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## **Factor H-Related Protein 4 Activates Complement by Serving as a Platform for the Assembly of an Alternative Pathway C3 Convertase via its Interaction with C3b**

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Running title: Interaction of CFHR4 with C3b allows C3 convertase formation

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**Background:** Complement factor H-related protein 4 (CFHR4) has been shown to interact with C3b.

**Results:** Binding of C3b to CFHR4 allows the formation of an active alternative pathway C3 convertase.

**Conclusion:** CFHR4 activates complement via the alternative pathway.

**Significance:** A novel function is identified for CFHR4 that stands in contrast with the complement inhibitory activity of factor H.

### **SUMMARY**

**Human complement factor H-related protein (CFHR) 4 belongs to the factor H family of plasma glycoproteins that are composed of short consensus repeat (SCR) domains. While factor H is a well-known inhibitor of the alternative complement pathway, the functions of the CFHR proteins are poorly understood. CFHR4 lacks SCRs homologous to the complement inhibitory domains of factor H and, accordingly, has no significant complement regulatory activities. We have previously shown that CFHR4 binds C-reactive protein via its most N-terminal SCR, which leads to classical complement pathway activation. CFHR4 binds C3b via its C-**

**terminus, but the significance of this interaction is unclear. Therefore, we set out to clarify the functional relevance of C3b binding by CFHR4. Here, we report a novel role for CFHR4 in the complement system. CFHR4 serves as a platform for the assembly of an alternative pathway C3 convertase by binding C3b. This is based on the sustained ability of CFHR4-bound C3b to bind factor B and properdin, leading to an active convertase that generates C3a and C3b from C3. The CFHR4-C3bBb convertase is less sensitive to the factor H-mediated decay compared to the C3bBb convertase. CFHR4 mutants containing exchanges of conserved residues within the C-terminal C3b binding site showed significantly reduced C3b binding and alternative pathway complement activation. In conclusion, our results suggest that, in contrast to the complement inhibitor factor H, CFHR4 acts as an enhancer of opsonization by promoting complement activation.**

Complement is a powerful effector system of innate immunity, with major roles in the elimination of microbes, inflammatory processes, disposal of cellular debris, and modulation of adaptive immunity (1). A multi-level regulation ensures complement activation on dangerous surfaces but prevents its harmful effects on host

cells and tissues. This is achieved by the expression of complement inhibitors on host cell surfaces and the absence of those on foreign or altered self surfaces, as well as by soluble regulators having distinct binding specificities for different surfaces (2).

The plasma glycoprotein factor H (FH)<sup>1</sup> is the major soluble inhibitor of the alternative complement pathway (AP) (3, 4). It prevents the formation of the AP C3 convertase C3bBb by blocking the binding of factor B (FB) to C3b, and accelerates the decay of existing C3 convertases by displacing Bb. It also acts as a cofactor for the serine protease factor I (FI) in the cleavage of C3b to inactive C3b (iC3b), which can no longer form a convertase (4, 5). By these mechanisms, FH inhibits the amplification of the complement cascade both in fluid phase and on the surface of host cells (3, 4).

FH, its splicing variant complement factor H-like protein 1 (CFHL1), and five complement factor H-related proteins (CFHR1-CFHR5), which are encoded by separate genes, constitute the human factor H protein family (6). FH is composed of 20 short consensus repeat (SCR) domains, CFHL1 contains SCRs 1-7 of FH, and the CFHR proteins consist of four to nine SCRs that are homologous to various domains of FH. Whereas CFHL1 shares the complement inhibitory activities of FH, the physiological roles of the CFHR proteins are less well understood.

CFHR4 is detected in human plasma as two different glycoproteins. The 86-kDa long isoform termed CFHR4A consists of nine SCRs (7). The ~45-kDa shorter isoform termed CFHR4B is composed of five SCRs that represent SCR1 and SCRs 6-9 of CFHR4A (Figure 1) (7, 8). In CFHR4A, SCRs 1-4 and SCRs 5-8 are highly related based on nucleotide and amino acid sequence identity, likely as a result of an intramolecular duplication (Figure 1) (7).

Like all CFHRs, both CFHR4 isoforms lack SCRs homologous to the N-terminal complement inhibitory domains SCRs 1-4 of FH and CFHL1. In agreement with this, no significant cofactor- and convertase decay accelerating activities have been reported for CFHR4B (9). The two most C-terminal domains of CFHR4A and CFHR4B are homologous to the C-terminal FH domains SCRs 19-20 that contain C3b/C3d binding sites. CFHR4B has been shown to bind

the C3d region of C3b via its C-terminal SCRs 4-5 (9, 10). However, except for a slight enhancement of the cofactor activity of FH in the presence of high (and rather non-physiological) CFHR4B concentrations (9), no significant functions have been associated with this C3b binding capacity. The CFHR4A isoform has not yet been characterized for its interaction with C3b and complement regulatory activity.

Recently, we described native pentameric C-reactive protein (CRP) as a ligand for CFHR4A and CFHR4B, and showed that this interaction via a binding site in SCR1 leads to enhanced opsonization of necrotic cells with CRP and promotes complement activation via C4-dependent pathways (11, 12).

This study was designed to investigate the significance of C3b binding by CFHR4. We describe a novel function for CFHR4 in complement activation by providing a platform for the assembly of an active AP C3 convertase. Thereby, and in marked contrast to FH, CFHR4 enhances complement activation via its interaction with C3b.

## EXPERIMENTAL PROCEDURES

### *Proteins, sera and antibodies*

Cloning and recombinant expression of CFHR4A as well as the CFHR4A deletion mutants SCR1-3, SCR2-4, SCR4-9, SCR5-7 and SCR8-9 were described (11, 12). CFHR4B, the CFHR4B mutants D221G and D221G/K290A, as well as CFHL1 were generated via gene synthesis (GenScript, Piscataway, NJ, USA) and subcloned into the baculovirus expression vector pBSV-8His (13). The proteins were expressed in *Spodoptera frugiperda* (Sf9) cells and purified by nickel affinity chromatography.

Complement components C3b, C3d, FH and FI as well as recombinant human CRP were purchased from Merck Biosciences (Schwalbach, Germany). C3 was from Complement Technology (Tyler, TX, USA). Factor D and properdin were from Quidel (TECOmedical, Bünde, Germany), human serum albumin (HSA) was from Sigma-Aldrich (Taufkirchen, Germany) and bovine serum albumin (BSA) was purchased from AppliChem (Darmstadt, Germany). Normal human serum (NHS) was purchased from Sigma-Aldrich and PAA Laboratories (Cölbe, Germany) or was

collected from healthy individuals after informed consent. FB-depleted serum was from Quidel and C2-depleted serum was purchased from Merck Biosciences.

A novel CFHR4 antiserum was raised by immunizing rabbits with the recombinant CFHR4A fragment SCR2-4. The histidine tag of SCR2-4 was cleaved off with recombinant bovine enterokinase light chain according to the manufacturer's protocol (New England Biolabs, Frankfurt am Main, Germany). Enterokinase was removed with trypsin inhibitor agarose (Sigma-Aldrich). Treatment of rabbits was conducted by BioGenes (Berlin, Germany) in accordance with German animal welfare regulations.

