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Factor H related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation

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abbreviations: CFHR1 complement factor H related protein 1, CFH complement factor H, SCR short consensus repeat

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Abstract

Homozygous deletion of a 84 kb genomic fragment in human chromosome 1 that encompasses the *CFHR1* and *CFHR3* genes represents a risk factor for hemolytic uremic syndrome (HUS) but has a protective effect in age related macular degeneration (AMD). Here we identify CFHR1 as a novel inhibitor of the complement pathway that blocks C5 convertase activity and interferes with C5b surface deposition and MAC formation. This activity is distinct from complement factor H and apparently factor H and CFHR1 control complement activation in a sequential manner. As both proteins bind to the same or similar sites at the cellular surfaces the gain of CFHR1 activity presumably is at the expense of CFH mediated function, i.e. inhibition of the C3 convertase. In HUS the absence of CFHR1 may result in reduced inhibition of terminal complex formation and in reduced protection of endothelial cells upon complement attack. These findings provide new insights into complement regulation on the cell surface and biosurfaces and likely define the role of CFHR1 in human diseases.

Introduction

The complement system is important for host innate and adaptive immunity and mounts a protective immune response to invading microbes¹. The alternative complement pathway is spontaneously activated, generates C3 convertases (C3bBb) that cleave the central component C3 to the anaphylactic peptide C3a and C3b^{2, 3}. C3b attached to a foreign surface binds Factor B and generates the C3 convertase (C3bBb), which enhances further complement activation resulting in opsonisation and phagocytosis of particles. Binding of an additional C3b molecule to the C3 convertase forms the C5 convertase (C3bBbC3b) of the alternative pathway. This convertase cleaves C5 and generates the potent chemoattractant C5a as well as C5b, which initiates the terminal complement pathway assembly⁴. C5b immediately undergoes conformational changes and binds C6 and C7 in a nonenzymatic manner. The assembled C5b67 complex is released from the convertase and attaches to lipid bilayers. Upon binding of C8 and C9 the lytic membrane attack complex (MAC) is formed^{3, 5}.

Once activated, this powerful defense system is tightly controlled on host cell surfaces by both, membrane anchored and surface attached soluble regulators. Proper and coordinated function of these regulators is essential for tissue integrity. Single genes mutations predispose to severe renal and retinal diseases, i.e. hemolytic uremic syndrome (HUS, OMIM #235400), membranoproliferative glomerulonephritis type II (MPGN II, OMIM #609814), or age related macular degeneration (AMD, OMIM #603075)^{6, 7}.

HUS is caused by occlusion of arterioles and capillaries in the kidney, due to endothelial cell and platelet damage⁸. MPGN II is a rare renal disease, with formation of dense deposits at the glomerular basement membrane and thickening of the peripheral capillary walls⁹. Similarly, the retinal disease AMD, which causes visual impairment of elderly people, is caused by deposits (drusen) which form on the Bruch's membrane, lead to atrophy of the retinal pigment epithelium (RPE) and choroidal neovascularization under the macular area¹⁰.

These diverse diseases are caused by defective local complement regulation and are associated with gene variations and mutations coding for complement components and regulators, such as complement factor H (CFH)^{8,11}. In addition deletion of a 84 kb genomic fragment on human chromosome 1, that leads to the loss of the complement factor H related genes 1 and 3 (*CFHR1* and *CFHR3*), is associated with both HUS¹² and AMD¹³. However, this chromosomal deletion has opposite effects, for HUS the deletion increases the risk for the disease and for AMD the deletion has a protective effect.

In aHUS the absence of *CFHR1* and *CFHR3* in plasma correlates with the presence of autoantibodies to CFH¹⁴. The autoantibodies bind to the C-terminal surface binding region of CFH¹⁵ and inhibit CFH surface attachment resulting in damage of endothelial cells as well as platelets¹⁶. The *CFHR1* plasma protein is composed of five short consensus repeats and is identified in two glycosylated forms. *CFHR1* β (42 kDa) has two and *CFHR1* α (37 kDa) has one attached carbohydrate side chain¹⁷⁻¹⁹. The high sequence identity of the three C-terminal SCRs of *CFHR1* and CFH (100, 100 and 98%) suggests related functions. *CFHR1* lacks cofactor and

decay accelerating activity²⁰ and the function is currently unknown. Given the opposing effects of CFHR1/CFHR3 deficiency in human diseases, we were interested to identify the function of CFHR1 protein. Here we identify CFHR1 as a regulator of the complement pathway that inhibits C5 convertase activity and MAC assembly.

Methods

Proteins and antibodies

Recombinant CFHR1 and deletion mutants CFHR1 SCRs 1-2 (CFHR1 /1-2) and CFHR1 SCR 3-5 (CFHR1/3-5) were expressed as described²¹. CFHR1/1-2 cDNA was cloned into vector pPICZ α B using specific primers CFHR1₁₋₂ forw. 5'TTTCTGCAGCCGAAGCAACATTTTGTGATTTTCAA3' and CFHR1₁₋₂ rev. 5'TTTTCTAGAGCAGTGGACCTGCATTTGGGAGGGGT3'. CFH/SCR11-15 was expressed in insect cells, as previously described^{14,15}. All proteins were expressed in *Pichia pastoris* and purified by Nickel chelate affinity chromatography¹⁴. Vitronectin was purchased from BD Biosciences, C3b, C5, C5b6, Factor H and Factor I from Merck Biosciences. C7, C8, C9 from Comptech. CFHR1 was purified from human plasma by HiTrap Heparin HP column (GE Healthcare) affinity chromatography. Pooled elution fractions were concentrated (Superdex 2000), separated by SDS-PAGE, CFHR1 was dissected from the gel, concentrated and dialyzed (1 x PBS pH 4.7). Mouse monoclonal antibody (mAb) C18²² (Alexis) was used to detect the C terminus of CFH and CFHR1. A novel CFHR1 mAb JHD10 was generated by immunizing mice with purified CFHR1-SCRs 1-2 fragments.

