Moss-Produced, Glycosylation-Optimized Human Factor H for Therapeutic Application in Complement Disorders

Stefan Michelfelder,* Juliana Parsons,† Lennard L. Bohlender,† Sebastian N.W. Hoernstein,† Holger Niederkrüger,‡ Andreas Busch,‡ Nicola Krieghoff,‡ Jonas Koch,‡ Benjamin Fode,‡ Andreas Schaaf,‡ Thomas Frischmuth,‡ Martin Pohl,* Peter F. Zipfel,§ Ralf Reski,¶ Eva L. Decker,† and Karsten Häffner*

*Department of Pediatrics and Adolescent Medicine, Faculty of Medicine, University of Freiburg Medical Center, Freiburg, Germany; †Plant Biotechnology, Faculty of Biology, University of Freiburg, Freiburg, Germany; ‡Greenovation Biotech GmbH, Freiburg, Germany; §Leibniz Institute for Natural Product Research and Infection Biology, Friedrich Schiller University, Jena, Germany; ¶BIOSS Centre for Biological Signalling Studies, University of Freiburg, Freiburg, Germany; and ¶FRIAS Freiburg Institute for Advanced Studies, University of Freiburg, Freiburg, Germany

ABSTRACT

Genetic defects in complement regulatory proteins can lead to severe renal diseases, including atypical hemolytic uremic syndrome and C3 glomerulopathies, and age-related macular degeneration. The majority of the mutations found in patients with these diseases affect the glycoprotein complement factor H, the main regulator of the alternative pathway of complement activation. Therapeutic options are limited, and novel treatments, specifically those targeting alternative pathway activation, are highly desirable. Substitution with biologically active factor H could potentially treat a variety of diseases that involve increased alternative pathway activation, but no therapeutic factor H is commercially available. We recently reported the expression of full-length recombinant factor H in moss (Physcomitrella patens). Here, we present the production of an improved moss-derived recombinant human factor H devoid of potentially immunogenic plant-specific sugar residues on protein N-glycans, yielding approximately 1 mg purified moss-derived human factor H per liter of initial P. patens culture after a multistep purification process. This glycosylation-optimized factor H showed full in vitro complement regulatory activity similar to that of plasma-derived factor H and efficiently blocked LPS-induced alternative pathway activation and hemolysis induced by sera from patients with atypical hemolytic uremic syndrome. Furthermore, injection of moss-derived factor H reduced C3 deposition and increased serum C3 levels in a murine model of C3 glomerulopathy. Thus, we consider moss-produced recombinant human factor H a promising pharmaceutical product for therapeutic intervention in patients suffering from complement dysregulation.


The glycoprotein complement factor H (FH) plays a central role in the regulation of the alternative pathway of complement activation (AP), and mutations as well as antibodies affecting FH function lead to severe renal diseases, like atypical hemolytic uremic syndrome (aHUS) or C3 glomerulopathies (C3Gs).1,2 Polymorphisms in FH are also associated with a frequent form of visual impairment, age-related macular degeneration.3

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Correspondence: Dr. Karsten Häffner, Department of Pediatrics and Adolescent Medicine, University of Freiburg Medical Center, Mathildenstrasse 1, 79111 Freiburg, Germany, or Dr. Eva L. Decker, Plant Biotechnology, Faculty of Biology, University of Freiburg, Schaeferstrasse 1, 79104 Freiburg, Germany. Email: karsten.haefner@uniklinik-freiburg.de or eva.decker@biologie.uni-freiburg.de

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In aHUS, FH dysfunction leads to uncontrolled complement activation and ultimately, formation of the C5b-9 terminal membrane attack complex on glomerular endothelial cells. This is accompanied by cell damage, glomerular thrombotic microangiopathy, and ARF, historically resulting in death or terminal renal failure in >60% of genetically determined patients. In C3G, which progresses to terminal renal failure in >50% of patients within 10 years, pathognomonic complement deposits are found in the glomerular basement membrane. Uncontrolled AP activation in C3G is caused by mutations in complement genes, especially membrane. Uncontrolled AP activation in C3G is caused by mutations in complement genes, especially FH.

Therapeutic treatment options for aHUS and C3G are limited and include plasmapheresis or substitution with fresh frozen plasma (FFP) and immunosuppressive treatment as well as renal transplantation, with a high risk of recurrence of the underlying disease.6–9 Among currently investigated complement targeting therapeutics, eculizumab, a humanized monoclonal anti-C5 antibody, blocks C5b-9 formation and has recently been approved for aHUS treatment.10–12 For C3G, no therapeutic regime has been established yet.7 The application of eculizumab in patients with C3G led to a partial response in only some patients.13

FH regulates the activation of C3 convertases in serum as well as on cell surfaces. It consists of 1213 amino acids with a molecular mass of 155 kD.14,15 SCRs -511, -700, -784, -804, -864, -893, -1011, and -1077 of the mannose-lectin pathway. The complement regulatory region of the pro-C3 convertase of factor H (FHplasma) resulted in the rapid normalization of plasma C3 levels and resolution of the glomerular C3 deposit.29 Additionally, FHplasma supplementation normalized serum-induced in vitro hemolysis in sera of patients with aHUS.24,30

Biopharmaceutical manufacturing is complex and labor and cost intensive. Recombinant FH has been expressed in mammalian cells,31 insect cells,32 and yeast.33 However, these approaches have yet to show therapeutic value.