Monoclonal anti-CFHR4 antibody (MAB5980) was purchased from R&D Systems (Wiesbaden, Germany). Horseradish peroxidase (HRP)-conjugated goat anti-human C3, which does not recognize C3d, was obtained from MP Biomedicals (Solon, OH, USA). Rabbit anti-human C3d was from Dako (Hamburg, Germany), goat anti-human FH from Complement Technology, goat anti-human CRP and anti-human FB from Merck. Monoclonal anti-properdin was purchased from Quidel. HRP-conjugated swine anti-rabbit, rabbit anti-goat and rabbit anti-mouse antibodies were from Dako.

#### *Microtiter plate binding assays (ELISA)*

Assays were conducted in Tris-buffered saline (TBS), pH 7.4, containing 140 mM NaCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> and 10 mM Tris ultra, using MaxiSorp microtiter plates (Nunc, Wiesbaden, Germany). Proteins were immobilized overnight at 4°C. For the washing steps, TBS containing 0.02% Tween-20 (Sigma-Aldrich) was used. Remaining binding sites were blocked with 3% dry milk in TBS containing 0.02% Tween-20 for 2 h at 20°C. If not stated otherwise, ligands/serum samples were added for 30 min at 37°C. Antibodies were added for 45 min at 20°C. TMB PLUS Substrate (Kem-En-Tec Diagnostics, Taastrup, Denmark) was used for the visualization and the absorbance was measured at 450 nm.

#### *Determination of CFHR4 plasma concentration*

CFHR4 plasma concentration was determined in sandwich ELISA using immobilized monoclonal mouse anti-CFHR4 as catch antibody and the CFHR4 rabbit antiserum as detection antibody.

Both the monoclonal antibody and the antiserum recognize the two CFHR4 isoforms (data not shown). Serum samples were diluted in TBS without CaCl<sub>2</sub> and MgCl<sub>2</sub> containing 10 mM EDTA to prevent complement activation. Calculation of CFHR4 content in serum samples was based on duplicate serial dilutions from two independent experiments. Results were obtained by four parameter logistic analysis of recombinant CFHR4A standard curves in SigmaPlot 10 (Systat Software, Erkrath, Germany).

#### *Assays with serum-opsonized yeast cells*

*Saccharomyces cerevisiae* strain ATCC9763 (American Type Culture Collection, Manassas, VA, USA) was cultured in YPD medium shaking over night at 30°C. Cells were harvested, washed three times with TBS and immobilized in microtiter plates for 30 min at 20°C. Saturation of the well surface with bound yeast cells was controlled by microscopic analysis throughout the experiment. Unbound cells were washed away and, after blocking, wells were incubated with TBS containing 5% NHS or with TBS only for 30 min at 37°C. After incubation with FH or CFHR4A (100 nM each), the binding of these proteins as well as the deposition of C3 fragments were analyzed by ELISA.

#### *Cofactor assays*

Cofactor activity of CFHR4A and enhancement of FH cofactor activity were determined in fluid-phase C3b cleavage assays. C3b (530 ng) and FI (90 ng) were incubated alone or in combination with FH (150 ng), CFHL1 (55 ng) and CFHR4A (1.3 µg) for 45 min at 37°C in a final volume of 15 µl in TBS. Reactions were stopped by the addition of reducing SDS-sample buffer. Samples were separated on 11% SDS-PAGE and subjected to Western blot analysis using HRP-conjugated anti-C3 antibody. A chemiluminescent detection kit (Applichem) was used to visualize the protein bands.

#### *Convertase assays*

C3bBb convertases were generated on C3b, immobilized at 5 µg/ml in microtiter plate wells, by the addition of FB (2 µg/ml), factor D (0.1 µg/ml), properdin (4 µg/ml) and BSA (10 µg/ml) in 50 µl for 30 min at 37°C. Decay acceleration was analyzed by the addition of

increasing concentrations of CFHR4A, FH and HSA for 30 min at 37°C. Remaining intact convertases were detected with anti-FB antiserum.

Convertases were assembled on immobilized CFHR4A or its deletion fragments SCR1-3 and SCR4-9 (100 nM) in the same way as described for the C3bBb convertase after preincubation with 5 µg/ml C3b for 30 min at 37°C. In some experiments, CFHR4A was incubated with 10 µg/ml CRP prior to the addition of C3b and building up the convertase. Activity of the convertases was measured by adding 10 µg/ml C3 for 1 h at 37°C and analyzing the supernatant for generated C3a fragments using a C3a ELISA kit (Quidel).

#### *Complement activation assays*

CFHR4A, CFHR4B, SCR1-3, SCR4-9, FH and HSA were immobilized in equimolar amounts (100 nM) and incubated with 5% NHS for 30 min at 37°C. To measure AP activation, TBS containing 3 mM MgCl<sub>2</sub> and 5 mM EGTA was used. Deposition of C3b, FB and properdin was measured by ELISA using the respective antibodies.

#### *Sequence analysis*

Protein sequences were aligned and analyzed with the MegAlign module of the Lasergene 7 software (DNASTAR, Madison, WI, USA) using the Jotun-Hein method.

## **RESULTS**

#### *CFHR4 serum concentration*

In order to establish the physiological concentration of CFHR4, we determined CFHR4 concentrations in eleven serum samples from single donors and in four different pooled sera. CFHR4 concentration in healthy single donor sera ranged from 6.5 µg/ml to 53.9 µg/ml with a mean value of 25.4 µg/ml. Similarly, the mean concentration of CFHR4 in the four tested pooled sera was 24.9 µg/ml (range 19.3-34.0 µg/ml).

#### *C3b binds to the C-terminal SCRs 8-9 of CFHR4A*

To investigate the relevance of C3b binding to CFHR4, we first analyzed the binding of C3b to CFHR4A, and compared it with the C3b binding to CFHR4B, which was previously shown to bind C3b and C3d (9, 10). C3b bound dose-dependently

to both CFHR4 isoforms and to FH immobilized in microtiter plates (Figure 2A). The binding of C3b to CFHR4A and FH was comparable, but a weaker C3b binding to CFHR4B was observed (Figure 2A). Both CFHR4 isoforms also bound inactivated C3b (iC3b) and C3d, but not C3c (data not shown).

Next, we mapped the binding sites for C3b using recombinant deletion mutants of CFHR4A. C3b only showed significant binding to CFHR4A deletion constructs that contained the C-terminal SCRs 8 and 9 (Figure 2B). A similar binding profile was observed for C3d (Figure 2C).

We also analyzed CFHR4 binding to surface-bound C3b as it would occur on naturally opsonized surfaces. Binding of CFHR4A to immobilized C3b was compared to that of FH using the respective antisera (the specificities of the antisera are shown in Figure S1A). In contrast to FH, a rather weak binding of CFHR4A to C3b and iC3b was observed (Figure S1B).

Because the immobilization on plate may affect C3b conformation and accessibility of binding sites, we analyzed, whether CFHR4A can bind to C3b that is bound covalently via its activated thioester bond to a natural surface. We used the yeast *Saccharomyces cerevisiae* as a complement activating surface (14). After exposure to normal human serum (NHS), the yeast cells were opsonized with C3 fragments as demonstrated by the strongly increased C3 signal compared to the buffer control (Figure S1C). CFHR4A and FH bound weakly even to non-opsonized yeast cells and this binding increased when the yeasts were opsonized (Figure S1D).