Serum probes

Normal human plasma (HP) was obtained from healthy volunteers, Jena, Germany, upon informed consent obtained in accordance with the Declaration of Helsinki. CFH depletion was performed by immunadsorbance as described¹². For C8 depletion: goat polyclonal anti C8 (Comptech) was used as described for CFH depletion. The study is approved by the ethic board of the Friedrich Schiller University of Jena. The board approved study of patient material (DNA and blood) for genetic and molecular

characterization of complement factor H and complement factor H family members in HUS, MPGN2 and AMD.

Cell culture and confocal microscopy

Human umbilical vein endothelial cells (HUVEC, ATCC CRL-1730) were cultivated as described²³. For confocal microscopy (LSM 510 META, Zeiss) HUVEC cells were grown on chamber slides (Nunc), incubated for 60 min with CFHR1 or CFH (100 µg/ml) and binding was visualized using mAb JHD10 (CFHR1) or polyclonal anti SCR1-4 (CFH)²⁴. C5 deposition on erythrocytes incubated in HP Δ CFH Δ C8 was detected with C5 mAb (Comptech).

Immunohistochemistry

Immunohistochemistry was performed on tissues derived from two human donor eyes (two female patients, 79 and 81 years of age) with neither history of clinically documented early AMD nor of morphologic evidence of ocular disease. The donor eyes were obtained at autopsy and were processed less than 15 hours after death. In addition, normal kidney tissue was obtained from two human adult donor kidneys that were not used for transplantation. Posterior eye poles and portions of decapsulated kidneys were embedded in optimal cutting temperature (OCT) compound and frozen in isopentane-cooled liquid nitrogen. Cryostat-cut sections (6 µm) were fixed in cold acetone, blocked with 10% normal goat serum, and incubated in a monoclonal mouse antibody to CFHR1 (JHD10) diluted 1:100 in phosphate-buffered saline (PBS) overnight at 4°C. Antibody binding was detected by Alexa 488-conjugated secondary antibodies (Molecular Probes, Eugene, OR). Nuclear counterstaining was performed

with propidium iodide. For preabsorption experiments the primary antibody was treated for 1 h either with CFHR1 or CFH.

Binding of CFHR1 to heparin, C3b, C5 and C5b6

MaxiSorp plastic plates (Nunc) were coated with heparin (500 units/well) or C3b (10 µg/ml), C5 or C5b6 (5 µg/ml) and incubated with CFHR1 (50 µg/ml) or CFH (75 µg/ml) dissolved in binding buffer B (10 nM Na₂HPO₄, 27 mM KCl, 1.4 M NaCl, 2% BSA, pH 7.4). Bound CFHR1 was detected with mAb C18 and bound CFH with SCRs 1-4 antiserum. In control experiments buffer B was added without proteins.

For identification of C5 binding site in CFHR1 mAb JHD10 or mAb C18 (15 µg/ml) were immobilized to a microtiter plate and used to catch CFHR1 (30 µg/ml). After washing, C5 or C5b6 (5 µg/ml) were added in gelatine veronal buffer (Sigma) and bound proteins were identified using a C5 mAb. CFHR1 specific antiserum was used to confirm binding of CFHR1 to the immobilized mAbs. C5 binding to CFH or CFHR1 was measured by immobilizing equimolar concentrations of CFH or CFHR1 to a microtiter plate. C5 binding to immobilized CFHR1 and CFH was assayed by incubation of increasing concentrations of C5 (1.5 to 50 µg/ml) for 1h and bound C5 was identified with mouse monoclonal antibodies (Quidel, 1:2000). In addition binding of C5 (50, 150, 200 and 400 nM) to immobilized CFHR1 or CFH/SCR10-13 (carboxylated dextran chip CM5, Biacore AB) was assayed by surface plasmon resonance in 75 mM PBS at a flow rate of 5 µl/min at 25 °C. Controls (binding of the fluid phase ligand to the uncoated surface) were subtracted .

Cofactor assay

Cofactor activity of heparin bound CFH was analyzed by measuring factor-I mediated degradation of C3b following SDS-Page and Western Blot analysis. For competition CFH immobilized to heparin coated microtiter plates (EpranEx™, Plasco) was incubated with CFHR1 (0.13 µg to 13.3 µg). Then C3b (2 µg) and CFI (0.28 µg) were added and cofactor activity was determined²⁴. Degradation products (β chain band and α'43 band) were analyzed by densitometry.

