

Complement factor H-related hybrid protein deregulates complement in dense deposit disease

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The renal disorder C3 glomerulopathy with dense deposit disease (C3G-DDD) pattern results from complement dysfunction and primarily affects children and young adults. There is no effective treatment, and patients often progress to end-stage renal failure. A small fraction of C3G-DDD cases linked to factor H or C3 gene mutations as well as autoantibodies have been reported. Here, we examined an index family with 2 patients with C3G-DDD and identified a chromosomal deletion in the complement factor H-related (CFHR) gene cluster. This deletion resulted in expression of a hybrid CFHR2-CFHR5 plasma protein. The recombinant hybrid protein stabilized the C3 convertase and reduced factor H-mediated convertase decay. One patient was refractory to plasma replacement and exchange therapy, as evidenced by the hybrid protein quickly returning to pretreatment plasma levels. Subsequently, complement inhibitors were tested on serum from the patient for their ability to block activity of CFHR2-CFHR5. Soluble CR1 restored defective C3 convertase regulation; however, neither eculizumab nor tagged compstatin had any effect. Our findings provide insight into the importance of CFHR proteins for C3 convertase regulation and identify a genetic variation in the CFHR gene cluster that promotes C3G-DDD. Monitoring copy number and sequence variations in the CFHR gene cluster in C3G-DDD and kidney patients with C3G-DDD variations will help guide treatment strategies.

Introduction

C3 glomerulopathy with dense deposit disease (C3G-DDD) pattern, previously termed dense deposit disease or membranoproliferative glomerulonephritis type II, is a rare kidney disorder that primarily affects children and young adults (1). Patients present with nephrotic or nephritic syndrome or with asymptomatic renal disease, which often progress to end-stage renal disease (ESRD), and the disease tends to recur in the transplanted kidney (1–3). Diagnosis of C3G-DDD is primarily based on a histological evaluation by electron microscopy of a kidney biopsy showing C3 staining and the absence of Igs and the presence of dense deposits along the glomerular basement membrane (GBM) with mesangial hypercellularity (4–6). In some patients with dense deposit disease, genetic causes in the form of mutations in genes coding for factor H or C3 (7–11) and allelic variants (11–15) as well as autoimmune forms were reported, including C3 nephritic factor (C3Nef), other forms of C3 convertase autoantibodies in patients' serum lacking C3Nef, and factor B autoantibodies (16–18). There is an ongoing discussion on how complement-related kidney diseases should be grouped, and what precise classifications are relevant for appropriate therapy, to predict transplant outcome and the response to complement-inhibitory drugs (2, 6, 18). Here, we define a novel genetic scenario for C3G-DDD, in which 2 related patients had a 24,804-nt fragment in the complement factor H-related (CFHR) gene cluster on chromosome 1q32 deleted.

This deletion resulted in expression of a CFHR2-CFHR5 hybrid plasma protein, which stabilizes the complement C3 convertase. The rapid reappearance of the hybrid protein in patient plasma after replacement or exchange therapy explains why the patient did not respond to this plasma therapy.

Results

Histology of kidney biopsies. We present 2 affected children of the index family (Figure 1I). Clinical information and development of disease are described in detail in the Methods section. Renal biopsies of both siblings revealed prominent C3c staining along the GBM and also partly within the mesangium, without relevant Ig deposits, by immunofluorescent microscopy. Electron microscopy of reprocessed, paraffin-embedded tissue performed in one case illustrated intense, partly ribbon-like dense deposits within and along the GBM and in the mesangium, which matched the histology and the immunofluorescence results (Figure 1, A–H). However, the quality of the electron microscopy was limited due to the procedure of reprocessing. Taken together, the diagnosis of these patients was C3 glomerulopathy, which, at least by electron microscopy, showed some morphological features of DDD. The findings may explain why both patients lost their renal functions and reached ESRD.

Complement analysis revealed decreased C3 and factor B levels and elevated Ba and soluble TTC (referred to herein as sC5b-9) levels (Figure 1J and data not shown), suggestive of alternative pathway activation and persistent C3 consumption. Assays to identify C3Nef, C3b, and factor B autoantibodies were negative (data not shown).

Conflict of interest: The authors have declared that no conflict of interest exists.

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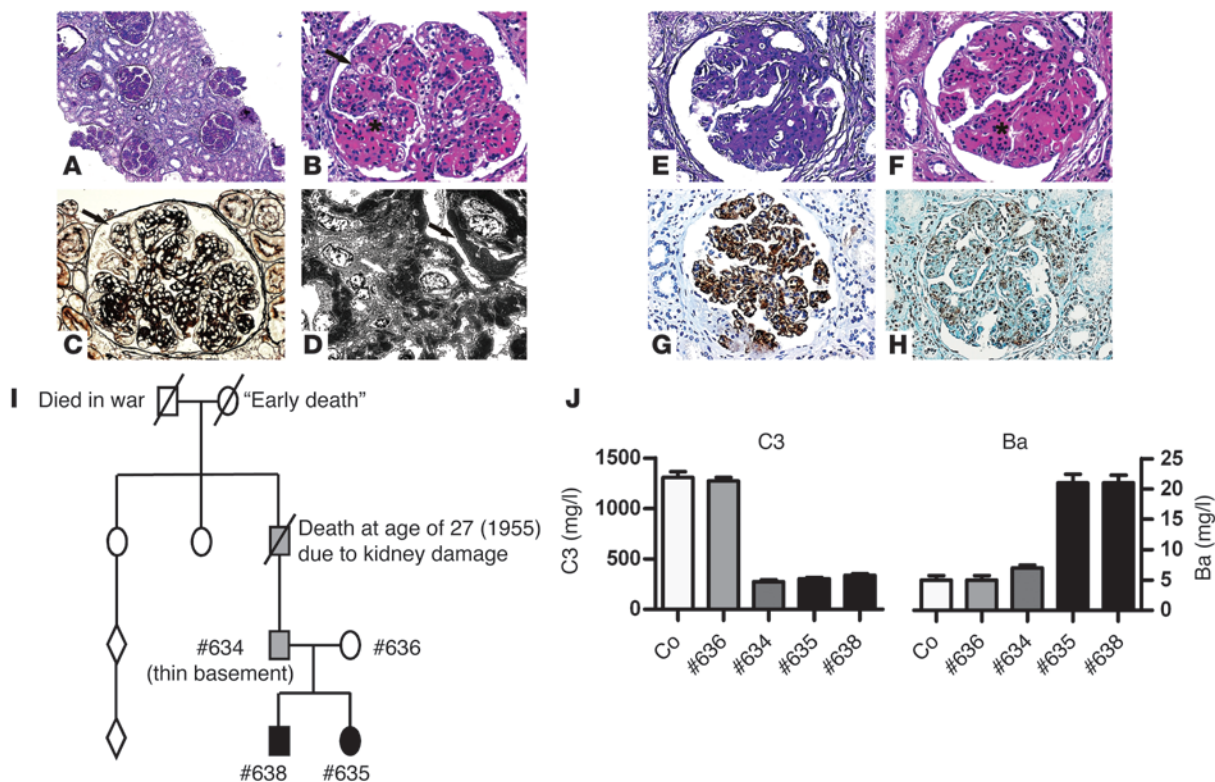


Figure 1

Histology of kidney biopsies and the plasma parameters of patients. (A–D) Kidney biopsy of patient no. 635. (A) PAS-stained kidney section illustrating prominent lobulation of the glomerular tuft, with mesangial proliferation. (B) H&E-stained kidney section with increased capillary tuft lobulation, prominent mesangial cell proliferation (asterisk), and numerous intracapillary foam cells (arrow). (C) Silver staining with double contours of the GBMs (arrow). (D) Electron microscopy of reprocessed paraffin-embedded material with abundant dense osmiophilic deposits in mesangium (white arrow) and linear dense deposits along the GBMs (black arrow). (E–H) Kidney biopsy of patient no. 638. (E) PAS-stained kidney section with prominent lobulation of the tufts and an increase in mesangial matrix and cellularity (asterisk). (F) H&E-stained kidney section, showing lobulation (asterisk). (G) C3c immunohistochemistry with intense mesangiocapillary positivity in the glomerulus. (H) TCC immunohistochemistry with a less intense positivity in the mesangium and also focally along the basement membranes. Original magnification, $\times 100$ (A); $\times 400$ (B, C, and E–H); $\times 2,156$ (D). (I) Pedigree of the Erlangen family. Two related C3 glomerulopathy patients (no. 635 and no. 638) and their father (no. 634) and mother (no. 636). (J) Complement C3 and Ba fragments in plasma of the 2 patients (no. 635 and no. 638) with low C3 (303 and 331 mg/l) and increased Ba serum levels (21.3 and 21.0 mg/l). The father (no. 634) had low C3 (275 mg/l) and normal Ba levels (7.0 mg/l). The mother and a healthy individual had normal C3 (1,309 and 1,273 mg/l) and Ba levels (5.0 mg/l).