#### *CFHR4A exhibits no significant complement inhibitory activities*

The capacity to enhance the cofactor activity of FH has been reported for CFHR4B (9). Therefore, we analyzed the CFHR4A isoform in fluid-phase cofactor assays (Figure S2A). CFHR4A displayed no own cofactor activity for the FI-mediated C3b cleavage (Figure S2A, lane 4) when compared with the control reaction containing C3b and FI only (Figure S2A, lane 1). Because weak cofactor activity has been reported for CFHR4B at very high and non-physiological concentrations (400 µg/ml) and extended incubation times (9), and also for the related CFHR3 (9, 15), we analyzed the cofactor activity of CFHR4A under

such conditions. Even after incubation of CFHR4A together with C3b and FI for five hours at 37°C, or at a concentration of 650 µg/ml (corresponding to 10 µM recombinant CFHR4A), no C3b degradation products were detected (data not shown). In comparison, C3b was readily cleaved in the presence of 10 µg/ml FH (67 nM) or 4 µg/ml CFHL1 (80 nM) as cofactors (Figure S2A, lanes 2 and 5). Although CFHR4A displays no own cofactor activity, it enhances the cofactor activity of FH. This is demonstrated by the weaker intensity of the C3b  $\alpha'$ -chain and the stronger intensity of the  $\alpha'$ -41 fragment when compared to the incubation with FH alone (Figure S2A, lane 3 in comparison to lane 2). Interestingly, CFHR4A had no detectable influence on the cofactor activity of CFHL1 (Figure S2A, lanes 5 and 6).

Next, we analyzed, whether CFHR4A has decay accelerating activity. In solid phase convertase stability assays, CFHR4A when applied up to a concentration of 20 µg/ml (308 nM) displayed no own decay accelerating activity and did not influence that of FH (Figures S2B and S2C).

Taken together, these and previously published data (9) indicate that CFHR4A and CFHR4B have no significant complement inhibitory functions at the level of C3b and the AP C3 convertase.

#### *CFHR4 activates the alternative complement pathway*

We have previously shown that CFHR4 activates C2/C4-dependent pathways via its interaction with CRP (12). To assess the role of CFHR4 in AP activation, we added human serum, diluted in Mg-EGTA buffer to allow AP activation only, to the immobilized CFHR4 isoforms. Incubation with 5% NHS led to a strong deposition of C3b on both CFHR4A and CFHR4B, whereas there was little increase in the anti-C3 signal on FH (Figure 3, white bars). No C3b deposition was detected for the CFHR4A deletion mutant SCR1-3 (containing the N-terminal CRP binding site), whereas strong C3b deposition, comparable to that of the whole proteins CFHR4A and CFHR4B, was detected for SCR4-9 (Figure 3). These results suggested that (i) CFHR4 activates complement via the AP and (ii) this activation is associated with the C-terminal part of the protein, which contains the C3b binding site.

Complement activation via the AP is dependent on the formation of the AP C3 convertase C3bBb, which assembles via the binding and activation of FB on C3b and is stabilized by properdin. We could indeed detect FB and properdin in parallel with the strong C3b deposition on both CFHR4 isoforms and the SCR4-9 deletion mutant. In contrast, the SCR1-3 deletion mutant lacking a C3b binding site showed no AP complement activation as demonstrated by the absence of C3b, FB and properdin (Figure 3). The convertase components were also not detectable on the control proteins FH and human serum albumin. Similarly, deposition of C3b on CFHR4A, CFHR4B and SCR4-9 was detected from C2-depleted human serum but not from FB-depleted serum (data not shown).

#### *CFHR4-bound C3b initiates the formation of an active AP C3 convertase*

Given the association of AP activation with the C-terminal part of CFHR4 that contains the C3b binding site, we asked, whether CFHR4-bound C3b may serve as a platform for the assembly of AP C3 convertases. To this end, CFHR4A and its deletion mutants SCR1-3 and SCR4-9 were immobilized on microtiter plates, preincubated with C3b, followed by the addition of factors D and B in the presence of properdin to allow the formation of a C3bBb convertase. Binding of FB to the CFHR4-bound C3b (FB does not bind itself to CFHR4, data not shown), indicating the assembly of the C3 convertase, was detected on CFHR4A and SCR4-9, but not on SCR1-3 (Figure 4A). To analyze, whether this CFHR4-C3bBb convertase is active, it was incubated with 10 µg/ml C3. Measurement of generated C3a indicated that this convertase indeed cleaves C3 into C3a and C3b and thus activates the AP, similar to the control C3bBb convertase that was assembled in parallel directly on immobilized C3b (Figure 4B).

We also analyzed, whether the CFHR4 ligand CRP influences the interaction of CFHR4 with C3b and thus interferes with the formation of the CFHR4-C3bBb C3 convertase. In competitive ELISA, the binding of C3b, which was added in a constant concentration to immobilized CFHR4A, was not inhibited by up to 10-fold concentrations of CRP and *vice versa* (data not shown). Likewise, CRP did not inhibit the assembly of the CFHR4A-

C3bBb convertase (data not shown). Thus, CRP and C3b can bind simultaneously to CFHR4A, allowing complement activation via the classical and the alternative pathways in parallel.

*The CFHR4-C3bBb C3 convertase shows resistance against the FH-mediated decay*

We next analyzed the stability of the CFHR4A-C3bBb convertase compared to the C3bBb convertase. To this end, both types of solid-phase convertases were assembled in microtiter plate wells, and their spontaneous decay over time as well as their decay in the presence of FH were measured. The spontaneous decay of both convertases was comparable (data not shown). However, the CFHR4A-C3bBb convertase showed resistance against the decay accelerated by FH compared to the C3bBb convertase (Figures 5A and 5B). Whereas the decay of the C3bBb convertase was strongly accelerated even at very low FH concentrations, higher amounts of FH were necessary to dissociate a similar amount of the CFHR4A-C3bBb convertase (Figure 5A). This effect was even more apparent when the decay of both convertases in the presence of a low FH concentration (2 µg/ml) was measured over time (Figure 5B). These results were confirmed by the measurement of C3a in the supernatants after adding C3 to the convertases. Generation of C3a from C3 by the C3bBb convertase was strongly reduced even at low FH concentrations (Figure 5C, white bars), whereas the inhibitory effect of FH on the activity of the CFHR4A-C3bBb convertase was significantly lower, i.e. requiring more FH to achieve the same effect (Figure 5C, black bars). At 20 µg/ml FH, C3a levels were reduced by approximately 40-50% for the CFHR4A-C3bBb convertase as compared to nearly 90% for the C3bBb convertase. In conclusion, the decay of the CFHR4A-C3bBb convertase is less efficiently accelerated by FH compared to that of the C3bBb convertase.

