour at 37°C to build up experimental C5 convertase (CVFBb). C5 (200 nM) was mixed with a 16-fold molar excess of test proteins and incubated for 30 minutes at room temperature, and lytic activity of the samples was measured after adding preassembled CVF convertases to C5/Inhibitor complexes to a mixture containing C6 (15 nM), C7 (15 nM), C8 (11 nM), C9 (24 nM), and 5 × 107 sEs (Fiebig, Idar-Oberstein, Germany) in GVB/Mg2+/EGTA buffer.

The inhibition of C5b-9 formation was followed using a modified protocol.4 In brief, C5b6 (0.7 nM) was incubated with 1.3 μM test protein for 5 minutes and added to C7 (9 nM), C8 (7 nM), C9 (15 nM), and 5 × 107 sEs. To measure the ability of MFHR1 to protect sE from serum-induced cell lysis, MFHR1 or eculizumab in GVB was diluted to a final concentration ranging from 0.3 to 100 nM. Next, 5 × 107 sEs were added, and the application of 20% serum of a patient with aHUS or FH-deficient serum started the reaction. For all lytic assays, reactions were stopped after incubation for 30 minutes at 37°C with GVB/EDTA buffer, and the supernatant was measured at 414 nm in a microplate reader.

Animals and Treatment Procedures
FH−/− mice, provided by Matthew Pickering, were generated as described.41 Animal procedures were performed in accordance with the guide for the care and use of laboratory animals published by the US National Institutes of Health and the German animal protection code, and they were approved by local authorities (Regierungspräsidium Freiburg G-13/86). Mice were injected intraperitoneally with 0.5 mg MFHR1 or hFH or an identical volume of PBS; blood samples were taken at serial time points, and renal tissue was taken after 24 hours. Serum C3 levels, immunostainings, and quantitative immunofluorescence were performed as previously described.28 Hematoxylin and eosin staining of kidney sections was performed using standard staining procedures. MFHR1 or hFH was detected using rabbit anti-FH1,4 after rabbit anti-goat Alexa 488-conjugated antibodies (Invitrogen). For immune detection of C5 in mouse serum, 20 μl serum diluted 1:50 in PBS was resolved by SDS-PAGE under nonreducing conditions and immunoblotted. C5 was detected with anti-C5 (Quidel) antiserum after HRP-conjugated anti-goat antibodies.

Statistical Analyses
All graphs and statistics were created using Prism5 (GraphPad Software, San Diego, CA). The IC50 values were calculated by x=log(x) data transformation, and curves were fitted using the nonlinear regression log (inhibitor) versus response (four parameters) setting. The goodness of the fit is indicated by R2 followed by the 95% confidence interval. One- or two-way ANOVA with Bonferroni multiple comparisons test was used for statistical evaluation as indicated. Statistical significance was defined as ***P<0.001, **P<0.01, and *P<0.05.

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