The moss Physcomitrella patens has been developed into a Good Manufacturing Practice–compliant production system on an industrial scale.34–37 In a proof of concept study, we showed the recombinant production of human FH in moss.38 To further the development of a moss-produced FH pharmaceutical, post-translational modifications have to be considered, especially N-glycosylation, which differs, to some extent, between plants and humans. Although both human and plant glycoproteins harbor diantennary complex-type N-glycans with the identical core structure GlcNAc2-Man1-GlcNAc2 (GnGn), there are two plant-specific sugar residues attached to this core, which are potentially immunogenic in mammals (reviewed in Bosch et al.39). These sugars, a xylose and a fucose, were completely abolished by genome engineering via homologous recombination, thus modifying the glycosylation pattern of moss for improved biopharmaceuticals.40–43

The reliability of the moss production system was acknowledged recently by the approval of a phase 1 clinical trial with the product moss-aGal for treatment of Morbus Fabry from Germany’s regulatory authority, the Federal Institute for Drugs and Medical Devices.44

Here, we describe the generation and production of moss-produced recombinant human factor H (FHmoss) in glyco-engineered moss strains. We proved complete glycosylation via mass spectrometry (MS) and characterized the regulatory activities of FHmoss in functional analyses, where it showed full bioactivity. Using sera of patients with aHUS, we showed the ability of FHmoss to inhibit serum AP activation as well as protect cells from disease-induced hemolysis. Additionally, in vivo administration of FHmoss increased serum C3 levels and significantly reduced glomerular C3 deposition in FH−/− mice, making it a candidate biopharmaceutical for patients suffering from complement dysregulation.

RESULTS

FHmoss Displays Complete Glycosylation

In a research and development production and purification process, FHmoss was extracted from bioreactor culture and purified via a two-step chromatography protocol, and reasonable yields of FHmoss were achieved (Supplemental Table 1). The quality of purified FHmoss was analyzed by SDS-PAGE and Coomassie staining, and the purity was approximately 85% as determined by band intensity densitometry, whereas...
very low amounts of host cell proteins were observed (Figure 1A). SDS-PAGE and Western blot analyses confirmed the pattern shown before in our proof of concept study for full-length FHmoss production with two distinct bands, which were comparable with those of FHplasma and had apparent molecular masses of approximately 140 kD and 120 kD in SDS-PAGE under reducing conditions (Figure 1A and B). To identify the nature of these two bands, we performed protein deglycosylation with PNGase F. SDS-PAGE showed that deglycosylation reduced the overall size of the bands, whereas the two distinct bands remained (Figure 1B). When we compared reduced with nonreduced protein, we observed only one band under nonreducing conditions with the size of the lower band (Figure 1C). Thus, we conclude that the lower FH band resulted from incomplete reduction under the conditions used. However, both FHmoss bands were separately subjected to MS

![Image of Figure 1](image-url)

**Figure 1.** Purified FHmoss is intact and completely N-glycosylated. (A) Characterization of FHmoss and FHplasma. Samples were separated under reducing conditions on SDS-PAGE and Coomassie stained. (B) Deglycosylation (degly.) of FHmoss and FHplasma. Samples treated with PNGase F (arrow) and untreated controls were separated under reducing conditions on SDS-PAGE and silver stained. MW, molecular mass marker. (C) Western blot of FHmoss and FHplasma under reducing and nonreducing conditions. (D) Summed MS1 scans showing complex GnGn-glycosylated FHmoss peptides. FHmoss was tryptically digested and analyzed by nano–LC-MS/MS on a Q-TOF instrument. Among the identified peptides, the glycopeptides 850–867 (IPCSQPPQIEHTINSSR; glycosylation site Asn864), 889–901 (ISEENETCTCYMGK; glycosylation site Asn893), and 1006–1018 (MDGASNVTCINSR; glycosylation site Asn1011) are displayed. Oxidized methionine is labeled with Mox. Shown m/z values correspond to the most abundant isotope peaks for each glycopeptide. The monoisotopic masses for all glycopeptides are listed in Table 1.
FHmoss binds to C3b, Heparin, and Host Cell Surfaces

After proving the structural integrity of FHmoss, we aimed to analyze binding characteristics that are essential to mediate the complement regulatory activity of FH. Binding to C3b is required for FH to control C3b amplification and AP activation. Therefore, FHmoss and FHplasma were compared for their ability to bind to immobilized C3b. In the ELISA-based assay, FHmoss specifically bound to C3b, similar to FHplasma (Figure 2A). Because the protective function of FH against complement attack of host cells is mediated via polyanionic surface structures, such as heparin sulfate chains, heparin binding was tested. Binding values of both FH variants to immobilized heparin were at comparable levels (Figure 2B).