*Mutation of conserved residues of the C3b binding site in CFHR4 significantly reduces C3b binding and AP complement activation*

The molecular basis of C3b binding to SCRs 19-20 of FH was analyzed recently (16, 17). All CFHR proteins contain domains that are homologous to these C-terminal FH domains as shown by alignment in Figure 6. Kajander et al.

propose that the FH C-terminus contains two C3b/C3d binding sites. Key residues of the SCR19 site, namely D1119 and Q1139, and also K1188, are conserved in the FH protein family, whereas T1184, R1203 and K1230 of the SCR20 site are found in FH and CFHR1 only (16) (Figure 6). We assumed that C3b binding by CFHR4 is likely mediated by the same conserved residues that make up the SCR19 C3b binding site in FH. To analyze this, we generated mutants of the CFHR4B isoform in which these key residues were substituted. CFHR4B D221G corresponds to the atypical hemolytic uremic syndrome-associated mutation D1119G in FH. In CFHR4B D221G/K290A, the lysine that corresponds to residue K1188 in FH was substituted for an alanine, additionally. Both mutations were shown to reduce the C3b binding capacity of FH (16–19).

The analysis of CFHR4B and its two mutant variants D221G and D221G/K290A by silver staining (calculated molecular weight of ~37 kDa each) after expression and purification is shown in Figure 7A. In comparison to the wild-type CFHR4B, C3b binding to the mutant proteins was reduced by approximately 50% for D221G and 90% for D221G/K290A (Figure 7B). The strongly reduced C3b binding also led to a significantly reduced AP complement activation in NHS diluted in AP-specific buffer by the D221G/K290A mutant (Figure 7C). The reduced C3b binding to the D221G mutant was still sufficient to activate the AP, leading to only a slightly reduced deposition of C3b, FB and properdin in this experiment (Figure 7C).

## DISCUSSION

Members of the factor H protein family have evolved through several gene duplications. All *CFHR* genes retained exons that encode SCRs homologous to the FH C-terminus. For most CFHR proteins interaction with C3b mediated by the C-terminal domains has been demonstrated (6). However, the relevance of this is rather unclear, and the mechanisms by which CFHRs may influence complement activation and regulation via their interaction with C3b are not well established.

C3b binding has been reported for CFHR1, CFHR3, CFHR4B and CFHR5 (9, 20–22). CFHR1, CFHR3 and CFHR4B were shown to

bind to the C3d part of C3b via their C-terminal domains (9, 10, 22). Here we have extensively analyzed the interaction of the long CFHR4 isoform CFHR4A with C3 fragments. Previous studies typically measured CFHR4B-C3b interactions using fluid-phase C3b (20). We found that both CFHR4 isoforms can bind soluble C3b, iC3b and C3d (Figure 2 and data not shown). Because CFHR4A contains additional domains compared to CFHR4B, in particular SCR4 that is related to SCR8 of CFHR4A and SCR4 of CFHR4B (88% amino acid sequence identity, Figure 1), which are implicated in C3b binding, we mapped the C3b/C3d binding domains of CFHR4A using several recombinant deletion mutants of this isoform (Figure 2). The stronger C3b binding to the SCR4-9 mutant compared to the SCR8-9 construct, and also to CFHR4A compared to CFHR4B (Figure 2B), as well as the lack of C3b binding to SCR2-4 suggest that SCR4 may contribute to the interaction of CFHR4A with C3 fragments, but does not mediate C3b binding itself.

Although sharing a relatively high sequence identity in general, the C-terminal domains of FH family proteins show differences, leading to functional diversification. Recently, the structural basis of C3b binding by FH via SCRs 19-20 has been resolved, indicating the presence of two binding sites (16, 17). Residues of these C3b-binding sites are differently conserved within the homologous domains in the FH protein family. Whereas the key residues of the SCR19 site (FH D1119, Q1139 and K1188) are found in all five CFHR proteins, those of the SCR20 site (FH T1184, R1203 and K1230) are all shared by CFHR1 only (and partly by CFHR2) (Figure 6) (16). Here, we show by site-directed mutagenesis that two residues in CFHR4, namely D221 and K290 in CFHR4B (corresponding to D468 and K537 in CFHR4A) that are homologous to key residues of the FH 'SCR19-site', are indeed involved in C3b binding (Figure 7B). The lack of a FH SCR20-homologue C3b-binding site in CFHR4 might explain its weak binding to surface-bound C3b. In FH, one of the key residues of the SCR19 C3b-binding site (K1188) lies in SCR20 (16). This might explain, why the FH SCR19-homologue SCR4 of CFHR4A showed no significant C3b/C3d binding in the SCR2-4

deletion mutant, which lacks an adjacent SCR20-homologue domain (Figures 1 and 2B).

Surface plasmon resonance studies to investigate the two closely related proteins CFHR4B and CFHR3 applied C3b immobilized on the surface of sensor chips (9). In our assays, we found no significant binding of CFHR4A to C3b immobilized non-specifically on a plastic surface (Figure S1B). Weak binding of CFHR4A was detectable on *S. cerevisiae* with C3b naturally deposited on the surface (Figure S1D). However, because binding of CFHR4A to the surface of opsonized yeasts was relatively weak and even weaker than that of FH, we conclude, that CFHR4 binding to surface-bound C3b is unlikely to be of major physiological significance. In line with this, the previously reported binding of recombinant CFHR4B to C3b-opsonized pneumococci was very weak (9).

For CFHR4B and CFHR3 a very weak cofactor activity for the FI-mediated cleavage of C3b at high, rather non-physiological concentrations has been reported (9). Recently, such an activity has been reported for CFHR3 at markedly lower concentrations (15). In addition, weak cofactor activity was also reported for CFHR5 (23). This is somewhat unexpected as all CFHRs lack domains homologous to the N-terminal complement inhibitory region of FH. All these studies missed to identify the mechanism(s) and the domains behind such activities. We have thoroughly analyzed the long isoform CFHR4A, which contains all the domains that make up the short isoform CFHR4B, in cofactor assays with concentrations and incubation times exceeding those of the initial study (9) and found no such activity (Figure S2A).

CFHR4B and CFHR3 were both shown to enhance the cofactor activity of FH and it was speculated that this activity might be based on conformational changes of the C3b molecule upon CFHR binding (9). We also found enhancement of the FH cofactor activity by the long isoform CFHR4A. This effect was, however, not evident when using CFHL1 instead of FH as cofactor (Figure S2A). In addition, in our assays, CFHR4A displayed no decay accelerating activity nor it influenced FH in this function (Figure S2).

Altogether, except for the weak FH cofactor enhancing activity also reported for CFHR4B, CFHR4A did not show any complement

inhibitory activity at the level of C3b under the studied conditions. Thus, CFHR4 lacks FH-like complement regulatory activities. The weak FH-cofactor enhancing activity of CFHR4 is unlikely to be of major physiological relevance, since (i) the plasma concentration of CFHR4 (~25 µg/ml) is significantly lower than that required for a detectable activity, and (ii) much less amounts of the potent complement inhibitors FH and CFHL1 than found in body fluids were sufficient for effective control of complement activation via C3b inactivation or decay acceleration.

Moreover, we observed complement activation rather than inhibition when CFHR4 was incubated with serum. Previously we have demonstrated that CFHR4 activates C2/C4-dependent pathways via its interaction with CRP (12). Here, we found that CFHR4 can initiate AP activation via the formation of active C3 convertases on CFHR4-bound C3b (Figures 3 and 4). Because complement is autoactivated in human plasma via the spontaneous hydrolysis of the thioester bond in C3 ('tick-over' mechanism) that generates trace amounts of C3b via the very short-lived C3(H<sub>2</sub>O)Bb initial AP C3 convertase (24, 25), the activation of the AP via CFHR4-bound C3b can occur independently from the activation of the classical pathway via CFHR4-bound CRP (12).