A novel chromosomal deletion in the CFHR2 gene. Copy number variation analysis in the *CFHR* gene cluster studied by multiplex ligation-dependent probe amplification (MLPA) revealed that both patients (no. 635 and no. 638) and their father (no. 634) had a heterozygous deletion of *CFHR2* exon IV on chromosome 1q32 (Figure 2A). Their mother (no. 637) and a healthy control had 2 allelic copies of *CFHR2* (Figure 2A).

The chromosomal breakpoint was identified by inverse-nested PCR using *EcoRI*-digested genomic DNA (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI171866DS1). The approximately 3,000-nt fragment amplified from the annealed genomic circle included 2 segments (Supplemental Figure 1B). The first segment, ending with . . . TGTC, matched to intron III of the *CFHR2* ending at position +8,918. The adjacent segment, beginning with ACAA . . . , initiated -12 nt prior to the transcription start site of the *CFHR5* gene (Figure 2B). Both patients and their father had the same chromosomal segment deleted. Deletion of this 24,804-nt segment, which spans from *CFHR2* intron III to the *CFHR5* gene, was confirmed

with genomic DNA from both patients by an amplification that covers the breakpoint locus using primers that anneal within the existing sequences in the *CFHR2* and the *CFHR5* genes and that covered the breakpoint (Supplemental Figure 1C).

This chromosomal deletion manifests on the protein level. Plasma of both patients had lower levels of *CFHR2* and *CFHR5* proteins as compared with normal human serum (NHS), which was in agreement with the presence of only 1 intact allele (Figure 2C, lanes 2 and 3, and data not shown). In addition, a new protein doublet with mobilities of about 70 and 65 kDa was identified (Figure 2C, lanes 2 and 3, arrowhead). Antibody reactivity and increased mobility upon N glycosidase F treatment revealed that these bands represented differently glycosylated forms of a *CFHR2*-*CFHR5* hybrid protein. The 70-kDa band had 3 and the 65-kDa band had 2 attached N-linked carbohydrate side chains (Figure 2 and Supplemental Figure 2).

The CFHR2_{1,2}-CFHR5 protein binds to C3b and competes with factor H for C3b binding. The *CFHR2*_{1,2}-*CFHR5* hybrid protein was recombinantly expressed in HEK 293 cells and purified from the supernatant by affinity chromatography (Supplemental Figure 3).

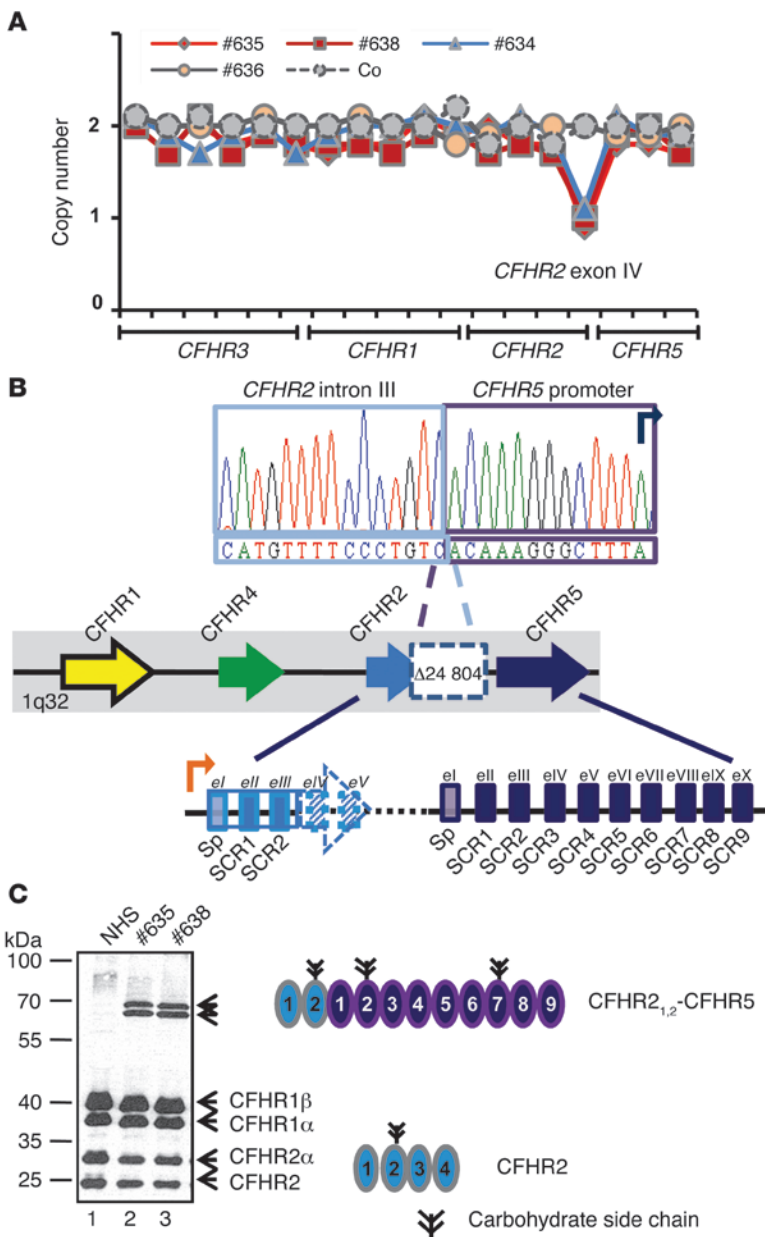


Figure 2

A novel chromosomal deletion in the *CFHR2* gene of 2 related patients with C3 glomerulopathy. **(A)** MLPA. DNA of patients (no. 635 and no. 638, red) and the father (no. 634, blue) showed a heterozygous deletion of *CFHR2* exon IV on chromosome 1q32. DNA of the mother (no. 636, orange) and a healthy individual (gray) presented with 2 copies. **(B)** Chromosomal breakpoint in the *CFHR2*-*CFHR5* region. The sequence of the PCR product generated from DNA of patients using inverse-nested PCR is shown. The presented nucleotides 1–15 correspond to intron III of *CFHR2* gene, and nucleotides 16–28 correspond to the 5' region of *CFHR5*. The structure of the *CFHR* gene cluster is presented with the indication of the deleted 24,804-nt-long segment (dashed box). The breakpoint spans from position +8,918 of *CFHR2* intron III to –12 in the *CFHR5* 5' UTR. Organization of the *CFHR2*-*CFHR5* genes. The exons coding for the signal peptide are in lighter colors and the deleted exons in the *CFHR2* gene are indicated by stippled boxes and dashed lines. **(C)** In serum of both patients, a novel protein doublet with mobilities of 70 and 65 kDa was detected (arrowheads, lanes 2 and 3). In addition, the positions of the 2 *CFHR2* isoforms are indicated. Note the lower intensity of *CFHR2* in patient sera as compared with NHS. The structures of the hybrid *CFHR2*_{1,2}-*CFHR5* protein and *CFHR2* are shown. Attached carbohydrates are indicated in black, and, alternatively, attached carbohydrates are indicated in gray.

This *CFHR2*_{1,2}-*CFHR5* hybrid protein bound to C3b with higher intensity as compared with *CFHR2* or *CFHR5* alone (Figure 3A). The recombinant *CFHR2*_{1,2}-*CFHR5* hybrid protein, as analyzed by microscale thermophoresis (NanoTemper) (19, 20), bound to fluid-phase C3b with a K_D of 96.3 nM, while factor H bound to C3b with a K_D of 735 nM (data not shown). Because *CFHR2*_{1,2}-*CFHR5*, *CFHR2*, and *CFHR5* contain carboxyterminal C3b-binding domains related to the recognition region of factor H (21–23), we analyzed by ELISA whether *CFHR2*_{1,2}-*CFHR5* as well as *CFHR2* or *CFHR5* influence the factor H–C3b interaction. *CFHR2*_{1,2}-*CFHR5* strongly reduced factor H binding to C3b and the effect was dose-dependent. The competitive effect of *CFHR2*_{1,2}-*CFHR5* was stronger than that of *CFHR2* and *CFHR5* and thus correlated with the high affinity of the hybrid protein for C3b binding (Figure 3B).