ELISA

To investigate CFHR1 regulation of the C3 convertase, the C3 convertase was generated by incubation of C3b (2µg/ml) and C3 (80 µg/ml) with Factor D (4 µg/ml) and Factor B (40 µg/ml) in activation buffer C (20 mM Hepes, 144 mM NaCl, 7 mM MgCl₂, 10mM EGTA, pH 7.4). Activity of C3 convertase was determined after incubation of constant amounts of C3 (80 µg/ml) and increasing amounts of CFHR1 (25 and 50 µg/ml), CFH (50 g/ml) or 25 µg/ml human serum albumine (HSA) by C3a generation. C3a concentrations were determined by ELISA (Quidel, USA). Sheep erythrocytes (1 x 10⁹) were incubated with C3b (10µg/ml) in veronal buffer over night at 4°C. C3 and C5 convertase was generated by incubation of erythrocytes for 40 min at 30°C with C3 (10 µg/ml), Factor D (5 µg/ml) and Factor B (10 µg/ml) in veronal buffer supplemented with Ni²⁺ and properdin (5 µg/ml). C5 convertase loaded erythrocytes were preincubated with recombinant or plasma derived CFHR1, CFH (50 µg/ml) or with BSA (50 µg/ml) prior to the addition of C5 (50 µg/ml). C5a generation was analyzed after 15 min by ELISA (DRG Diagnostics).

Erythrocyte lysis assay

Increasing concentrations of CFHR1 (5 to 160 $\mu\text{g/ml}$) or CFH, vitronectin or HSA were added to CFH and CFHR1 depleted plasma (30%) and incubated at 37⁰C for 15 minutes with about 2×10^7 sheep erythrocytes in activation buffer C. Supernatants were recorded at 415 nm. In similar experiments generation of complement activation products C3a and C5a was followed by ELISA (Quidel, DRG Diagnostics).

Hemolysis of chicken erythrocytes, which are more sensitive for MAC induced hemolysis, was investigated in complement inactivated (20 mM HEPES, 144 mM NaCl, 10 mM EDTA, pH 7.4) defHP with constant concentrations of C5b6 (5 ng/ml), increasing amounts of CFHR1 (25 to 100 $\mu\text{g/ml}$), CFH or BSA. In addition C5b6 complexes (5 ng/ml) were preincubated with either recombinant CFHR1 (50 $\mu\text{g/ml}$), purified CFHR1 (12.5 $\mu\text{g/ml}$), vitronectin (12.5 $\mu\text{g/ml}$) or BSA (12.5 $\mu\text{g/ml}$) in 20 mM HEPES, 144 mM NaCl, 10 mM EDTA, pH 7.4 for 5 min at 20⁰C. Lysis of sheep erythrocytes (2×10^7) was followed after addition of C7 (final concentration 1 $\mu\text{g/ml}$) C8 (0,2 $\mu\text{g/ml}$) and C9 (0.2 $\mu\text{g/ml}$) by absorbance at 415 nm. To compare similar molar amounts of the plasma purified CFHR1 with vitronectin, C5b6 complexes (5 ng/ml) were preincubated with increasing concentrations (1 to 300 nM) of either plasma purified CFHR1, vitronectin or BSA for 5 min at 20⁰C. After addition of C7 (final concentration 1 $\mu\text{g/ml}$), C8 (0,2 $\mu\text{g/ml}$) and C9 (0.2 $\mu\text{g/ml}$) the mixture was added to sheep erythrocytes (2×10^7) and incubated for 30 min at 37⁰C. Percentage of cell lysis (absorbance at 415 nm) was calculated by the formula: $[(\text{sample} - \text{background}) / (100\% \text{ lysis} - \text{background})] \times 100$, where background hemoglobin release was obtained from sheep erythrocytes incubated in buffer only, and 100 % lysis was achieved using distilled water.

Flow cytometry

CFHR1/CFHR3 deficient plasma was depleted of CFH and C8 (HP $_{\Delta$ CFH $_{\Delta}$ C8) by immunoaffinity chromatography and added to sheep erythrocytes in the presence or absence of 50 μ g/ml CFHR1 in 20mM HEPES, 250 mM Mannitol, 8 mM MgCl₂, 10 mM EGTA, pH 7.4. At each time point the sample was transferred to ice cold buffer with 1% w/v BSA with protease inhibitor mix (Complete Inhibitor Mix, Roche). C5 was detected by using C5 mAb (Comptech).

Binding of serum derived CFHR1 to HUVEC cells was investigated by incubating HUVEC cells, which have been grown serum free for 3 days, in 25 % normal human serum with mAb JHD10.

Immunoprecipitation

Monoclonal C5 antibodies (Comptech) were immobilized to Protein A sepharose beads (GE Healthcare) by incubation over night at 4⁰C. Antibody loaded beads were washed three times in PBS (1x) and incubated for 2 hours at 4⁰ C with 50% NHP. The beads were washed five times in PBS (1x) and bound antibodies and proteins were eluted with 0.1M glycine/0.5 M NaCl, pH 2.7. Eluates were separated by SDS-PAGE, transferred to a membrane and CFHR1 was detected with a polyclonal antiserum and C5 with a rabbit polyclonal antiserum in combination with a secondary horse-radish-peroxidase conjugated antiserum.

Statistical analysis

Statistics were analyzed with Student's *t*-test, P values less than 0.05 were considered significant. Statistical analysis of defined groups was performed with Jonckheere-Terpstra trend test²⁵, p values were calculated for the two-sided test of no trend.

Results

CFHR1 binds to C3b, C3d, heparin and to human cells

The three C-terminal SCRs of CFHR1 display almost sequence identity to the central C3b and surface binding region of CFH, i.e. SCRs 18-20 (Fig. 1A). This homology suggests related functions. Therefore binding of CFHR1 to the ligand C3b was investigated. CFHR1 binds to C3b and CFH, that harbors three interaction sites for C3b, showed more pronounced binding (Fig. 1B).