Activation of the AP through C3b bound by complement activatory host molecules might represent a mechanism to enhance opsonization and phagocytosis. Recently, properdin was shown to provide a platform for such a *de novo* convertase via C3b binding and thereby to initiate complement activation on target surfaces (26, 27). We propose that CFHR4 can similarly activate the AP on surfaces where it is able to bind. CFHR4 was shown to bind to necrotic cells (11) and also to certain pathogens, such as *Fusobacterium necrophorum* (28) and the yeast *Candida albicans* (29). In our experiments both CFHR4A and FH bound weakly to *S. cerevisiae* cells (Figure S1D). By activating complement, CFHR4 could contribute to the opsonization and enhanced opsonophagocytosis of microbes and damaged host cells, provided that its interaction sites for ligands that mediate complement activation are still accessible in its surface-bound state. This was shown for CRP which is recruited by CFHR4 to the surface of necrotic cells (11).

The assembly of alternative forms of C3 convertases, like the herein reported CFHR4A-C3bBb C3 convertase, might be relevant in light of their stability and regulation by complement inhibitors, such as FH. As we have demonstrated, the CFHR4A-C3bBb C3 convertase displays certain resistance against the FH-mediated decay (Figure 5). This could ensure enhanced complement activation and C3b deposition on target surfaces on the one hand, and a limited proceeding of the complement cascade to the terminal steps due to the binding of FH, and possibly other regulators, on the other hand. Such a scenario could occur on surfaces where both CFHR4 and FH bind (e.g., late apoptotic and necrotic cells) (11, 30), and would allow sufficient complement activation and C3b deposition without inflammation until the particle is eliminated via phagocytosis.

Previously we have shown that CFHR4 binds pentameric CRP both at low concentrations (such as in the plasma of healthy individuals) and at acute-phase concentrations (e.g., in the plasma of sepsis patients) and thereby activates complement (12). Here, we found that C3b- and CRP-mediated complement activation by CFHR4 could take place simultaneously, as the two ligands do not interfere with the binding of each other and CRP did not interfere with C3 convertase formation and activity (data not shown). This is explained by the spatially separated C3b and CRP binding sites within CFHR4. Because C3 fragments generated via CRP-mediated complement activation can potentially bind back to CFHR4, it is possible that AP C3bBb convertases are formed secondary to CRP binding and amplify CFHR4-mediated complement activation.

In conclusion, we identify a new function for CFHR4 in the activation of complement via the AP by allowing the assembly of an active C3 convertase. Thus, CFHR4 appears as a molecule that is able to recruit CRP to specific surfaces (11) and also to bind fluid-phase C3b, and through these two mechanisms can activate complement via different pathways and enhance local opsonization. These capacities distinguish CFHR4 as a complement activatory molecule that enhances target opsonization from the complement inhibitor FH. It will be interesting to see, whether some of the other CFHR proteins share this ability



of CFHR4 to activate AP via their interaction with C3b.

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## FOOTNOTES

<sup>1</sup> The abbreviations used are: AP, alternative pathway; CFHL1, complement factor H-like protein 1; CFHR, complement factor H-related protein; CRP, C-reactive protein; FB, factor B; FD, factor D; FH, factor H; FI, factor I; FP, properdin; HSA, human serum albumin; NHS, normal human serum; SCR, short consensus repeat

## FIGURE LEGENDS

**FIGURE 1. Domain composition of the CFHR4 isoforms and FH.** The SCR domains of the two CFHR4 isoforms are aligned to the most related domains in FH based on amino acid sequence identity. The numbers above the CFHR4 domains show the amino acid sequence identity to the corresponding FH domain in percentage. Domains of CFHR4 are homologous to the FH domains SCRs 6, 8, 9, 19 and 20. SCRs 1 and 6-9 of CFHR4A are identical to the SCRs that represent the shorter CFHR4B isoform (indicated by shading). CFHR4A contains four additional SCRs (SCRs 2-5) that differ in amino acid sequence from the SCRs that are common in both isoforms. The CFHR4 isoforms lack domains that are homologous to the N-terminal complement regulatory region (SCRs 1-4) of FH, but contain SCRs that are homologous to the FH C-terminus. SCRs 19-20 of FH and SCRs 4-5 of CFHR4B have been shown to bind C3b and C3d. Both CFHR4 isoforms harbor a binding site for native pentameric CRP in their SCR1.

**FIGURE 2. C3b binding to CFHR4A is mediated by SCRs 8-9.** Binding of C3b to the immobilized CFHR4 isoforms and CFHR4A deletion mutants was analyzed in ELISA. **A.** C3b was added in increasing concentrations to FH, CFHR4A and CFHR4B immobilized in equimolar amounts (50 nM). C3b binding was detected with a HRP-conjugated C3 antibody. Data shown are means + SD from duplicates of three independent experiments. **B.** Binding sites for C3b within CFHR4 were mapped with CFHR4A deletion mutants representing SCRs 1-3, 2-4, 5-7, 4-9 and 8-9. The full-length isoforms and the deletion constructs were immobilized at 250 nM concentration. Binding of 25 µg/ml C3b was detected as in A. Background binding to BSA was subtracted. Data shown are mean values + SD of triplicates from three independent experiments. **C.** Binding of C3d (5 µg/ml) to the immobilized CFHR4 isoforms and CFHR4A deletion constructs was analyzed as described in B. Bound C3d was detected with a rabbit human C3d antiserum. Data show mean values + SD of triplicate measurements from three independent experiments.

**FIGURE 3. CFHR4 activates the alternative complement pathway.** CFHR4A, its deletion mutants SCR1-3 and SCR4-9, CFHR4B, FH and human serum albumin (HSA) were immobilized in microtiter plates at 100 nM and incubated for 30 min at 37°C with 5% NHS in TBS containing Mg<sup>2+</sup> and EGTA to allow alternative pathway complement activation only. Deposition of C3b and binding of factor B (FB) and properdin were measured by ELISA. Data shown are mean values + SD from three independent experiments performed in duplicates.

**FIGURE 4. An active alternative pathway C3 convertase assembles on CFHR4-bound C3b.** The ability of CFHR4A to serve as a platform for the formation of an active C3 convertase from purified complement components was analyzed in solid-phase assays. **A.** CFHR4A and its deletion mutants SCR1-3 and SCR4-9 were immobilized at 100 nM in microtiter plate wells and incubated with C3b (30 min, 37°C). Unbound C3b was washed away and FB, factor D (FD), properdin (FP) and BSA were added for 1 h at 37°C to allow convertase formation. After washing, remaining FB as a measure of intact convertases was detected. Means + SD from duplicates of two independent experiments are shown. **B.**

The CFHR4A-C3bBb convertase was generated as described in A and was incubated with 10 µg/ml C3 or buffer for 1 h at 37°C. The supernatants were analyzed for generated C3a using a C3a ELISA kit. The C3bBb convertase formed on immobilized C3b was used as a control. Data shown are mean values + SD from two independent experiments performed in duplicates.