Furthermore, the ability of FHmoss to bind to endothelia was analyzed by testing binding to human umbilical vein endothelial cells (HUVECs). After incubation with FHmoss, FHplasma, or controls (Δx/ft and buffer), FH attached to the cells was analyzed by flow cytometry. The mean fluorescence values observed were 49.02 for FHmoss and 25.78 for FHplasma, whereas binding of the Δx/ft extract (8.28) was comparable with the buffer control (7.18), indicating that the binding capacity of FHmoss to endothelial cells is slightly enhanced to that of FHplasma (Figure 2C). Surface attachment of FHmoss to HUVECs was also visualized by immunofluorescence microscopy. Binding of FHmoss was detected with an Alexa 488–labeled antibody, resulting in green signals at the cell surface (Figure 2D, left panel). These results show that FHmoss binds to human cell surfaces.

**Table 1. Major N-glycan structures and corresponding calculated mass-to-charge ratios of FH glycopeptides**

<table>
<thead>
<tr>
<th>Glycan Structure</th>
<th>Major FH glycopeptide (AA, N-terminal)</th>
<th>Mass-to-Charge Ratio (M+nH+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man4</td>
<td>LCPPPPQIPNSHNMoxTTT</td>
<td>[M+4H+] 744.5715</td>
</tr>
<tr>
<td>Man5</td>
<td>IPCSQPPQIEHGTINSSR</td>
<td>[M+4H+] 748.5947</td>
</tr>
<tr>
<td>Man6</td>
<td>ISEEENTTCYMGK</td>
<td>[M+3H+] 779.6179</td>
</tr>
<tr>
<td>Man7</td>
<td>ISEEENTTCYMoxGK</td>
<td>[M+3H+] 800.6406</td>
</tr>
<tr>
<td>Man8</td>
<td>MDGASNVTCINSR</td>
<td>[M+3H+] 831.6633</td>
</tr>
<tr>
<td>Man5Gn</td>
<td>IPCSQPPQIEHGTINSSR</td>
<td>[M+3H+] 860.6877</td>
</tr>
<tr>
<td>Man6Gn</td>
<td>ISEEENTTCYMGK</td>
<td>[M+3H+] 889.7104</td>
</tr>
<tr>
<td>Man7Gn</td>
<td>ISEEENTTCYMoxGK</td>
<td>[M+3H+] 918.7331</td>
</tr>
<tr>
<td>Man8Gn</td>
<td>MDGASNVTCINSR</td>
<td>[M+3H+] 957.7557</td>
</tr>
<tr>
<td>Man8GnX</td>
<td>IPCSQPPQIEHGTINSSR</td>
<td>[M+3H+] 986.7793</td>
</tr>
<tr>
<td>Man8GnXF</td>
<td>ISEEENTTCYMGK</td>
<td>[M+3H+] 1015.8020</td>
</tr>
<tr>
<td>Man9</td>
<td>M(AF)b</td>
<td>[M+4H+] 1044.8253</td>
</tr>
<tr>
<td>Man9Gn</td>
<td>ISEEENTTCYMGK</td>
<td>[M+3H+] 1073.8480</td>
</tr>
<tr>
<td>Man9GnX</td>
<td>MDGASNVTCINSR</td>
<td>[M+3H+] 1102.8717</td>
</tr>
</tbody>
</table>

An oxidized methionine is labeled with Mox, and the charge state of a glycopeptide is shown by \([\text{M+nH}^+]\). AA, amino acid; MGn, terminal mannose on one antenna.
Because FH is an essential cofactor for FI-mediated proteolysis of C3b to control the AP amplification cascade, we compared FHmoss and FHplasma for their cofactor activity in a fluid-phase assay. After adding FHmoss or FHplasma to C3b and FI, we observed a concentration-dependent cleavage of the C3b $\alpha^\prime$-chain into the typical $\alpha^\prime68$, $\alpha^\prime46$, and $\alpha^\prime43$-kD fragments, whereas the C3b $\beta$-chain remained unaffected. Cleaved $\alpha^\prime$-chain fragments were already detectable after the addition of 1.6 nM (5 ng) FH (moss or plasma derived), showing comparable cofactor activities (Figure 3A). In addition to its cofactor activity, FH controls complement activation by dissociation of C3 convertase (C3bBb) complexes. Therefore, decay acceleration activity of both FH variants was compared in a solid phase–based ELISA. The addition of either FHmoss or FHplasma resulted in a dose-dependent dissociation of Bb from the preformed C3 convertases, showing similar decay acceleration activity for moss- and plasma-derived FH (Figure 3B).