CFHR1 utilizes the C-terminus for both C3b and heparin binding: Surface plasmon resonance showed that deletion mutant CFHR1/3-5 (Supplementary Fig. 2A, blue line), but not CFHR1/1-2 (black line) bound to immobilized C3b and to heparin (Supplementary Fig. 2A and Fig. 2B). The binding affinity of the C-termini of CFHR1 and of CFH were determined by surface plasmon resonance. The affinity of CFHR1/3-5 to C3b is $K_D = 6.4 \times 10^{-6}$ M and that of CFH/18-20 is $K_D = 2.6 \times 10^{-6}$ M. Thus the C-terminus of CFHR1 binds C3b with lower affinity than that of CFH (Supplementary Fig. 2C and Fig. 2D).

Binding of CFHR1 to cell surfaces

Binding of CFHR1 to human cells was investigated by flow cytometry. HUVEC cells were incubated in normal human plasma (HP) and binding of native, plasma derived CFHR1 was detected with the novel CFHR1 specific mAb JHD10 (Fig. 1C). Binding of recombinant CFHR1 to HUVEC, as well as to epithelial cells (ARPE-19) was confirmed by confocal microscopy. On the cell surface CFHR1 showed a patchy distribution (Supplementary Fig. 3A, panels I and II). In addition CFHR1 bound to

C3b treated rabbit erythrocytes (Supplementary Fig. 3A, panel III). The secondary antibodies alone showed no binding (Supplementary Fig. 3A, panels Ia to IIIa). The C-terminus of CFHR1 mediates cell binding, as the deletion mutant CFHR1/3-5, but not CFHR1/1-2, bound to ARPE-19 cells (Supplementary Fig. 3B). Thus CFHR1 binds via the C-terminal binding region to C3b and to human cells.

CFHR-1 binding to kidney and retinal tissue

Having demonstrated that CFHR1 binds cell surfaces, we analyzed *in vivo* CFHR1 expression in human retinal and ocular tissues by immunohistochemistry. In renal tissue CFHR1 was detected in the lining of blood vessels (green fluorescence), particularly of large arteries and of afferent and efferent arterioles at the vascular poles of glomeruli (Fig. 1D, panels I). In the posterior segment of a retina, CFHR1 staining was specifically localized to Bruch's membrane and the choriocapillaries of the choroids (panels IV). Pre-incubation of the antibodies with CFH did not affect reactivity (panel III and VI), but preincubation with CFHR1 (panel II and V). Thus CFHR1 is present at the surface of endothelial cells and at the Bruch's membrane.

CFHR1 and CFH recognize overlapping binding sites at the cell surface

The almost identical C termini and the conserved binding characteristics of CFHR1 and CFH suggested coordinated, as well as competitive binding. Therefore simultaneous binding to cell surfaces was investigated. HUVEC cells were incubated with CFHR1 and CFH. Surface bound CFHR1 was visualized by red- and bound CFH by green fluorescence (Fig. 1E, panels I and II). A merge of the two images revealed overlapping binding as indicated by the yellow fluorescence (panel III).

To investigate whether CFHR1 replaces CFH and reduces local CFH mediated activity, heparin bound CFH was competed with increasing concentrations of CFHR1 and cofactor activity was assayed by analyzing the generation of the C3b degradation products α' 68 and α' 41/43. CFHR1, by replacing CFH, reduced the local regulatory activity as demonstrated by the lower amount of C3b degradation fragments (Fig. 1F, upper panel). Densitometric analyses showed reduction of the α' 43 band (as demonstrated by the α' 43 / β 75 ratio) when equal molar amounts of CFHR1 and CFH were used (Fig. 1F, lower panel, column 3). This effect demonstrates that CFHR1 may reduce local CFH mediated complement control.

CFHR1 regulates complement pathway activation

Binding of CFHR1 to C3b suggested a unique regulatory function(s) on the level of C3 convertase. To identify such an activity a hemolytic assay^{12,26,27} was used. Sheep erythrocytes representing non activator surfaces remain intact when incubated in human plasma (HP). However, these cells are lysed, when incubated in complement active HP depleted of CFH and CFHR1 (HP Δ CFH). In this system the absence of CFH results in erythrocyte lysis and addition of CFH has a dose dependent protective effect (Fig. 2A, grey squares). The role of CFHR1 on complement activation was analyzed in this serum. Addition of CFHR1 to HP Δ CFH reduced lysis of sheep erythrocytes dose dependently (Fig. 2A, black triangles) and HSA showed no effect (open squares). The protective effect of CFHR1, used at 80 μ g/ml was 13 % and that of CFH was 75 %. The inhibitory activity of CFHR1 was comparable to the known terminal pathway inhibitor vitronectin (Fig. 2A, compare black triangles and grey diamonds).

The regulatory role of CFHR1 on the complement pathway was investigated for the C3 convertase. C3 convertases were formed *in vitro* by incubating C3 with Factor B and Factor D and after addition of C3 convertase activity was monitored by C3a generation. CFHR1 (25 and 50 $\mu\text{g/ml}$) did not significantly affect C3a levels (Fig. 2B, columns 5 and 6), demonstrating that CFHR1 does not affect the C3 convertase. In contrast CFH (50 $\mu\text{g/ml}$) strongly inhibited C3a generation (Fig. 2B, column 7). Thus CFHR1 does not affect the C3 convertase and likely acts downstream of this convertase.