**FIGURE 5. The CFHR4A-C3bBb C3 convertase is less efficiently regulated by FH.** The regulation of the convertase assembled on CFHR4-bound C3b by FH was analyzed in ELISA. **A.** Both the C3bBb and the CFHR4A-C3bBb convertases were assembled in microtiter plate wells as described in Figure 4. After exposure to increasing FH concentrations for 30 min at 37°C, intact convertases were detected with anti-FB. Wells incubated with buffer only were set to 100%. Data shown are means + SD from two independent experiments performed in duplicates. **B.** The spontaneous decay and the decay of both types of convertases in the presence of 2 µg/ml FH were measured over time by detecting remaining FB. The remaining convertases after decay in the presence of FH are shown as percent of the spontaneous decay of the respective convertases. Mean values + SD of duplicates from two independent experiments are shown. **C.** C3a generation by the two different convertases after incubation with buffer or FH for 30 min at 37°C was compared by analyzing the supernatants as described in Figure 4B. Data are mean values + SD from duplicates of two independent experiments.

**FIGURE 6. Alignment of all SCRs of the FH family proteins that are homologous to SCRs 19-20 of FH.** All CFHR proteins contain domains homologous to the C-terminal SCRs 19 and 20 of FH that harbor binding sites for C3b/C3d. Within SCRs 19-20 of FH two binding sites for C3b/C3d have been described (16). The FH SCR19 C3b-binding site is comprised of residues located within SCR19 and SCR20. The residues D1119, Q1139 and K1188 of this site were shown to be particularly important for the interaction with C3b. All three residues of this site are conserved within the related domains in the FH protein family. The most important amino acids of the second C3b-binding site formed exclusively by residues of SCR20 are T1184, R1203 and K1230. In contrast to the SCR19 site, these residues are not conserved within the FH protein family and all three are present only in CFHR1. This suggests that a single C3b-binding site exists within CFHR4, which involves residues homologous to the FH SCR19 site.

**FIGURE 7. Analysis of CFHR4B C3b-binding site mutants.** Mutations within the putative C3b binding site of CFHR4 were analyzed using mutated variants of the short isoform CFHR4B. **A.** Silver staining of recombinantly expressed CFHR4B, CFHR4B D221G and CFHR4B D221G/K290A. CFHR4B is composed of five SCRs and the recombinant protein has a molecular weight of approximately 37 kDa. **B.** Dose-dependent binding of C3b to immobilized CFHR4B and the CFHR4B mutants D221G and D221G/K290A. C3b binding was detected with a HRP-conjugated C3 antibody. Data shown are means + SD from three independent experiments performed in duplicates. **C.** The CFHR4B C3b-binding mutants were analyzed in complement activation assays. The immobilized proteins were incubated with 5% NHS in TBS containing Mg<sup>2+</sup> and EGTA to allow AP complement activation only. Formation of the AP C3 convertase was measured by the deposition of C3b, FB and properdin (FP) as in Figure 3. Data shown are means + SD from three independent experiments performed in duplicates.