FHmoss Normalizes Complement Dysregulation in Sera of Patients with aHUS

Therapeutic approaches in patients with aHUS aim to prevent activation of the terminal complement cascade. Therefore, we explored the potential of FHmoss to suppress C5b-9 formation in serum of patient 1 with aHUS carrying a heterozygous R1215Q mutation in FH using the Wieslab Complement System Alternative Pathway ELISA. Serum of this patient shows normal AP activity. However, the addition of increasing

Figure 2. FHmoss interacts with C3b, heparin, and host cell surfaces. (A) Relative binding of FHmoss and FHplasma to immobilized C3b measured by ELISA. (B) Relative binding of FHmoss and FHplasma to heparin measured by ELISA. Controls represent unspecific binding of FHmoss or FHplasma to BSA- or PBS-coated wells. Unspecific response of putative moss-derived contaminants was tested using a sample of the parental moss strain $\Delta xt/ft$. Data represent mean values ± SD from $n=3$ experiments. The values obtained for the binding of FHplasma to C3b or heparin were set to one. (C) Binding of FHmoss to the surface of HUVECs analyzed by FACS. FHmoss (red line), FHplasma (blue line), moss parental strain $\Delta xt/ft$ (black line), and buffer negative control (gray shadow). (D) Binding of FHmoss to the surface of HUVECs analyzed by immunofluorescence microscopy and visualized using FH-specific antibodies and secondary Alexa 488–labeled antibodies (green; upper panel). FHmoss is colocalized with wheat germ agglutinin (Alexa 633 labeled; red), which recognizes sialic acid and N-acetylglucosamine residues predominantly found on the plasma membrane (lower panel). Nuclear staining was performed by HOECHST (blue). The purified extract of the parental moss strain $\Delta xt/ft$ was included as control. Scale bar, 50 $\mu$m.

FHmoss Exhibits Cofactor Activity and Decay Acceleration Activity

Because FH is an essential cofactor for FI-mediated proteolysis of C3b to control the AP amplification cascade, we compared FHmoss and FHplasma for their cofactor activity in a fluid-phase assay. After adding FHmoss or FHplasma to C3b and FI, we observed a concentration-dependent cleavage of the C3b $\alpha^\prime$-chain into the typical $\alpha^\prime68$, $\alpha^\prime46$, and $\alpha^\prime43$-kD fragments, whereas the C3b $\beta$-chain remained unaffected. Cleaved $\alpha^\prime$-chain fragments were already detectable after the addition of 1.6 nM (5 ng) FH (moss or plasma derived), showing comparable cofactor activities (Figure 3A). In addition to its cofactor activity, FH controls complement activation by dissociation of C3 convertase (C3bBb) complexes. Therefore, decay acceleration activity of both FH variants was compared in a solid phase–based ELISA. The addition of either FHmoss or FHplasma resulted in a dose-dependent dissociation of Bb from the preformed C3 convertases, showing similar decay acceleration activity for moss- and plasma-derived FH (Figure 3B).
BASIC RESEARCH

C3b, F1, or Fh or with the extract of the parental moss strain mean values of complexes. Negative control was performed without adding factor D. Data represent OD at 450 nm values of control wells (without Fh) were set to 100% intact C3bBb association of these complexes was determined by measuring the relative amount of Fb.

was added to preformed C3 convertase (C3bBb) complexes (Fb/factor D), and dis- ciently promotes C3 convertase decay. Fhmoss or Fhplasma in indicated amounts was added to preformed C3 convertase (C3bBb) complexes (FB/factor D), and dis- sociation of these complexes was determined by measuring the relative amount of Fb. OD at 450 nm values of control wells (without Fh) were set to 100% intact C3bBb complexes. Negative control was performed without adding factor D. Data represent mean values ± SD from n=3 experiments.

concentrations of Fhmoss to serum of patient 1 inhibited formation of active C5b-9, thus showing that Fhmoss inhibits the AP in patient serum after LPS-induced activation (Figure 4).

Next, we assayed if Fhmoss affects AP activity on host surfaces on erythrocytes. Sheep erythrocytes (sE), like human erythrocytes, express glycosaminoglycans on their surfaces and are normally protected from complement-mediated lysis by plasma-derived Fh. Incubation of sE with FH-depleted serum leads to AP-mediated cell surface complement activation, C5b-9 formation, and subsequent hemolysis. The addition of Fhmoss or Fhplasma specifically and dose dependently protected sE from complement-mediated lysis (Figure 5B), confirming that Fhmoss has the ability to reverse an inherent regulatory defect if supplemented to this patient serum.

Fhmoss Reduces Glomerular C3 Accumulation and Regulates Serum AP Activity in a Murine C3G Model

Glomerular C3 deposition and low serum C3 levels are characteristic pathologic abnormalities in patients with C3G. Fh−/− mice also develop abnormal glomerular C3 accumulation and low serum C3 levels due to AP overactivation, which can be re- voked by human Fh supplementation, thus providing a useful C3G model for testing therapeutic efficiency of recombinant Fh. To test whether Fhmoss regulates AP activation in vivo, either 0.5 mg Fhmoss or 0.5 mg Fhplasma was injected intravenously into Fh−/− mice. As determined by glomerular C3 immunostaining, Fhmoss significantly reduced C3 staining in the glomeruli after 24 hours compared with PBS-treated mice (Figure 6, A and B).