To analyze whether the *CFHR2*_{1,2}-*CFHR5* hybrid protein also binds to C3b and competes with factor H binding in serum,

patient serum either alone or mixed with NHS was absorbed by C3b and bound plasma proteins were eluted, followed by Western blotting. Again *CFHR2*_{1,2}-*CFHR5* as well as *CFHR5* bound to C3b, and the binding intensity of both proteins correlated directly with the protein levels in serum (Figure 3C). Although factor H levels were equivalent in the 4 input samples (Figure 3C), in the presence of high concentrations of *CFHR2*_{1,2}-*CFHR5*, factor H bound with lower intensity to C3b. Thus, the *CFHR2*_{1,2}-*CFHR5* hybrid influenced factor H binding to C3b and factor H binding correlated inversely with the serum concentration of the hybrid protein (Figure 3C, lanes 1–4).

*CFHR2*_{1,2}-*CFHR5* stabilizes the C3 convertase and influences dissociation of the convertase by factor H. Based on the high binding affinity of *CFHR2*_{1,2}-*CFHR5* to C3b and the competition with factor H, we next assayed how *CFHR2*_{1,2}-*CFHR5* affected convertase activity and factor B cleavage for the assembled C3 convertase

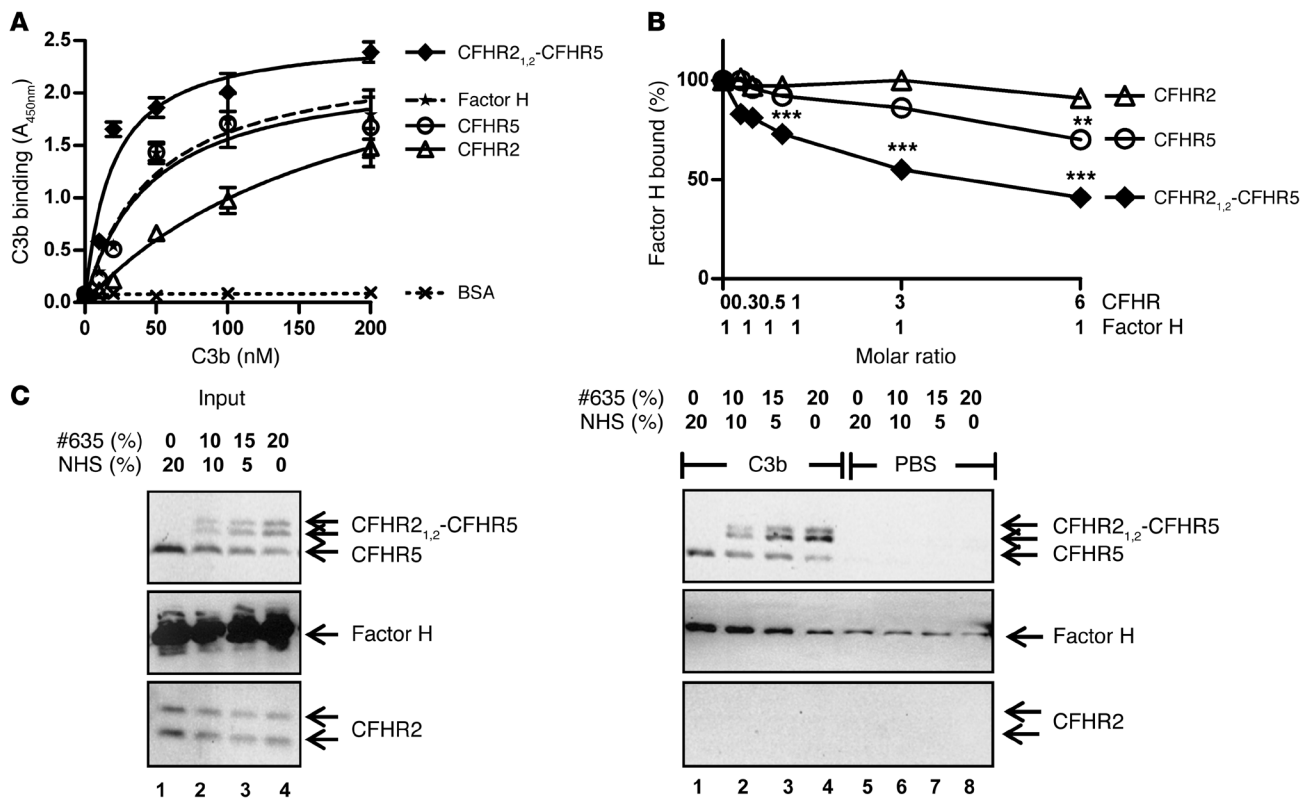


Figure 3

The CFHR2_{1,2}-CFHR5 hybrid protein interacts with C3b. (A) Recombinant CFHR2_{1,2}-CFHR5 binds with higher intensity to the C3 activation products C3b than CFHR5 and CFHR2. CFHR2_{1,2}-CFHR5 also binds with higher intensity than factor H to C3b. BSA represents no interaction. (B) The competition effect of recombinant CFHR2_{1,2}-CFHR5 on factor H binding to immobilized C3b was assayed by ELISA. CFHR2_{1,2}-CFHR5 reduced factor H binding to immobilized C3b, and bound H was detected with factor H mAb T13. Factor H interaction with C3b alone was set to 100%; CFHR2 and CFHR5 were used in addition, and these 2 proteins showed minor effects on factor H binding. **P ≤ 0.01, ***P ≤ 0.001. (C) Serum-derived CFHR2_{1,2}-CFHR5 binds to and influences binding of factor H to C3b. Protein input. NHS alone or in combination with sera of patient no. 635 (20%:0%, 10%:10%, 5%:15%, 0%:20%, NHS/serum of patient no. 635) were separated by SDS-PAGE and detected by Western blotting. The 4 probes were absorbed to the immobilized C3b at a final serum concentration of 20%. CFHR2_{1,2}-CFHR5 and CFHR5 bound to C3b correlated with input levels (lanes 1–4). However, factor H binding to C3b correlated inversely with the levels of CFHR2_{1,2}-CFHR5 (lanes 1–4). No binding of CFHR2 was detected in this set up (lanes 1–4). The blot shown is representative of 3 independent experiments.

(24). To this end, convertase-attached Bb was assayed by Western blotting. CFHR2_{1,2}-CFHR5 enhanced C3 convertase formation, and the effect was dose-dependent (Figure 4A, lanes 1–3). CFHR2, or CFHR5 used at the same concentrations, had minor effects (Figure 4A, lanes 4–6 and 7–9, respectively). In addition, we assayed whether CFHR2_{1,2}-CFHR5 influences factor H-mediated dissociation of the C3 convertase and analyzed the remaining attached Bb. At a concentration of 50 nM, CFHR2_{1,2}-CFHR5 reduced factor H-mediated dissociation as a consequence of stabilizing convertases (Figure 4B, lanes 1–3).

To quantitate the enhancement in C3 convertase assembly and stability in presence of CFHR2_{1,2}-CFHR5, Bb attachment was measured by ELISA. At a concentration of 50 nM, CFHR2_{1,2}-CFHR5 enhanced convertase formation by 69%, while at the same concentration, CFHR2 and CFHR5 enhanced the assembly by 3% and 19%, respectively (Figure 4C). Thereafter, the influence of factor H on CFHR2_{1,2}-CFHR5-stabilized convertases was measured. The rate of convertase dissociation was slower as compared with the convertase in the absence of CFHR2_{1,2}-CFHR5, and Bb levels dropped by 34% (Figure 4C). The effect on the decay was

confirmed when the activation fragment Ba was analyzed in the supernatant. CFHR2_{1,2}-CFHR5 increased Ba generation, and the hyperactivity of the convertase was limited by factor H (Figure 4D, lanes 1–3). Thus, CFHR2_{1,2}-CFHR5 stabilized the C3 convertase of the alternative pathway, enhanced convertase formation, and, by limiting access for the regulator factor H, attenuated dissociation of the convertase.