CFHR1 regulates the C5 convertase of the alternative pathway

CFHR1 binds C3b and inhibits alternative complement activation prior to MAC assembly and insertion. Therefore we hypothesized that CFHR1 inhibits the C5 convertase of the alternative pathway. To this end sheep erythrocytes were incubated in complement active $\text{HP}_{\Delta\text{CFH}}$ and C3a as well as C5a generation was determined. CFHR1 showed a dose dependent protective effect on erythrocyte lysis, did not affect C3a generation, but reduced C5a generation (Fig. 3A). Thus CFHR1 inhibits the C5 convertase of the alternative pathway.

Having demonstrated that CFHR1 inhibits the C5 convertase, we next aimed to characterize this inhibitory effect in more detail. To this end sheep erythrocytes were incubated in CFH depleted, complement active human plasma which in this case was depleted for CFH and C8 ($\text{HP}_{\Delta\text{CFH}\Delta\text{C8}}$). Surface deposition of C3b and C5b was analyzed by flow cytometry. CFHR1 (10, 20 and 40 $\mu\text{g/ml}$) did not, but CFH (10, 20 and 40 $\mu\text{g/ml}$) strongly reduced C3b deposition (compare Fig. 3B, solid red and blue lines). However, CFHR1 (10, 20 and 40 $\mu\text{g/ml}$) reduced C5b deposition by 40, 50 and

60% (Fig. 3B, red stippled line). In the presence of CFH which acts on the C3 convertase no further progression to the level of C5 convertase occurs (Fig. 3B, stippled blue line). These results show that CFHR1 controls complement activation at the level of C5.

The inhibitory effect of CFHR1 on C5b but not C3b generation was further investigated by analyzing surface deposition of C5/C5b and C3b on sheep erythrocytes by microscopy. Again CFHR1 inhibited C5/C5b-, but not C3b surface deposition (Fig. 3C, panels II and V). The C3 convertase inhibitor CFH affected both C3b and C5/C5b deposition (Fig. 3C, panels III and VI). These results confirm that CFHR1 inhibits the C5 convertase of the alternative pathway and prevents C5 cleavage, as demonstrated by reduction of C5/C5b deposition and C5a generation.

To confirm the CFHR1 inhibitory effects on the C5 convertase, the C5 convertase was generated *in vitro* using purified complement components. Activity of the convertase was demonstrated by an increase of C5a levels upon incubation of convertase loaded sheep erythrocytes with C5 (Fig. 3D, column 2). Both recombinant and plasma purified CFHR1 (Supplementary Fig. 1C) reduced C5a generation (Fig. 3D, columns 3 and 4). Similarly C5 cleavage was reduced by CFH (column 5) but not by BSA (Fig. 3D, column 6). These results confirm that CFHR1 inhibits the C5 convertase and reduces C5 cleavage.

CFHR1 binds C5 and C5b6

CFHR1 inhibits the alternative pathway C5 convertase, therefore direct binding of CFHR1 to C5 and to the activation product C5b6 was studied. CFHR1 was captured

with mAbs that either bind to the C-terminus (C18) or the N-terminus (JHD10). C5 and C5b6 bound to CFHR1, which was immobilized via the C-terminus and which has the N-terminal SCRs accessible (Fig. 4A, columns 1 and 2). Reduced binding of C5 or C5b6 to CFHR1 was observed when CFHR1 was immobilized via JHD10, which binds the N-terminal epitope and has the C-terminus accessible (Fig. 4A, columns 5 and 6). These results indicate that the N-terminal region of CFHR1 binds C5 and C5b6.

To analyze interaction of CFHR1 with C5 in plasma, CFHR1/C5 complex formation in plasma was analyzed by immunoprecipitation. Normal human plasma was incubated with a C5 monoclonal antibody which was bound to a protein A matrix. Bound complexes were eluted from the matrix, separated by SDS-PAGE, transferred to a membrane and C5 and CFHR1 were identified by Western blotting. CFHR1 forms complexes with C5 as demonstrated by the presence of both proteins CFHR1 and C5 in the immunoabsorbed sample (Fig. 4B, lane 2; compare upper panel for C5 and lower panel for CFHR1). An eluate derived from a CFHR1 or C5 lacking sample showed the absence of either protein (Fig. 4B, lane 1, co).

Interaction between CFHR1 and C5 was further confirmed by surface plasmon resonance and by ELISA. CFHR1 showed binding to C5 as indicated by dose dependent association and reduced dissociation of CFHR1 to C5 (black lines). In contrast to CFHR1 the CFH fragment including SCR10-13 (grey lines) did not interact with C5 (Fig. 4C). C5 binding to CFHR1 or CFH was also measured by ELISA and demonstrated dose dependent binding to CFHR1 but no interaction with CFH (Fig. 4D). Thus the interaction with C5 represents a distinct characteristic of CFHR1.