We next explored the influence of Fhmoss on serum C3. As determined by quantitative ELISA, serum C3 levels increased in Fhmoss- (205±68 µg/ml) and Fhplasma-treated animals (270±46 µg/ml) 2 hours after Fh administration, whereas Fhmoss-treated mice reached peak C3 values at 6 hours (220±54 µg/ml) that remained stable until 24 hours; Fhplasma-treated mice reached peak C3 values after 24 hours (639±174 µg/ml) (Figure 6C). C3 level in untreated Fh−/− mice was 49±0.8 µg/ml; in normal wild-type mice, it was 1275±122 µg/ml. Highest serum FH levels were detected 10 minutes after injection at comparable values (Fhmoss: 452±36 µg/ml; Fhplasma: 447±50 µg/ml). Thereafter, FH levels diminished in both groups but more rapidly for Fhmoss (2 hours: Fhmoss: 22±12 µg/ml; Fhplasma: 279±96 µg/ml), indicating that Fhmoss is removed faster from the fluid phase (Figure 6D). Antibodies to human Fh were tested in sera of FH and PBS-treated mice, and no antibodies were detected (data not shown).

DISCUSSION

Treatment options for the devastating diseases aHUS and C3G are limited, and therefore, new therapeutic approaches are needed. Here, we describe the production of biologically active glycosylation-optimized Fhmoss in the moss P. patens. We
showed full bioactivity of FHmoss and proved its ability to control complement activation in serum of patients with aHUS as well as a C3G mouse model.

Eculizumab treatment leads to a complement blockade at the C5 convertase level. FHmoss might offer a safe treatment option for the therapeutic control of complement activation, because it has some considerable advantages compared with eculizumab. First, the substitution of a physiologic protein might imply a low risk of allergic or antibody reactions. Second, because FH acts in the fluid phase and on self but not on foreign surfaces, there might be a lower risk of infection in the treated patients.\textsuperscript{15} Third, the correction of the primary AP imbalance at the level of C3b and the C3 convertase might be beneficial, especially in C3G, where C3-cleavage products still deposit in the kidney, under eculizumab or C5 blockade, respectively.\textsuperscript{29,47,48}

Therefore, the establishment of a production system for recombinant FH is highly desirable. Moss is an established system for the large-scale production of pharmaceutical proteins.\textsuperscript{34,35,37,44,49} Additionally, precise genome editing via homologous recombination\textsuperscript{50–52} allows the engineering of the glycosylation pattern of moss for improved biopharmaceuticals.\textsuperscript{40–43} N-glycosylation plays an important role in protein structure, stability, and functionality.\textsuperscript{53} Moreover, differences in the glycosylation pattern compared with the native protein can not only affect the \textit{in vivo} activity but also, cause immunogenicity and subsequently, a decline of product efficacy as well as clinical complications.\textsuperscript{54} As higher eukaryotes, plant cells synthesize complex proteins and perform post-translational modifications similar to human cells. Unlike insect cells and yeast,
which display N-glycans with mainly paucimannosidic and high-mannose structures, respectively.\textsuperscript{55} Plant cells are able to produce N-glycans of the complex type with the core sugar structure GnGn, which are identical to those in humans.\textsuperscript{56,57} Differences caused by plant-specific sugar residues have been eliminated successfully by gene targeting of the responsible glycosyltransferases.\textsuperscript{43} The FH\textsuperscript{moss} produced in this glycosylation-optimized moss line was evaluated for its N-glycosylation pattern and showed a complete elimination of plant-specific sugars.

In addition to excellent in vitro activity, FH\textsuperscript{moss} induced a reduction of C3 deposition in glomeruli of the FH\textsuperscript{2/2} mice and increased serum C3 levels. We observed a reduction of FH\textsuperscript{plasma} and FH\textsuperscript{moss} from sera within the first 2 hours after intravenous injections, which was more pronounced for FH\textsuperscript{moss} and might be caused by endothelial surface binding. The faster removal of FH\textsuperscript{moss} from the serum might also be attributed to a lack of terminal sialic acids, which are common on mammalian glycoproteins, including human FH,\textsuperscript{14} but generally missing in plants. FH\textsuperscript{moss} N-glycans carry terminal GlcNAc residues, which are recognized by the Man/GlcNAc receptor (e.g., on macrophages) and thus, could be subjected to rapid clearance.\textsuperscript{58,59} Sialylation of plant glycoproteins was proven in transient approaches.\textsuperscript{60} Therefore, we assume that moss glyco-engineering toward terminal sialylation is feasible.
In conclusion, glycosylation-optimized FHmoss binds to heparin and C3b equivalently to FHplasma as well as cell surfaces, thereby potentially protecting host cells from AP over-activation. We were able to show full AP regulation capacity, because FHmoss shows cofactor activity and in addition, accelerates the decay of C3bBb complexes as efficiently as FHplasma. Moreover, addition of FHmoss to the serum of a patient with aHUS efficiently abolished C5b-9 formation, and as shown by hemolysis assays, FHmoss protected sE from complement-mediated lysis caused by serum of a patient with aHUS. Finally, systemic application increased serum C3 levels and reduced glomerular C3 depositions in a murine C3G model.