**Figure 1**

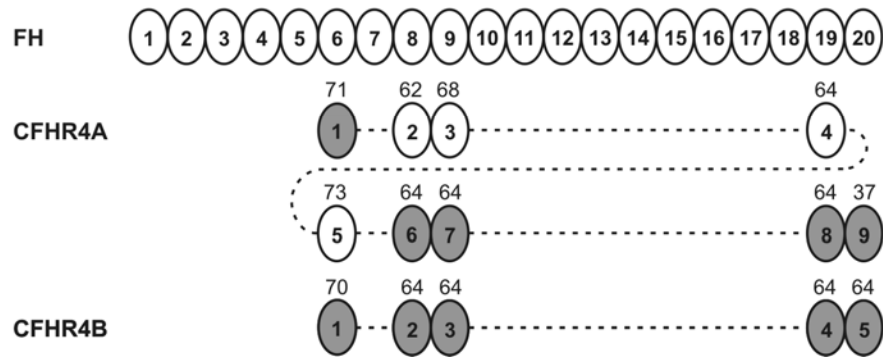


Figure 2

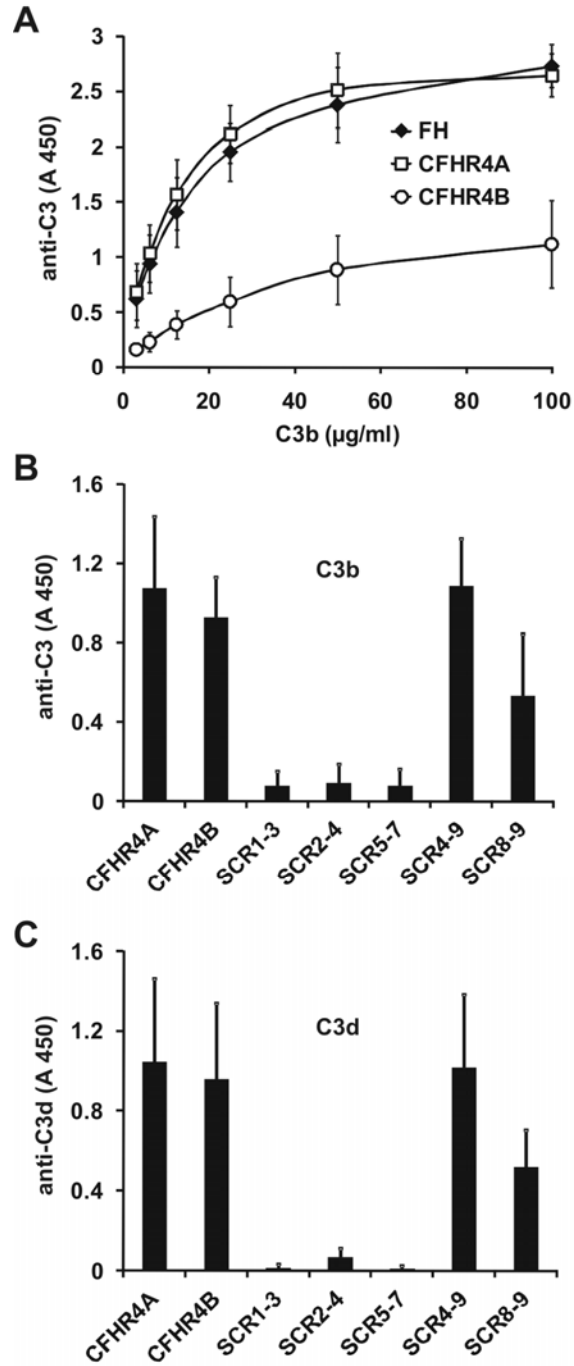


Figure 3

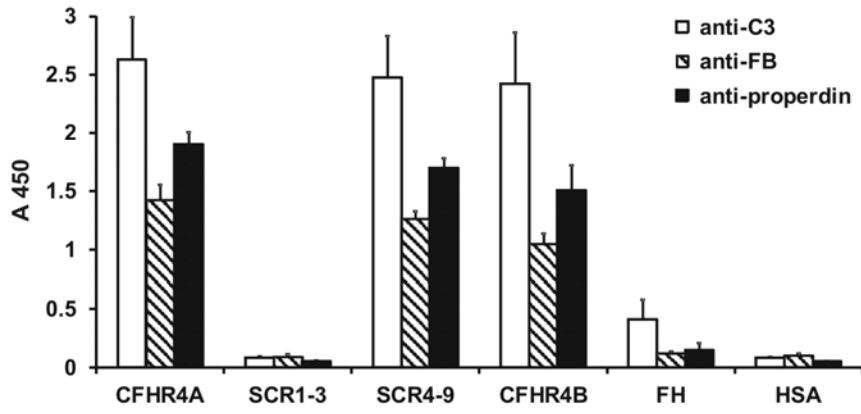




Figure 4

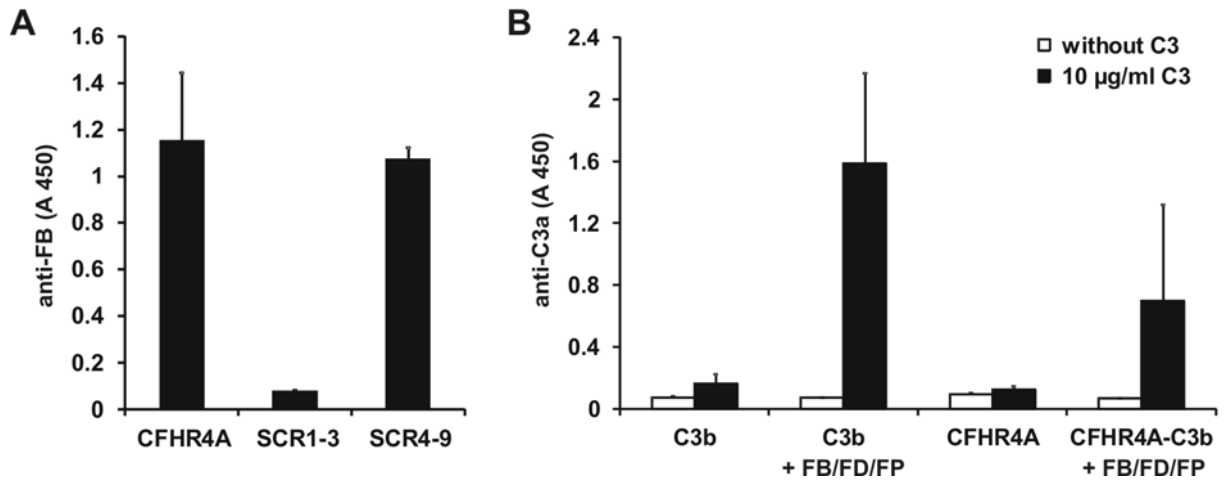
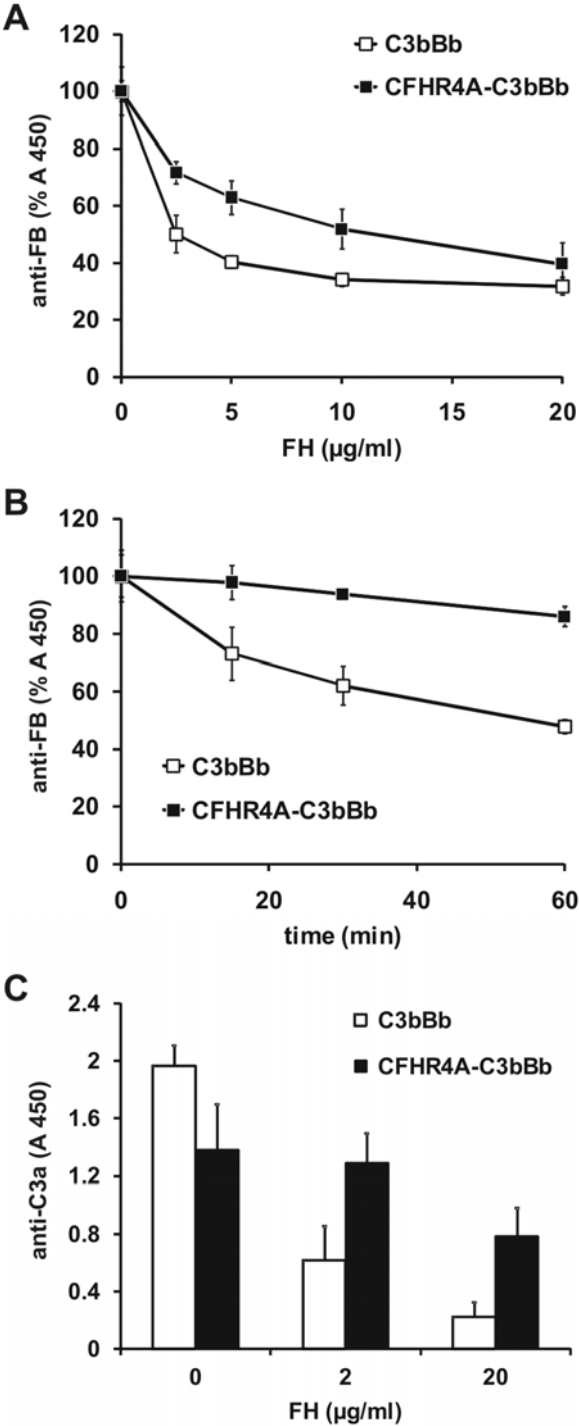
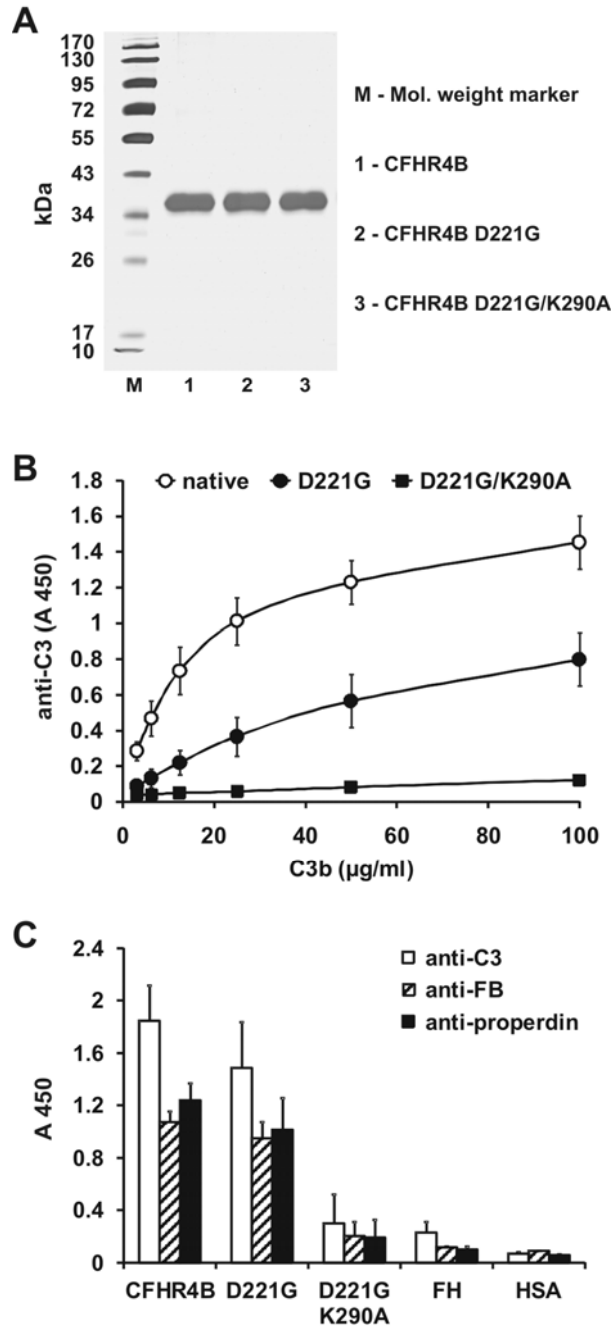