CFHR1 prevents non enzymatic assembly of MAC

Cleavage of C5 is the last enzymatic reaction of the complement cascade and MAC complexes are formed by conformational changes and protein assembly. Therefore we asked whether CFHR1 might inhibit MAC assembly and deposition on the lipid bilayer. To this end chicken erythrocytes were incubated with increasing amounts of CFHR1 together with C5b6 and added to non lytic, complement inactive HP deficient of CFHR1 and CFHR3, which was used as a source for C7 to C9. MAC formation was followed by assaying lysis of erythrocytes. CFHR1 inhibited lysis in a dose dependent manner, at 70 $\mu\text{g/ml}$ FHR1 reduced lysis by 38 % (Fig. 5A, red triangles). This inhibitory effect was specific for CFHR1 and was neither observed for CFH nor for BSA (Fig. 5A, blue diamonds and black squares, respectively). These data identify a second regulatory function of CFHR1: inhibition of MAC assembly or formation.

This complement regulatory activity was confirmed for native plasma derived CFHR1 (Supplementary Fig. 1B, lanes 3 and 6). Sheep erythrocytes, that are incubated with the terminal complement components C5b6, C7, C8 and C9 are lysed (Fig. 5B, column 1). Preincubation of C5b6 complexes with recombinant (50 $\mu\text{g/ml}$) or native CFHR1 (12.5 $\mu\text{g/ml}$) proteins inhibited hemolysis (Fig. 5B, columns 2 and 3). Similarly preincubation of C5b6 complexes with the MAC inhibitor vitronectin (12.5 $\mu\text{g/ml}$) inhibited hemolysis (Fig. 5B, column 5). In this set up CFH (12.5 $\mu\text{g/ml}$) (column 4) or BSA (12.5 $\mu\text{g/ml}$) (Fig. 4D, column 6) had no effect. The inhibitory activity of plasma purified CFHR1 and vitronectin on MAC assembly were compared. Both proteins were added to C5b6 and the terminal components C7, C8 and C9 and

then to erythrocytes. Plasma derived CFHR1 and vitronectin showed similar inhibitory effects on sheep erythrocyte lysis (Figure 5C). These data demonstrate that both, recombinant and plasma derived CFHR1 block MAC formation.

CFHR1 and CFHR3 deficient serum reduces viability of human nucleated cells

As CFHR1 and CFHR3 deficiency increases the risk for aHUS¹² we asked whether the lack of CFHR1 in plasma may result in enhanced complement activation on the surface of nucleated human cells. To this end the metabolic activity of human endothelial cells (HUVEC) upon complement challenge was monitored after uptake of the non fluorescent substrate resazurin by following the intracellular conversion to the fluorescent dye resorufin. Cellular vitality results in a typical metabolic response which is followed by fluorescence generation (Supplementary Fig. 4, HP). However, when cells were incubated in complement active, CFHR1/CFHR3 deficient HP (defHP), derived from three healthy individuals, cell viability was reduced, as shown by the slower turnover of the substrate (Supplementary Fig. 4, defHP). When plasma from three CFHR1/CFHR3 deficient HUS patients was assayed cell vitality and metabolic activity was significantly reduced (Supplementary Fig. 4, defHP,- aAb,HUS). These data indicate that the absence of CFHR1 and CFHR3 results in reduced cell vitality, likely due to inappropriate control of complement activation at the surface of nucleated human endothelial cells.

Discussion

Here we identify CFHR1 as a novel human complement regulator. CFHR1 is a human plasma protein, composed of five SCR domains, with two functional regions. The N-terminus, i.e. SCRs 1-2 binds C5 and C5b6 and the C-terminus, i.e. SCRs 3-5, binds C3b/C3d, heparin and to host cells. CFHR1 is a complement regulator that controls the activity of the C5 convertase and also assembly and membrane insertion of MAC. This is - to our knowledge - the first description of a regulator of the C5 convertase which does not affect the C3 convertase. CFHR1 and CFH have almost identical C-terminal surface binding regions and the two proteins bind to the same ligands, i.e. C3b and heparin and colocalize at the surface of endothelial cells (Figure 1C and 1D). This simultaneous binding suggests a sequential and coordinated action at the cell surface. As CFHR1 mutations and absence of the protein in plasma is linked to renal and retinal diseases, such as HUS and AMD the characterization of CFHR1 as a complement regulator deepens our understanding on the molecular mechanisms leading to pathology.

The CFHR1 cDNA identified in 1991 represented the first member of the group of CFH related plasma proteins^{17,18,29}. Each of the known five CFHR proteins is encoded by a unique gene, which are located adjacent to the *Factor H* gene on human chromosome 1³⁰. The five CFHR proteins show immune crossreactivity, and individual domains have significant sequence identity to domains of Factor H. The two N-terminal SCRs of CFHR1 (i.e. SCRs 1-2) show 36 and 45 % sequence identity to SCRs 6 and 7 of CFH. The three C-terminal SCRs are almost identical with the C terminus of CFH, except for the residues L290 and A296 in SCR 5 of CFHR1, which correspond to S1191 and V1197 in SCR 20 of CFH. The CFHR1 plasma

concentration is about 70 to 100 $\mu\text{g/ml}$ (1.6 to 2.4 μM) (data not shown) and thus comparable to the concentrations of the terminal complement components C5 to C9. Due to its association with high density lipoproteins (HDL)¹⁹ the concentration of CFHR1 in the circulation might actually be higher.