CFHR2_{1,2}-CFHR5 activates alternative pathway in patient's serum. Plasma addition is a therapeutic option for membranoproliferative glomerulonephritis type II and DDD (25). After patient no. 635 received a plasma infusion (540 ml fresh frozen plasma), Ba levels remained high (21 mg/l) and C3 levels remained low. Also, AP50 and CH50 remained low (Table 1), and C3d levels increased transiently from 38 mU/l prior to treatment to 71 mU/l after treatment. Similarly, sC5b-9 levels increased from 694 ng/ml prior to plasma infusion to 1,799 ng/ml directly after plasma infusion (Table 1). Thus, plasma addition triggered complement activation.

To demonstrate directly that the lack of response to plasma addition was due to stabilization of the C3 convertase by CFHR2_{1,2}-CFHR5 in patient's plasma, serum of patient no. 635 was mixed in vitro with

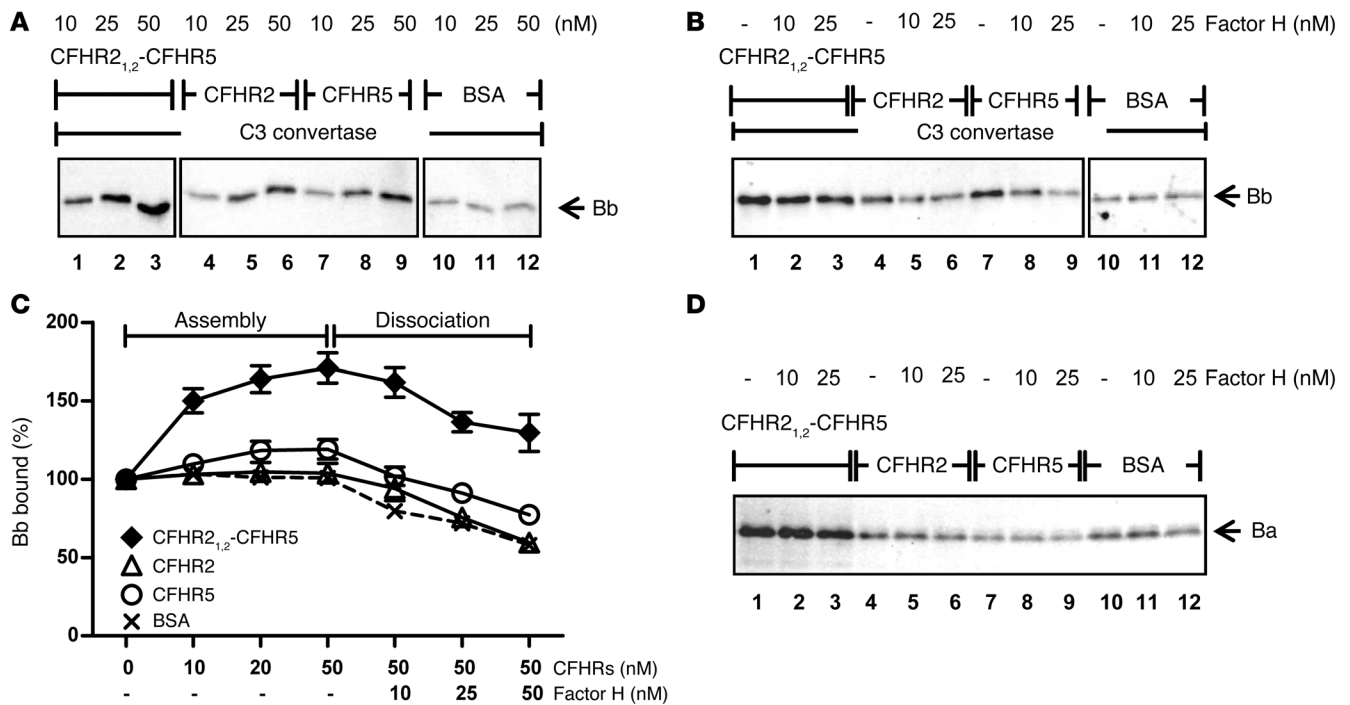


Figure 4

The CFHR2_{1,2}-CFHR5 hybrid protein deregulates the C3 convertase. (A) The alternative pathway C3 convertase C3bBb (Mg²⁺) was assembled, and attached Bb was measured by Western blotting. CFHR2_{1,2}-CFHR5 (10, 25, and 50 nM) increased Bb attachment and thus convertase formation (lanes 1–3), and CFHR2 and CFHR5 added at the same concentration had less effect (lanes 4–6 and 7–9, respectively). BSA had no effect (lanes 10–12). (B) Factor H–mediated convertase dissociation was dose-dependent for all 3 situations. In presence of CFHR2_{1,2}-CFHR5, more C3 convertases were formed and more resistant to factor H–mediated decay. (C) The convertase assembly and stability in presence of CFHR2_{1,2}-CFHR5, CFHR2, or CFHR5 were quantitated by ELISA. With CFHR2_{1,2}-CFHR5, convertase formation was enhanced by 69% and more resistant to factor H–mediated decay. The convertase assembly with BSA was sent to 100%. (D) Ba levels were determined in the supernatant after reaction. Ba levels, which were high in the presence of CFHR2_{1,2}-CFHR5 (50 nM) (lane 1), were slightly reduced when factor H was added (10 and 25 nM, lanes 2 and 3), the amount of Ba was higher as compared with the set up when factor H was added to C3 convertase in the absence of CFHR2_{1,2}-CFHR5 (BSA; lane 10–12). CFHR2 and CFHR5 alone did not influence the Ba levels significantly (lane 4–6 and lane 7–9). Blots shown in A and B were run in the same gels but were noncontiguous.

NHS at 2% increments and complement activation was followed on the level of C3 and C5 convertase activity and for terminal complement complex (TCC) formation. Mixing serum of patient no. 635 with NHS restored C3 levels dose dependently (Supplemental Figure 4), but C3a generation showed a triphasic response. In the first phase, until ratios of 12%:8% patient serum/NHS were reached, C3a levels increased linearly and dose dependently. At a ratio of 10%:10% patient serum/NHS, thus when the hybrid CFHR2_{1,2}-CFHR5 was diluted by 50%, C3a generation exceeded the levels obtained with NHS alone by about 20%. Thus, the CFHR2_{1,2}-CFHR5–stabilized C3 convertase cleaved the newly added substrate C3 and activated complement. Further dilution of patient’s serum (9%:11% patient serum/NHS, data not shown, and 8%:12% patient serum/NHS) and of CFHR2_{1,2}-CFHR5 decreased C3a levels (phase II). At ratios of 8%:12% patient serum/NHS and those with increased NHS, C3a generation increased in a normal dose-dependent manner (phase III) (Figure 5A). A rather similar response was observed for C3b deposition (Figure 5A), but TCC formation was less affected (Figure 5A). The activity of the C5 convertase, as assayed by C5a generation, increased dose dependently (Figure 5A).

The enhanced activity of the stabilized C3 convertase was confirmed when patient’s serum was supplemented directly with the substrates of the C3 convertase, i.e., C3 and factor B (Supplemen-

tal Figure 5). The stabilizing effect on the serum C3 convertase by CFHR2_{1,2}-CFHR5 was apparent when the serum was supplemented with only C3 and C3 was processed. Higher convertase activity was

Table 1

Complement parameters prior to and after plasma infusion in patient no. 635

	Plasma addition			Normal
	Prior	After	24 hours after	
	04.03.2012	04.03.2012	04.04.12	
CH50 (%)	57	72	58	65–135
AP50 (%)	10	10	12	60–140
C3 (mg/ml)	0.27	0.32	0.43	0.89–1.87
C3d (mU/l)	38	71	41	<40
C5b-9 (ng/ml)	694	1,799	569	<320
C4 (mg/ml)	0.20	0.19	0.27	0.12–0.33

Patient no. 635 received plasma infusion (540 ml fresh frozen plasma), and complement parameters were followed prior to treatment (Prior), directly after plasma treatment (After), and 24 hours after treatment (24 hours after). Substitution with 540 ml plasma did not significantly improve complement activity or AP50 and CH50 levels, and C3 levels remained low. C3d and particularly soluble C5b-9 levels increased upon treatment.