Figure 5





**Figure 7**



## SUPPLEMENTARY FIGURE LEGENDS

**FIGURE S1. Binding of CFHR4 to surface-bound C3b is weak compared to that of FH.** Binding of CFHR4A to surface-bound C3b was analyzed in ELISA. **A.** Analysis of the antibodies that were used to detect CFHR4A and FH. Binding of the antibodies to CFHR4A, FH and human serum albumin (HSA) immobilized at 100 nM is shown. Data represent mean values + SD of duplicates from two independent experiments. **B.** Binding of 50 nM CFHR4A and FH to C3b, iC3b and C3c immobilized on microtiter plates at a concentration of 50 nM was detected with the respective antibodies. Data are mean values + SD from two independent experiments performed in triplicates. **C, D.** *S. cerevisiae* yeast cells were immobilized on microtiter plate wells at a saturating concentration followed by blocking of non-specific binding sites and incubation with 7.5% NHS for 30 min at 37°C. Opsonization with C3 fragments was confirmed using an anti-C3 antibody (C) and binding of 100 nM CFHR4A and FH to serum-opsonized and untreated yeast cells was measured using the respective antisera (D). Data are means + SD from two independent experiments performed in duplicates.

**FIGURE S2. Analysis of complement regulatory functions of CFHR4A.** The role of CFHR4A in complement regulatory functions such as cofactor and convertase decay accelerating activities was analyzed. **A.** In a fluid-phase cofactor assay, C3b was incubated with FI alone (lane 1) or in the presence of FH (lane 2), CFHL1 (lane 5), CFHR4A (lane 4) and in the presence of CFHR4A in combination with FH or CFHL1 (lanes 3 and 6) for 45 min at 37°C. The samples were analyzed by SDS-PAGE and Western blotting using a HRP-conjugated anti-C3 antibody. FI-mediated cleavage of the C3b  $\alpha'$ -chain resulted in characteristic fragments with mobilities of 68, 43 (iC3b<sub>1</sub>) and 41 (iC3b<sub>2</sub>) kDa and in the diminishing of the intensity of the  $\alpha'$ -band. The proteins were used in molar ratios of 3:1:1:1:20 (C3b:FI:FH:CFHL1:CFHR4A) corresponding to 35, 6, 10, 4 and 90  $\mu$ g/ml, respectively. A representative experiment out of four independent repeats is shown. **B.** The AP C3 convertase C3bBb was assembled on C3b immobilized in microtiter plate wells by the addition of FB, FD, properdin and BSA for 1 h at 37°C. Remaining FB as a measure of intact convertases was detected after separate incubations with increasing concentrations of FH, CFHR4A and HSA for 30 min at 37°C. Data shown are mean values + SD of two independent experiments. **C.** C3bBb convertases assembled in microtiter plates as described in B were incubated with constant concentrations (2.5  $\mu$ g/ml) of FH and increasing concentrations of CFHR4A (up to 20  $\mu$ g/ml, corresponding to a molar ratio FH:CFHR4A of 1:20) or buffer only for 30 min at 37°C. Intact convertases were detected with anti-FB. A representative of two independent experiments is shown.

Figure S1

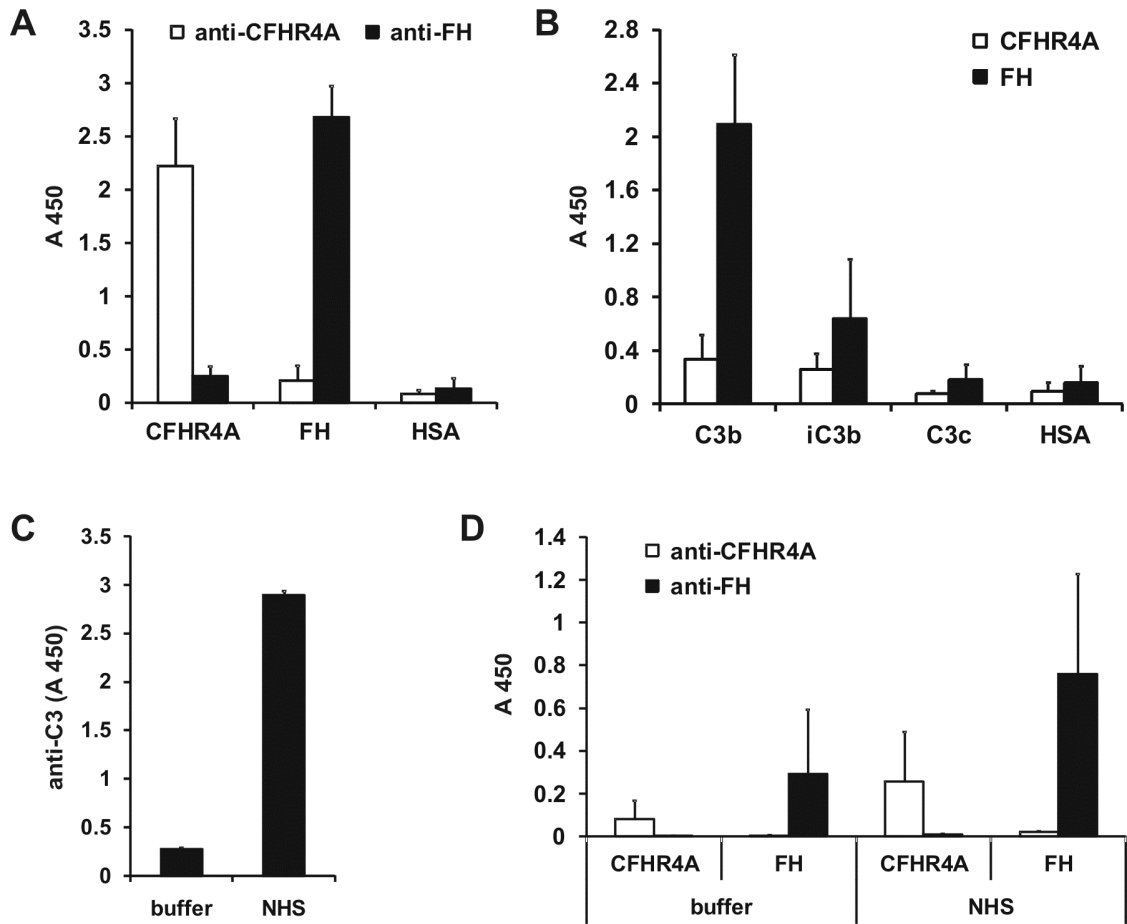


Figure S2