CFHR1 is a complement regulator acting in the late alternative and early terminal complement pathway:

1. CFHR1 is –to our knowledge– the first human regulator of the C5 convertase of the alternative pathway which does not inhibit the C3 convertase. CFHR1 at physiological concentrations inhibits C5 cleavage and prevents C5a generation (Fig. 3). This effect is in agreement with C3b and C5 binding, suggesting that CFHR1 binds C3b and C5 simultaneously and thus may contact the C5 convertase (C3bBbC3b) and the substrate C5 at the same time^{4,31}. CFHR1 regulates C5 convertase activity and inhibits further complement activation. The physiological effects are inhibition of C5a and C5b generation. Thus formation of the potent anaphylactic peptide C5a is blocked and also MAC complex formation and cytolysis^{31, 32}.

2. CFHR1, but not CFH inhibits assembly of C5b6(7) complexes and prevents surface attachment (Fig. 3, 4 and 5). CFHR1 may act in concert with the soluble terminal pathway inhibitors clusterin³⁴ and vitronectin³⁵. Binding and inhibitory activity on the C5b6 complexes is independent of convertase activity (Fig. 5). These complement regulatory functions could also be the reason why pathogens like

Candida albicans, *Aspergillus fumigatus* or *Pseudomonas aeruginosa* bind CFHR1 to their surfaces^{36,37,38}.

3. The C-terminus of CFHR1, similar to that of CFH, binds to C3b, heparin and to cell surfaces (Fig. 1, supplementary Fig. 2). Thus CFHR1 likely has the capacity to discriminate between self and foreign surfaces. The three C-terminal SCRs of CFHR1 bind immobilized C3b with lower affinity as compared to the three C terminal SCRs of CFH (K_D 6.4×10^{-6} vs 2.6×10^{-6} M) (supplementary Fig. 2C and Fig 2D). The lower affinity of CFHR1 to C3b confirms previous results, that demonstrate reduced heparin- and cell surface binding of a HUS associated CFH mutant, which encompasses the CFHR1 specific residues at position 1191 and 1197^{21,39}. CFHR1 competes of CFH at a heparin surface and thus reduces local CFH mediated regulatory activity (Fig. 1F). CFHR1 may replace CFH at surfaces and reduction of CFH mediated C3 convertase inhibition is for the gain of C5 regulatory activity. This balance may explain the opposing effects of CFHR1 deficiency, risk in HUS versus protection in AMD.

CFHR1 and CFHR3 deficiency is a predisposing factor for aHUS¹², correlates with the presence of autoantibodies to CFH¹⁴, and defines a new subgroup of aHUS in children termed DEAP HUS (deficiency of CFHR proteins and CFH autoantibody positive)¹⁴. The risk effect of CFHR1 deficiency in aHUS suggests that the coordinated action of the two regulators CFHR1 and CFH is required for surface integrity in situations of complement stress. Both CFHR1 and CFH inhibit complement activation, prevent cell lysis of sheep erythrocytes and protect human endothelial cells (Fig. 2 and supplementary Fig. 4). Thus the absence of CFHR1 may

result in enhanced complement activation on host cell surfaces leading to endothelial cell- or platelet damage and to pathology (supplementary Fig. 5). This effect is in agreement with the recent detection of CFHR1 in dense deposits of patients with dense deposit disease (MPGNII), suggesting that CFHR1 plays a role in this renal disease⁴⁰. However, additional investigations with more patients samples are necessary to define the patho-mechanism.

Deficiency of CFHR1 and CFHR3 is a risk factor in HUS, however has a protective effect in AMD. Currently the reasons for these opposing effects are poorly understood. Based on the presented data we hypothesize that the unique functions of CFHR1 and CFH are responsible for this difference. CFH is a regulator of the C3 convertase and promotes degradation of C3b and opsonisation of a particle with C3b which results in phagocytosis. CFHR1 controls the later steps of the complement activation, regulates C5 convertase activity and early MAC assembly. Thus CFHR1 blocks C5a formation and consequently inhibits inflammation. Therefore, in the absence of CFHR1 and CFHR3, local CFH binding and activity is increased resulting in enhanced iC3b deposition and likely phagocytosis of opsonized particles. In the retina this scenario may be advantageous for the clearance of cellular debris. The prevalence especially of AMD is growing on the background of increasing longevity of the population. Starting to resolve the functional role of the disease associated protein CFHR1 and of CFHR3 is a further step to define the underlying biological mechanism of the complement system for the development of disorders like AMD, HUS and MPGN.

The authors declare that no conflict of interest exists.

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Authors contributions

S.H., A.H., N.L., U.W., H-M.D, K.G., S.S., T.E., S.Hä, M.M performed experiments and discussed results, R.W. generated the monoclonal antibody JHD10, U.SCH-SCH. performed immunohistology , P.F.Z. and C.S. conceived and directed the study and wrote the manuscript.