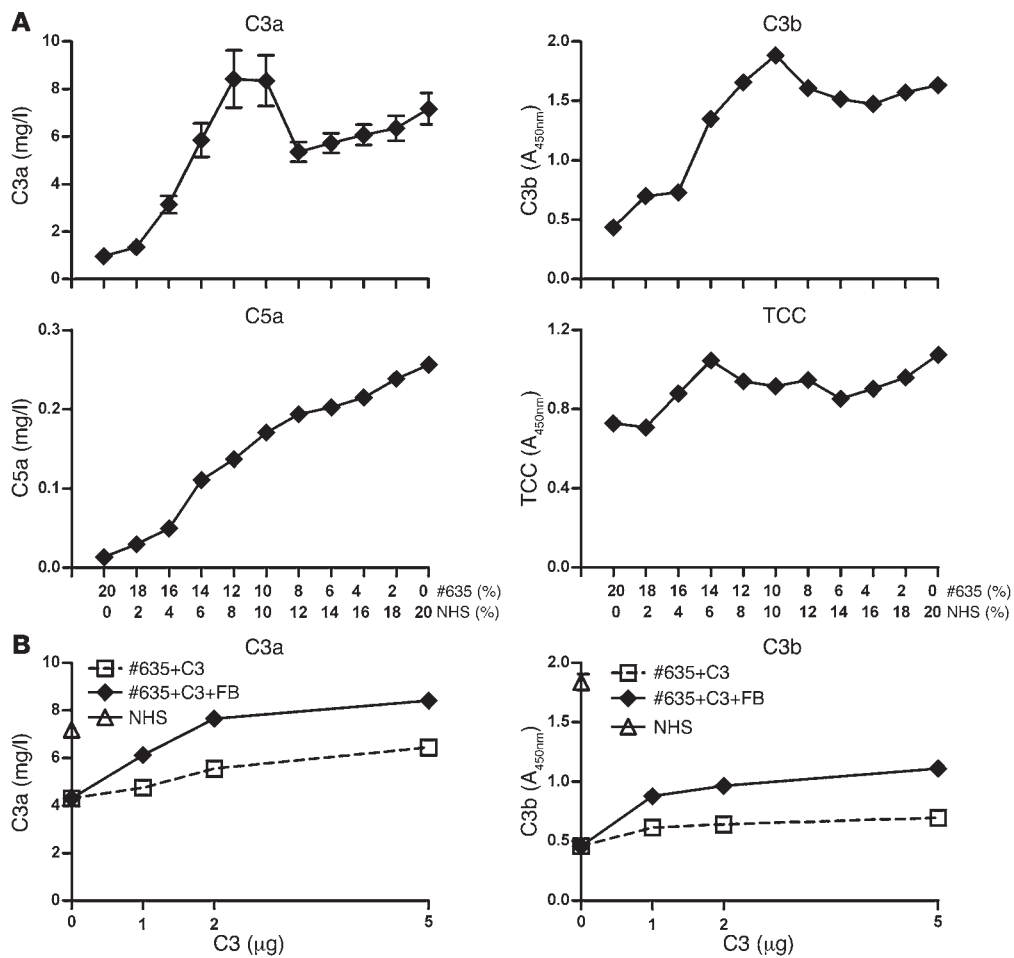


Figure 5

Enhanced C3 convertase activity in serum of patient no. 635. (A) Serum of patient no. 635 was mixed with NHS at 2% increment. Following incubation, complement activation was followed on the level of the C3 convertase by measuring C3a release and C3b deposition and on the level of C5 convertase activity by measuring C5a release and TCC surface deposition. Upon increase of NHS (20%:0%–12%:8%, serum of patient no. 635/NHS), C3a generation increased. This increase correlated with C3b deposition. At ratios of 12%:8% and 10%:10%, C3a generation and C3b deposition reached and exceeded the levels obtained achieved with NHS alone. With further increase of NHS levels and reduced patient no. 635 levels (8%:12%–0%:20%), C3a and C3b generation were decreased, and starting from ratios of 8%:12% to 0%:20%, the 2 parameters increased linearly. C5a levels showed a continuous increase and TCC surface deposition was less affected. (B) Serum of patient no. 635 (20%) was supplemented with the substrate C3 or with the combination of C3 and factor B. Following incubation for 1 hour, C3a generation was followed by ELISA and C3b surface deposition was monitored. The levels of C3a and C3b generated in NHS are indicated on the y axis. Note that in the C3 factor B–substituted serum C3a levels were higher than in NHS.

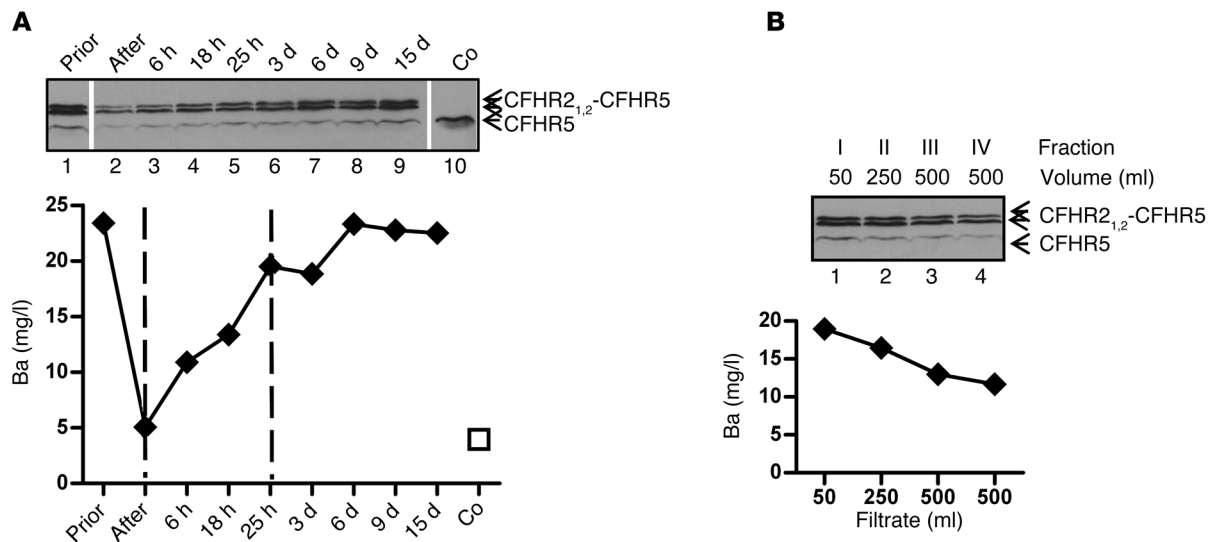
achieved when the serum was supplemented with both C3 and factor B and formed new convertases, thus confirming the activating and stabilizing effect of CFHR2_{1,2}-CFHR5 (Figure 5B). These in vitro results explain the detrimental effect of plasma substitution.

Response to plasmapheresis in patient. According to these results, 6 months later, intervention by plasmapheresis was initiated to lower the levels of the deregulating CFHR2_{1,2}-CFHR5 hybrid protein. Plasmapheresis decreased CFHR2_{1,2}-CFHR5 levels (Figure 6A, lanes 1 and 2). CFHR2_{1,2}-CFHR5 was present in the filtrate, thus confirming reduction of the protein in plasma (Figure 6B, lanes 1–4). However, 25 hours after plasmapheresis plasma, levels of the CFHR2_{1,2}-CFHR5 protein increased substantially and reached initial levels after 6 days.

In parallel, Ba levels, as a complement activation marker, were followed. Plasma Ba levels (23.1 mg/l, prior to treatment) dropped directly after plasmapheresis by 75% (8.0 mg/l) (Figure 6A). After

treatment, Ba levels increased rapidly, and, within 25 hours, Ba levels almost reached starting levels (about 80%). This rapid increase in complement activation suggested an efficient in vivo synthesis of CFHR2_{1,2}-CFHR5 and/or of the substrate proteins C3 and factor B. The correlation of increasing CFHR2_{1,2}-CFHR5 and elevated Ba plasma levels in vivo after plasmapheresis confirmed the enhancing effect of CFHR2_{1,2}-CFHR5 on the C3 convertase and the impaired complement regulation.