Taken together, our results show that FHmoss is completely glycosylated, is devoid of potentially immunogenic plant-specific N-glycans, and has full biologic activity in vitro and in vivo, making it a candidate biopharmaceutical with clinical potential for therapeutic interventions in patients suffering from complement dysregulations.

CONCISE METHODS

Strain Development, Plant Material, and Cell Culture

The DNA sequence coding for human complement FH (NCBI reference NM_000186.3) without the native signal peptide was synthesized as N-terminal translational fusion to a plant signal peptide by GeneArt. The FHmoss-producing strains were obtained by PEG-mediated transformation of protoplasts generated from the Δxtt/ft moss line, a double knockout for the α1,3 fucosyltransferase and the β1,2 xylosyltransferase genes as described earlier. Transformation was done with excised and purified DNA fragments containing an expression cassette for FH driven by the promoter of the PpActin5 gene and the npIII gene under control of the constitutive Cauliflower mosaic virus 35S promoter. After G418 selection, plantlets regenerated from single cells underwent two consecutive rounds of screening for levels of FH production, resulting in the confirmation of the highest producing strains.

For protein analysis, in vitro activity tests, and in vivo experiments, cultivation of FH-expressing moss lines was done as described previously with few modifications. The cultivation duration was 16 days.

Purification of FHmoss

For chromatographic purification, FHmoss was extracted, concentrated, and adjusted to phosphate buffer (PB; 10 mM Na₂HPO₄ × 2H₂O and 1.76 mM KH₂PO₄ [pH 7.4]) supplemented with 1 M NaCl by tangential flow filtration (30-kD cutoff; regenerated cellulose membrane) and loaded onto a PhenylSepharose 6 FF HS Column (GE Healthcare, Uppsala, Sweden) equilibrated with PBS and 1 M NaCl (pH 7.4). FHmoss was eluted with elution buffer (PB containing no additional NaCl) in a single step of five column volumes (cv) 100% B. Appropriate fractions were pooled, diluted with PB to a conductivity of ≤10 mS/cm, and loaded onto a HitTrap Heparin HP Column (GE Healthcare) equilibrated with PB supplemented with 68 mM NaCl. FHmoss was eluted using increasing amounts of elution buffer C (PB and 1 M NaCl): ten cv 10% C followed by a linear gradient of 10%–30% C in 20 cv followed by a wash with 100% C. FHmoss containing fractions were pooled and further concentrated using Vivaspin Turbo 15 (PES membrane; Sartorius, Göttingen, Germany). During concentration, buffer was adjusted to PBS. Samples from the parental moss line Δxtt/ft were purified as described for FHmoss and served as control extract.

Protein Analyses and Western Blotting of FHmoss

ELISA was performed as described before using the mAb T13 and a rabbit polyclonal antibody or the Complement Factor H ELISA Kit by Hycult Biotech (Uden, The Netherlands). FHmoss was detected by SDS-PAGE and Western blot using polyclonal goat antibody against human FH (Complement Technology; Tyler) and a rabbit anti-goat IgG (GE Healthcare). Deglycosylation with PNGase F (Promega Corp.) was performed following the manufacturer’s instructions. Mouse serum C3 levels were measured by C3 ELISA kit provided by ABCAM (Cambridge, United Kingdom) following the manufacturer’s instructions. Serum FH levels were quantified by ELISA as described above.

MS Analyses

For MS analysis, the protein was reduced in loading buffer (BioRad, Munich, Germany) with 50 mM dithiothreitol for 5 minutes at 95°C and subsequently alkylated using iodacetamide at room temperature. SDS-PAGE was carried out on 4%–15% gradient SDS polyacrylamide gels (Mini-PROTEAN TGX; BioRad), and gels were stained with PageBlue (Thermo Fisher Scientific). Bands corresponding to FH and monitored by Western blot as described in Büttner-Mainik et al. were cut, and proteolytic digestion and extraction of peptides were carried out as described. Peptides were measured with an ESI-Q-TOF (Agilent 6250) coupled to a 1200 Agilent Nano HPLC-Chip Cube Interface. An HPLC-Chip packed with Zorbax SB C18 (3 μm inner diameter and 150-mm length; trapping column: 40 nl and 5-μm particle size) was used. Peptides were eluted with a linear acetonitrile gradient at a flow rate of 300 nl/min. MS/MS analysis was performed using data-dependent acquisition mode using three precursors for MS/MS (standard enhanced model; CID). Raw data analysis was performed using MassHunter Workstation version B.04.00 Build 4.0.479.9, Service Pack 2. Additional MS/MS analysis was done on an LTQ Orbitrap XL Mass Spectrometer (Thermo Fisher Scientific) as described. Raw data processing of all MS/MS data was performed using Mascot Distiller V2.5.1.0 (Matrix Science). Database searches were performed using Mascot Daemon V2.4 (Matrix Science) against a reverse concatenated P.pastoris database (version 1.6 protein models) and the human FH sequence (Uniprot: P08603). Simultaneously, an in-house database containing sequences of known contaminants (e.g., human keratins and trypsin) was used. Glycopeptide identity was confirmed via inspection of the presence of the Gn-specific reporter ions on MS/MS spectra. The following search parameters were used for Q-TOF measurements: peptide mass tolerance, 50 ppm; MS/MS mass tolerance, 0.05 Da; 13C, 0; enzyme, semitrypsin or no enzyme specification with maximal two
missed cleavages; and variable modifications, Gln→pyroGlu (N-terminal Q) −17.026549 D, oxidation (M) +15.994915 D, Asn_{deam}_Asp −0.984016 D, Hydroxyproline (P) +15.994915 D, and carbamidomethyl (C) +57.021464 D as fixed modification. For the Orbitrap measurements, the same search parameters were used, with a peptide mass tolerance of 10 ppm and an MS/MS mass tolerance of 0.8 D. The Mascot searches of the Orbitrap measurements were filtered with Scaffold4 software (version 4.2.1; Proteome Software, Inc., Portland, OR) using the ProteinProphet and the PeptideProphet.66,67 The threshold was 99% ProteinProphet and 90% PeptideProphet and to at least two assigned peptides.