Modulation and inhibition of complement activation in patient’s serum. Patients with atypical hemolytic uremic syndrome and some, but not all, patients with DDD respond to therapy with the C5-blocking complement inhibitor eculizumab (26–28). Given that CFHR2_{1,2}-CFHR5 causes defective complement regulation on the level of the C3 convertase and has a minor effect on the C5 activation, we aimed to define whether eculizumab is effective

**Figure 6**

Responses to plasmapheresis in patient no. 635. **(A)** Effect of plasmapheresis. CFHR2_{1,2}-CFHR5 and Ba levels were analyzed at the indicated times after plasmapheresis. Directly after plasmapheresis, CFHR2_{1,2}-CFHR5 and Ba levels dropped by about 75%. The levels increased within the first 25 hours and reached the original concentration within 6 days. The blot shown was run in the same gel but was noncontiguous. **(B)** CFHR2_{1,2}-CFHR5 and Ba levels in filtrate fractions. The volume in ml of each fraction is indicated.

in patient's plasma (29). Eculizumab added in vitro to serum of patient no. 635, which was mixed with NHS (10% plus 10%), did not influence C3a generation (Figure 7A). C3b surface deposition was increased to about 130% (Figure 7A). This effect was unexpected, as eculizumab acts downstream of the C3 convertase. Eculizumab blocked C5a generation, the effect was dose-dependent, and TCC deposition was reduced by 37% in the patient's serum and by 56% in NHS (Figure 7A). Eculizumab showed a rather similar effect when patient serum was supplemented with the substrates C3 and factor B (data not shown). Eculizumab – as expected – had no effect on the overacting C3 convertase in patient's serum but blocked C5 cleavage and influenced TCC generation. Thus, it remains questionable whether the patients will respond to this type of complement inhibition.

In order to test other therapeutic options, the complement inhibitors soluble CR1 (sCR1) (30) and a tagged variant of the C3 inhibitor compstatin (31, 32) were tested. Tagged compstatin, i.e., CompCFH15-20, which blocks C3 activation at damaged self surfaces did not affect C3a generation (Figure 7B) but blocked C3b surface deposition by 73% (Figure 7B). In this case, eculizumab, used at a lower concentration, did not affect C3a generation or C3b surface deposition. However, sCR1, when added to mixed serum of patient no. 635, reduced C3a generation by 75% (Figure 7B) and also blocked C3b deposition to background levels (Figure 7B). Thus, sCR1 blocks the action of the CFHR2_{1,2}-CFHR5-stabilized C3 convertase and might be a potent treatment option for the patients.

Discussion

Here, we identify a genetic cause for C3G-DDD in form of a heterozygous, paternally inherited deletion of a 24,804-nt chromosomal segment in the *CFHR* gene cluster on human chromosome 1q32. The transcript derived from this mutant allele has exons I–III of *CFHR2* linked to all exons of *CFHR5*. In plasma, the translated and secreted hybrid CFHR2_{1,2}-CFHR5 protein stabi-

lizes and deregulates the alternative complement pathway C3 convertase. CFHR2_{1,2}-CFHR5, which binds with high affinity to C3b, prolongs the half-life of the convertase. This results in enhanced complement activation, consumption of the substrates C3 and factor B, and increased plasma levels of the activation marker Ba. Second, CFHR2_{1,2}-CFHR5 blocks binding or limits access of the endogenous complement inhibitor factor H and thereby reduces factor H-mediated decay acceleration of the convertase. The existence of such an overactive and deregulated convertase explains the low C3 levels in plasma of both patients, which were reduced by 80%, and also the increased levels of activated fragment Ba and of sC5b-9 through alternative pathway. This deregulation of C3 convertase by CFHR2_{1,2}-CFHR5 defines the pathogenic principle, as the overactive C3 convertase causes continuous complement activation in fluid phase and also locally causes C3b deposition along the GBM. This ultimately results in thickening of the GBM and glomerular damage.

The identification of this novel genetic deletion and of a novel hybrid protein in plasma of 2 patients with C3G-DDD initiated an intense and iterative discussion and exchange between the clinicians and the laboratory in order to define an optimal treatment and to characterize the pathological mechanism. Following the identification of the genetic deletion and the C3-stabilizing effects of CFHR2_{1,2}-CFHR5 in 2 related patients, one patient received plasma replacement therapy, with the concept of reducing the levels of the hybrid protein and also substituting CFHR2 as well as CFHR5 plasma levels. However, this treatment resulted in enhanced complement activation and increased Ba as well as sC5b-9 levels. Consequently, in the laboratory, we characterized in more detail how CFHR2_{1,2}-CFHR5 deregulates the complement convertase C3. CFHR2_{1,2}-CFHR5 is a C3 convertase-stabilizing protein that causes substrate depletion in patient's plasma and makes the C3 convertase refractory for inhibition and decay by factor H. It explains the accelerated complement activation during plasma infusion and

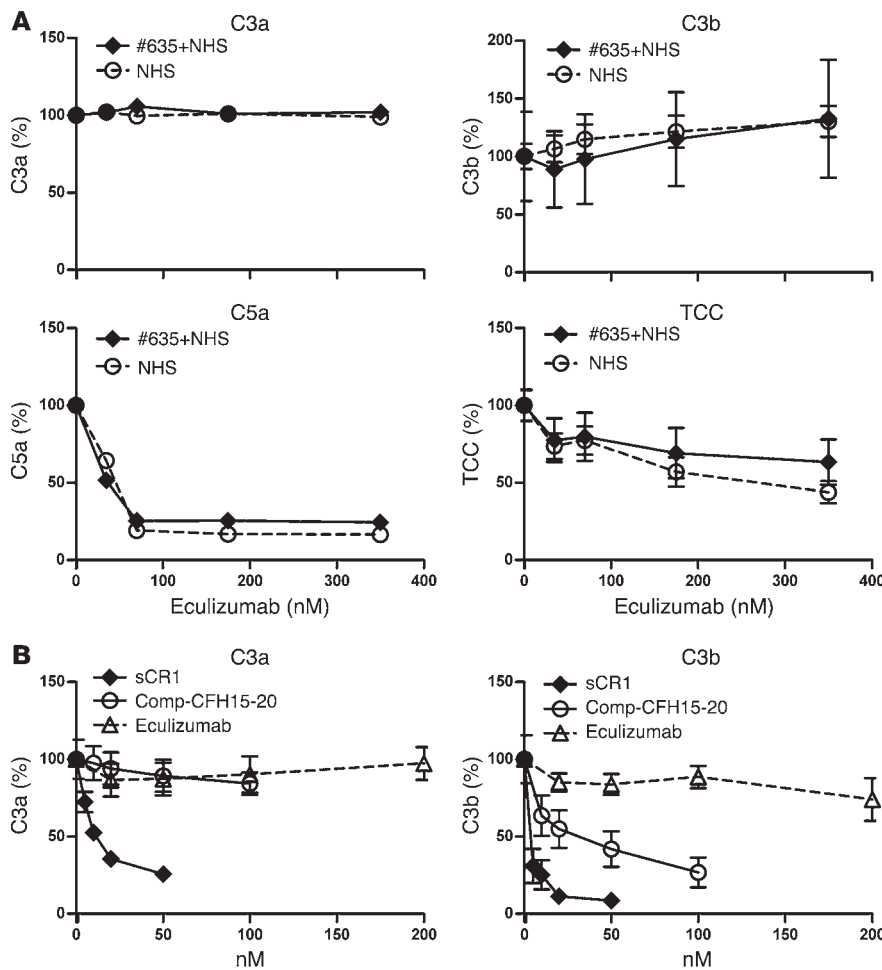


Figure 7

Modulation and inhibition of complement activation in serum of patient no. 635. (A) Effect of eculizumab in mixed patient serum. Eculizumab (35–350 nM) was added to serum of patient no. 635 mixed with NHS (10% each) or to NHS alone. Eculizumab did not affect C3a generation but augmented C3b deposition when used at 350 nM. Eculizumab blocked C5a generation and TCC deposition. The effect of mixed serum or NHS alone was set to 100%. (B) Effect of C3 convertase inhibitors and eculizumab in mixed patient serum. sCR1, when added to patients serum at 50 nM, inhibited C3a generation by 75% and C3b deposition by 92%. Comp-CFH15-20 used at 100 nM affected C3a generation by 16% but reduced C3b deposition by 73%. Eculizumab used at 200 nM showed no inhibition. The C3a and C3b levels detected in mixed serum in the absence of an inhibitor were set to 100%.

the low C3 and high Ba levels in patient’s plasma. Therefore, plasmapheresis was next initiated in the clinic in order to reduce the levels of the deregulating hybrid protein. Directly after pheresis, the plasma levels of CFHR2_{1,2}-CFHR5 decreased by 80%, and the important biomarkers C3 increased and Ba levels decreased. However, this response was short lived; after 25 hours, plasma levels of CFHR2_{1,2}-CFHR5 and complement activation were already close to the starting levels. The presence of a highly active C3 convertase in the patient’s plasma explains the failure of plasma therapy.

Next, the effect of additional complement inhibitors, i.e., eculizumab, tagged compstatin that targets the C3 convertase to the surface, and sCR1, was assayed for their effects on CFHR2_{1,2}-CFHR5-stabilized C3 convertase in patient’s plasma. In serum of patients in which plasma has been substituted, eculizumab, which acts on the level of the C5 convertase, did not influence C3a generation or C3b deposition. Tagged compstatin blocked C3b deposition but did not affect C3a generation. However, sCR1 blocked C3a generation and C3b deposition.

sCR1 and compstatin, which both affect the C3 convertase of the alternative pathway (33–35), influence the CFHR2_{1,2}-CFHR5-stabilized C3 convertase in substituted plasma of the index patients differently. These in vitro results suggest that sCR1 can be used to block CFHR2_{1,2}-CFHR5-mediated deregulation.

Characterizing how the hybrid protein overactivates and deregulates the alternative pathway C3 convertase is relevant for therapy

and intervention. Plasma levels of CFHR2_{1,2}-CFHR5, C3, and the Ba activation fragment are important biomarkers with which to follow ongoing complement activation. The 2 patients in this study have reached ESRD, are on dialysis, and are listed for renal transplantation. Therefore, these 3 parameters will be carefully evaluated and quantitated prior to, during, and after kidney transplantation to monitor efficacy of therapy and to provide an optimal outcome. An alternative therapeutic option – which is at the moment theoretical – is to develop mAbs that are specific to CFHR2_{1,2}-CFHR5 but do not bind to and do not influence the functions of intact CFHR2 and CFHR5 proteins and to use these specific mAbs for immune absorption of the hybrid protein.

In conclusion, we identified and characterized a CFHR2_{1,2}-CFHR5 hybrid protein and identified the pathogenic mechanism of this hybrid protein for C3G-DDD. These findings provide insights into the role of CFHR proteins for complement C3 convertase regulation. In agreement with previous reports, we have shown defective C3 convertase action in C3G-DDD due to overactivation and deregulation, which are caused by defective factor H (7, 9) or C3Nef (36) as well as C3 convertase and factor B autoantibodies in the absence of C3Nef (18). Here, we show that complement dysregulation due to a hybrid CFHR protein resulted from a deletion in the CFHR gene cluster. Recently, patients from Cyprus with CFHR5 nephropathy were reported (37–39). These patients present with a duplication in the CFHR5 gene and express a CFHR5 variant



composed of 11 SCRs and which has SCRs1-2 duplicated (40). In addition, a mutant CFHR1 protein with internal duplication of exons II-IV in the *CFHR1* gene (41) and a CFHR3/CFHR1 hybrid protein (42) have been associated with C3 glomerulopathy. These new genetic scenarios show that *CFHR* gene mutations and the rearrangements within the instable regions in the *CFHR* gene cluster cause C3 glomerulopathy and related kidney pathologies. This demonstrates that duplications within the N-terminal oligomerization domains of CFHR proteins can cause multimerization, increased binding, and competition with factor H to C3b. In addition, our findings also show that the CFHR2_{1,2}-CFHR5 hybrid protein activates and stabilizes the C3 convertase in fluid phase, thus causing augmented C3 consumption in plasma of the 2 presented patients. These low C3 levels differ from normal C3 plasma levels in the other patients with CFHR1- or CFHR5-related C3 glomerulopathy. Taken together, copy number and sequence variations in the *CFHR* gene cluster should be monitored in patients with kidney disease. Whether CFHR glomerulopathy will define a unique group of kidney disorders or whether patients with *CFHR* gene mutations represent distinct kidney pathologies among the growing spectra of C3 glomerulopathies needs to be evaluated.

Methods

Patients. The index family has 2 affected children. In 1987, at the age of 2, patient no. 635, a girl, suffered from urinary tract infection followed by proteinuria and hematuria. Similar symptoms were also found in her older brother (patient no. 638). No other clinical signs were noted, and serology for antinuclear antibody and antineutrophil cytoplasmic autoantibody was negative. The proteinuria was steroid resistant, increased slowly over the years, and developed into nephrotic syndrome. In 2000, renal biopsies of both siblings revealed prominent C3c staining along the GBM and also partly within the mesangium but no relevant immunoglobulin staining. Electron microscopy in one reprocessed paraffin-embedded biopsy showed intense partly ribbon-like, electron-dense deposits within and along the GBM and in the mesangium. The observed images were interpreted as evidence of C3 glomerulopathy in both cases. In the case analyzed by electron microscopy, some features were reminiscent of DDD. Renal function decreased in late adolescence in both siblings. The sister and brother reached ESRD at 20 years of age and 23 years of age, respectively, and both are treated by peritoneal dialysis. Their parents were considered healthy, although the father showed a low degree of proteinuria and hematuria and presents with relatively normal renal function through the present time (Figure 11). In 1955, their paternal grandfather died of kidney failure at the age of 27. In children and family members, complement parameter C3, Ba, and sC5b-9 levels and C3Nef, C3b, and factor B autoantibodies were analyzed (18, 36, 43).

Genetic analysis. Genomic DNA from the patients, family members, and healthy control individuals was extracted from peripheral blood using the PAXgene Blood DNA Kit (Qiagen). Abnormal copy numbers of factor H and genes encoding factor H-related proteins, CFHR3, CFHR1, CFHR2, and CFHR5, were assayed by MLPA, which was done with the SALSA MLPA P236-A1 ARMD Kit (MRC-Holland) (44). Data were analyzed by the GeneMarker software v1.85 (SoftGenetics).

The breakpoint of the deletion in *CFHR2-CFHR5* gene region was identified with long-distance inverse-nested PCR (45). Genomic DNA of both patients and a control individual was digested with *EcoRI* (New England Biolabs) according to specifications in the known sequence. Digested DNA was heated to 65°C for 15 to 20 minutes to inactivate the restriction enzyme and circularized by self-ligation in 100 µl reaction mixture under low concentration (0.1–1.0 ng/µl reaction) at 16°C for 16 hours with T4

DNA ligase (New England Biolabs) (45). The self-ligated products were purified using the Invisorb DNA Cleanup (Invitrogen). PCR amplification of the deletion breakpoint was performed by using 2 pairs of primers, QCL-1/QCR-1 and QCL-2/QCR-2 (Supplemental Table 1). Primers QCF-3/QCR-3 were designed immediately upstream of the breakpoint and in CFHR5 intron I to amplify the bridge in patient DNA (Supplemental Table 1). Amplified PCR fragments were separated by capillary electrophoresis on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems) using the size standard ROX 500 (Applied Biosystems).

Plasma analysis. Plasma proteins from patients and a healthy individual were separated on SDS-PAGE under nonreducing conditions and blotted onto nitrocellulose membrane (Waterman) (46). The membranes were incubated with CFHR1-CFHR2-reacting antiserum (CFHR1 polyclonal antibody [pAb]; generated in house) (47, 48), followed by HRP-conjugated secondary antibodies (swine anti-rabbit; DAKO). Plasma was treated with N glycosidase F (Roche Diagnostics) and separated by SDS-PAGE electrophoresis, and, after transfer, the membrane was treated with CFHR2-CFHR5-reacting antiserum (generated in house).