C3b- and Heparin-Binding Assay

The ability of FHmoss to interfere with C3b or soluble heparin was determined by ELISA as described before.21 For C3b ELISA, 1 µg purified C3b (Complement Technology) diluted in PBS (Gibco; Life Technologies, Darmstadt, Germany) was coated on Maxisorb Plates (Nunc; Thermo Fisher Scientific). Unspecific binding sites were blocked with 2% BSA (Thermo Scientific) in PBS for 2 hours at 37°C. For heparin ELISA, 5 µg heparin sodium salt (Sigma-Aldrich) was diluted in PBS and coated onto heparin-binding plates (BD Biosciences, Heidelberg, Germany). Wells were washed with assay buffer (20 mM HEPES, 130 mM NaCl, and 0.1% Tween20 [pH 7.3]), and unspecific binding sites were blocked with 1% BSA in assay buffer for 2 hours at 37°C. After washing, 125 ng FHmoss or the same volume of Δxt/ft extract at 37°C for 30 minutes. The proteolytic cleavage of C3b catalyzed by FH and FI was analyzed by SDS-PAGE on 8% gels followed by Coomassie staining. The ability of FHmoss to displace Bb from the C3bBb convertase complex was measured by an ELISA adapted from McRae et al.69 and Eberhardt et al.70. In brief, purified C3b (2.5 µg/ml in PBS; Complement Technology) was immobilized on Maxisorb Plates, and the C3bBb complex was generated by adding 400 ng FB (Merck, Darmstadt, Germany) and 25 ng factor D (Merck) in phosphate buffer containing 2 mM nickel chloride, 25 mM sodium chloride, and 4% BSA. After incubation for 2 hours at 37°C and washing, FHplasma or FHmoss at various doses was added to the preformed convertase complex for 30 minutes at 37°C. FH-mediated dissociation of the convertase was monitored by determination of intact C3bBb complexes by measuring the amount of C3b-bound Bb detected by FB-specific antibodies (1:2000 in PBS and 0.1% BSA; Merck) followed by HRP-conjugated rabbit anti-goat (1:5000 in PBS and 0.1% BSA; DAKO). After washing, TMB Substrate was added, and the OD at 450 nm was determined.

Cellular Binding Assay

HUVECs were cultivated after manufacturer’s instructions (PromoCell, Heidelberg, Germany). For flow cytometry, HUVECs were grown under serum-starved conditions for 24 hours. After blocking with 1% BSA in 0.5× PBS, cells were incubated with 10 µg FHplasma or FHmoss for 1 hour at 37°C. Control experiments were performed using the same volume of an identically processed purified extract from Δxt/ft or without recombinant protein. After washing, cells were stained using goat polyclonal FH-specific antibody (Complement Technology) after three washing steps and incubation using a rabbit anti-goat Alexa 488-labeled antibody (Invitrogen, Darmstadt, Germany). Finally, nonviable cells were counterstained using 7-amino-actinomycin D (Sigma-Aldrich), and viable cells were processed by flow cytometry using an FACS (FACSCalibur; Becton and Dickinson, Heidelberg, Germany) and Cylogic analysis software. For binding experiments using immunofluorescence microscopy, HUVECs were grown as described above and plated onto fibronectin-coated (Sigma-Aldrich) coverslips (Langenbrink, Emmendingen, Germany). After fixation in 3% paraformaldehyde (Science Services) and blocking, cells were incubated with 10 µg FHmoss or the same volume of Δxt/ft extract. After washing, cell-bound FH was stained using a goat polyclonal FH-specific antibody in blocking buffer after intensive washing and incubation with a rabbit anti-goat Alexa 488-labeled antibody. Nuclear and membrane counterstaining was performed by using Hoechst 33342 (Sigma-Aldrich) and Alexa 633 conjugate of wheat germ agglutinin (Thermo Scientific). Cells were covered with PermaFluor Aqueous Mounting Medium (Thermo Scientific) on glass slides, and fluorescence staining was visualized with appropriate filters using an AX10 Microscope (Carl Zeiss GmbH, Jena, Germany) and Axiosview software.