Protein expression and purification. Full-length CFHR5 cDNA sequence was amplified from a plasmid pPICZαB-CFHR5 (generated in house) using primers CFHR5-F/CFHR5-R. Restriction sites for endonucleases *KpnI* and *XbaI* (New England Biolabs) are underlined in Supplemental Table 1. The amplified fragment was cloned into the pCDNA4/To/*myc*-His B expression vector (Invitrogen). Hybrid CFHR2_{1,2}-CFHR5 was generated by insertion of CFHR2 exon I-III using domain swapping, according to the manufacturer's instructions (Stratagene), with primers CFHR2-5-F/CFHR2-5-R (Supplemental Table 1 and ref. 49). Recombinant CFHR2_{1,2}-CFHR5 and CFHR5 were expressed in HEK 293 cells (ACC305) by transient transfection with polyethylenimine (jetPEI, Polyplus) (50, 51). Secreted CFHR2_{1,2}-CFHR5 and CFHR5 were purified from the culture supernatant by affinity chromatography. Supernatants were applied onto a Hitrap NHS-activated HP column (GE Healthcare) coated with CFHR1 pAb. Purified proteins were concentrated by Amicon Ultra-15 filters (Millipore Corporation) and dialyzed against Dulbecco's phosphate-buffered saline (DPBS, Lonza). Recombinant CFHR2 was used as a control protein and was expressed in *Pichia* system (52).

Protein interaction studies — C3b binding and competition assay. Recombinant CFHR2_{1,2}-CFHR5, CFHR2, CFHR5, factor H (CompTech), or BSA, each at 100 nM, were bound onto MaxiSorp microtiter plates (F96 MaxiSorp, Nunc-Immuno Module) at 4°C overnight. After blocking with DPBS containing 2% BSA for 1 hour, C3b (CompTech, 10–200 nM) was incubated for 1 hour at room temperature and bound C3b was detected with C3b mAb (Fitzgerald). OD values were measured at 450 nm (47).

Competition between factor H and the CFHR2_{1,2}-CFHR5 hybrid protein bound to C3b was evaluated by ELISA (47, 53). C3b was immobilized and blocked as above, and factor H alone (25 nM) or in combination with CFHR2_{1,2}-CFHR5 at increasing amounts (0–150 nM), reflecting the molar ratios of CFHR2_{1,2}-CFHR5 to factor H from 0:1 to 6:1, was incubated at 37°C for 1 hour. Bound factor H was detected with mAb T13 (generated in house). OD values were measured at 450 nm. The CFHR2_{1,2}-CFHR5-factor H competition happening on a native approach was determined in serum. Serum of patient no. 635 and NHS were combined under different percentages (serum of patient no. 635/NHS = 0%:20%, 10%:10%, 15%:5%, 20%:0%) and incubated on surface-immobilized C3b at 37°C for 1 hour, and the bound proteins were separated by SDS-PAGE under nonreducing conditions and visualized by Western blotting. The membrane was cut according to the protein size and blotted with appropriate antibodies. CFHR2_{1,2}-CFHR5 and CFHR5 were detected with CFHR5 pAb (R&D Systems), factor H was detected with factor H polyclonal antiserum (CompTech), and CFHR2 was detected with CFHR1-CFHR2-reacting antiserum.



Alternative pathway C3 convertase activity. The influence of CFHR2_{1,2}-CFHR5 on the C3 convertase of the alternative pathway was investigated using a solid-phase C3 convertase stability assay (47). The C3 convertase, C3bBb (Mg²⁺), was assembled by coating C3b (5 µg/ml) onto microtiter plates. Then, factor B (2.5 µg/ml, CompTech), factor D (0.5 µg/ml, CompTech), and factor P (2 µg/ml, CompTech), together with the CFHR2_{1,2}-CFHR5 (at 10, 25, and 50 nM), were added and incubated for 20 minutes at 37°C. Following incubation, the formed C3 convertases were eluted and separated by SDS-PAGE, and the amount of attached Bb was evaluated by Western blotting with factor B pAb (CompTech).

The effect of factor H for decay acceleration of C3 convertases assembled and stabilized by CFHR2_{1,2}-CFHR5 or by CFHR2 and CFHR5 was assayed. C3 convertases were formed in the presence of constant amounts of CFHR2_{1,2}-CFHR5, CFHR2, or CFHR5 (50 nM) with increasing factor H (0–50 nM) by incubation at 37°C for 20 minutes, and remaining bound Bb was analyzed by Western blotting with factor B pAb. For a quantitative evaluation of the effect of CFHR2_{1,2}-CFHR5, CFHR2, or CFHR5 on convertase assembly and the influences of dissociation by factor H, similar experiments were done and the amount of attached Bb was determined by ELISA with factor B pAb; the OD values were measured at 450 nm. In addition, Ba levels were determined in the supernatant after adding factor H to the assembled convertases. To this end, after the reaction, supernatant was separated by SDS-PAGE and transferred to a membrane and the Ba fragment was identified by factor B antiserum.

Deregulation of alternative pathway. Deregulation of CFHR2_{1,2}-CFHR5 on the alternative pathway was analyzed using an optimal version of an assay described by Roos et al. and others (54, 55) with serum of patient no. 635. Microtiter plates were coated with LPS (10 µg/ml, Sigma-Aldrich) for activation through the alternative pathway overnight at 4°C and blocked for 1 hour with 2% BSA. Serum of patient no. 635 was mixed with NHS at a 2% increment, and total C3 serum levels were measured upon mixing using the Human C3 ELISA (Immunology Consultants Laboratory). Serum fractions were preincubated for 15 minutes at 37°C in MgEGTA buffer and, thereafter, added to the preparative wells at 37°C for 1 hour. C3a and C5a in supernatant were quantitated with the C3a Plus and C5a Enzyme Immunoassay (QUIDEL). C3b deposition and TCC formation on the surface were measured by ELISA at 450 nm using C3b mAb (Fitzgerald) and C5b-9 mAb (DAKO), respectively.

To reach close to normal substrate levels in patient serum, 20% serum of patient no. 635 was supplemented with substrate C3 (1, 2, 5 µg; CompTech) alone or in combination with factor B (0.5, 1, 2 µg). The C3 serum levels were quantitated with Human C3 ELISA, and the factor B levels were measured by Western blotting. Serum fractions were treated as described above. C3a and C5a levels and C3b and TCC deposition were measured.

Response to plasma therapy in patient no. 635. Patient no. 635 received a plasma infusion (540 ml fresh frozen plasma), and complement parameters, including the total serum alternative hemolytic complement (AP50) and the classic hemolytic complement (CH50) tests as well as CFHR2_{1,2}-CFHR5 hybrid protein levels and Ba, C3, C3d, and sC5b-9 levels, were followed prior to, directly after, and 24 hours after plasma treatment as described above. Six months later, intervention by plasmapheresis was initiated (total of 3 liters electrolyte solution substituted with 5% human serum albumin). The levels of the CFHR2_{1,2}-CFHR5 hybrid protein and Ba, as the marker of complement activation, were followed in patients' plasma prior to and directly after treatment for up to 15 days. In addition, these markers were evaluated in the filtrate fractions obtained by plasmapheresis. For determination of CFHR2_{1,2}-CFHR5, serum and the filtrate obtained upon plasmapheresis were separated by SDS-PAGE and transferred to a membrane, and the CFHR2_{1,2}-CFHR5 was identified by Western blotting with CFHR5 pAb. The Ba levels were determined by Ba Enzyme Immunoassay (QUIDEL).

In vitro effect of inhibitors on complement activation. Eculizumab (35–350 nM, ALEXION) was added to serum of patient no. 635 mixed with NHS (10% each) or to NHS alone. Following incubation, complement activation was followed on the level of the C3 convertase and C5 convertase activity as described above. In addition, sCR1 (5–50 nM; R&D Systems) and a tagged compstatin that fuses to factor H SCR 15–20 (Comp-CFH 15–20, 10–100 nM; generated in house) were added to serum of patient no. 635 mixed with NHS (10% each), respectively. Serum fractions were preincubated for 15 minutes at 37°C in MgEGTA buffer and, thereafter, added to the LPS-coated wells at 37°C for 1 hour. Following incubation, the activated C3a levels in supernatant were quantitated with C3a Plus Enzyme Immunoassay (QUIDEL). After washing, the C3b deposition on the surface was measured by ELISA at 450 nm using C3b mAb.

Statistics. Statistical analyses of the data shown in Figure 1J; Figure 3, A and B; Figure 4C; and Figures 5–7 were done by Student's 2-tailed *t* test for unpaired data. Results represent mean ± SEM of 3 independent experiments. The *P* value ≤ 0.05 was considered to be statistically significant. All blots are representative of 3 independent experiments.

Study approval. This study was approved by the Ethical Board of the Friedrich Schiller University Jena. The patients provided written informed consent for participation in this study.

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