Cofactor Activity and Decay Acceleration Activity

The complement regulatory activity of FHmoss was measured in a fluid-phase cofactor assay as described previously.68 Briefly, C3b and FI (Complement Technology) were incubated with FHmoss, FHplasma, or the same volume of Δxt/ft extract at 37°C for 30 minutes. Serum samples were collected before infusion of FFP. This study was approved by the Ethics Committee Freiburg, and written informed consent was provided for sample collection and subsequent analysis.
FH Inhibition of AP Activity in Human Sera

The ability of FHmoss to inhibit the AP activity in human sera was determined by the Wieslab Complement System Alternative Pathway Enzyme ELISA Kit according to the manufacturer’s instructions (Euro-Diagnostica, Malmo, Sweden). Briefly, FHmoss or FHplasma was diluted in serum of a patient with aHUS and incubated in presence of AP-specific buffer in wells precoated with LPS. After washing, active C5b-9 formation was detected with alkaline phosphatase-conjugated mAb, recognizing the C9 neoantigen formed during C5b-9 assembly, followed by incubation with alkaline phosphatase substrate solution for 30 minutes. The amount of complement activity was calculated on the basis of the OD at 405 nm measured in a Synergy HT Microplate Reader.

Hemolytic Assay

The hemolytic assay was performed to measure the ability of FHmoss to protect sE from serum-induced cell lysis as described before.30 FH-depleted serum was obtained from Complement Technology. Heat-inactivation of FHmoss was performed at 95°C for 30 minutes. Briefly, \( 5 \times 10^7 \) freshly prepared sE (Fiebig, Idar-Oberstein, Germany) were diluted to a final volume of 25 \( \mu l \) in GVB/Mg\(^{2+}\) per EGTA buffer (Complement Technology). FH in varying amounts or controls were diluted to a final volume of 15 \( \mu l \) of GVB/Mg\(^{2+}\) per EGTA buffer, and 10 \( \mu l \) serum was added. Subsequently, the reaction mix was incubated at 37°C for 30 minutes and stopped by adding 200 \( \mu l \) GVB/EDTA buffer. After centrifugation, the OD of the supernatants was measured at 414 nm in a Synergy HT Microplate Reader, and corresponding blank values (without serum) were subtracted from each value.

Animals and Administration of FHmoss and FHplasma to FH\(^{-/-}\) Mice

FH\(^{-/-}\) mice, provided by Matthew Pickering (Centre for Complement and Inflammation Research, Imperial College London, London, United Kingdom), were generated as described.46 All procedures involving animals were conducted 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 approved by local authorities (Regierungspräsidium Freiburg G-13/86); 0.5 mg FHmoss or FHplasma in 100 \( \mu l \) final volume or 100 \( \mu l \) PBS was injected intravenously into the tail vein of FH\(^{-/-}\) mice. Blood was collected via tail vein at serial time points after injections, and serum was separated via centrifugation after clotting for storage at \(-80^\circ\)C. Mice were euthanized after 24 hours, and kidneys were collected into PBS and snap frozen for storage at \(-80^\circ\)C. Sera of mice were screened for FH antibodies using an ELISA method, analogous to that used to detect anti-FH IgG autoantibodies in humans.73

Immunostaining of Mouse Renal Sections for Murine C3

Ten-micrometer cryosections from mouse kidneys were cut on a Leica CM 3050S Cryostat, mounted on SuperFrost Plus Glass Slides (Langebrink), and stored at \(-80^\circ\)C. After fixation in 4% paraformaldehyde solution, sections were permeabilized in PBS and 0.3% Tween 20, and C3 was detected using a goat anti-mouse C3 after a second rabbit anti-goat Alexa 488-conjugated antibody (Invitrogen). After washing, slides were mounted in VECTASHIELD HardSet Mounting Medium (Vectorlabs) and covered with glass coverslips. Sections were visualized using an AX10 Microscope (Carl Zeiss GmbH) and Axiovision software. For quantitative immunofluorescence staining, mean fluorescence intensity of three glomeruli per section was determined by using Image J Software (National Institutes of Health) and expressed in arbitrary fluorescence units. Data were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Groups were compared using one-way ANOVA with Bonferroni multiple comparisons test as indicated.

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DISCLOSURES

R.R. is a founder of Greenovation Biotech GmbH (Freiburg, Germany) and currently serves on its advisory board. H.N., A.B., N.K., J.K., B.F., A.S., and T.F. are staff members of this company. Greenovation Biotech GmbH develops and markets mass-based biopharmaceuticals.

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