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Figure Legends

Figure 1: CFHR1 binds to C3b and cells and competes with CFH

(A) CFHR1 is composed of five SCR domains. SCRs 1 and 2 show 42 and 34 % sequence identity to SCRs 6 and 7 of Factor H (CFH). The three C-terminal SCRs show 100, 100 and 98 % sequence identity to SCRs 18-20 of CFH which comprise the C-terminal surface binding region. **(B)** Equimolar concentrations of CFHR1 (25 $\mu\text{g/ml}$) and CFH (75 $\mu\text{g/ml}$) bind to immobilized C3b. Data represent mean values \pm standard deviations from three separate experiments. Control represents binding of antibodies to immobilized BSA **(C)** Plasma derived CFHR1 (red curve) binds to HUVEC cells. Cells were incubated in human plasma and bound CFHR1 was visualized with specific mAb (JHD10) by flow cytometry. Control: cells were treated with the secondary antibody alone (black curve). **(D)** Immunofluorescence staining of CFHR1 (green fluorescence) in renal and retinal human tissue. CFHR1 is detected at the lining of renal (panel I) and ocular (panel IV) blood vessels including large arteries, afferent and efferent arterioles associated with glomeruli (panel I), or the choriocapillaries (panel IV) as well as Bruch's membrane (nuclear counterstain: propidium iodide; original magnification x100). Preabsorption of mAb JHD10 with CFHR1 blocks reactivity (panels II, V). In contrast preabsorption with CFH (panels III, VI) does not affect reactivity and demonstrates specificity of signals in panel I and IV. Thus CFHR1 is present at the surface of endothelial cells and at the Bruch's membrane. Inlays represent magnifications. Autofluorescence of lipofuscin containing cells appears yellow. **(E)** HUVEC cells were incubated with a combination of CFHR1 (100 $\mu\text{g/ml}$) and CFH (100 $\mu\text{g/ml}$). After addition of the appropriate secondary antibodies bound proteins were identified by confocal laser scanning microscopy. CFHR1 binding was detected with the CFHR1 specific mAb JHD10 in combination

with a secondary anti mouse antibody labeled with Alexa 647 (red fluorescence) (panel I) and binding of CFH with a polyclonal antiserum specific for the N-terminal domains of CFH (anti SCR1-4) together with a secondary goat antirabbit antibody labeled with Alexa 488 (panel II) (green fluorescence). An overlay of panels I and II (panel III) reveals colocalization of the two regulators as indicated by the yellow signal. Binding of primary (JHD10) and secondary antibodies showed no signal (control; panel IV). All cells were stained with DAPI to visualize the cell nucleus (bar = 20 μm). **(F)** CFHR1 competes with Factor H for heparin binding and thus affects the regulatory activity at surfaces. Constant amounts of Factor H (10 $\mu\text{g/ml}$) were bound to immobilized heparin and CFHR1 was used at increasing concentrations (0.1 – 20 $\mu\text{g/ml}$) as competitor. Following competition C3b and Factor I were added. After incubation for 30 min the supernatant was removed, separated by SDS-PAGE, transferred to a membrane and C3b and degradation fragments were visualized with C3 antiserum (upper panel). The mobility of the α' and β chain as well as the degradation fragments are indicated. A densitometric analysis as determined by the ratio of the α' 43 band and the β chain is shown in the lower panel. A representative experiment out of three separate experiments is shown.

Figure 2: CFHR1 is a regulator of the alternative pathway of complement

(A) Hemolysis of sheep erythrocytes in the presence of CFHR1 and CFH depleted normal human plasma ($\text{HP}_{\Delta\text{CFH}}$). Sheep erythrocytes represent non activator surfaces when incubated in complement active human plasma. However when the same cells are incubated in $\text{HP}_{\Delta\text{CFH}}$, these cells represent activator surfaces and are lysed. Factor H acts as a surface protector and reverts the effect (grey squares). Addition of CFHR1 (5 to 160 $\mu\text{g/ml}$) results in a reduction of erythrocytes lysis, which indicates a

regulatory effect of this protein in complement control (black triangles). Similar inhibition of hemolysis is observed with vitronectin (grey diamonds). HSA has no effect on hemolysis (open squares). Data show one representative out of three experiments. A_{415} , absorbance at 415 nm. **(B)** CFHR1 does not affect C3a generation of an *in vitro* assembled C3 convertase. C3 was incubated with Factor D and Factor B and C3 convertase activity was analyzed by comparing C3a prior (column 3) and after addition of C3 (column 4). Addition of CFHR1 (25 or 50 $\mu\text{g/ml}$) did not significantly effect C3a generation (columns 5 and 6). CFH (50 $\mu\text{g/ml}$) strongly effected C3 convertase activity (column 7). Addition of human serum albumin (HSA) did not affect C3a generation (column 8). C3 mAb which react with C3a (1 $\mu\text{g/ml}$, standard) (column 2) did not react with an empty well (co). A representative result of three independent experiments is shown and standard deviations are given. A_{490} , absorbance at 490 nm.

Figure 3: CFHR1 regulates C5 convertase activity, binds to C5 and C5b6, and inhibits binding of C5b6 to the cell surfaces

(A) Sheep erythrocytes were incubated in complement active CFH/CFHR1 depleted HP ($\text{HP}_{\Delta\text{CFH}}$) in the presence or absence of CFHR1 (18 to 300 $\mu\text{g/ml}$). Lysis was recorded after 30 min. In addition the concentration of C3a (grey squares) and C5a (red triangles) was assayed in the supernatant. Data represent mean values of three separate experiments and standard deviations are indicated. *, $P < 0.05$, ** $P < 0.005$ versus control. **(B)** Effect of CFHR1 on C3b (solid line) and C5/C5b (stippled line) deposition on the surface of sheep erythrocytes. Sheep erythrocytes were incubated in $\text{HP}_{\Delta\text{CFH}-\Delta\text{C8}}$ plasma and deposition of C3b and C5b was assayed in the presence of increasing concentrations of CFHR1 (10, 20 and 40 $\mu\text{g/ml}$, red solid and stippled

lines) and CFH (10, 20 and 40 μ g/ml, blue solid and stippled lines) by flow cytometry. Data represent mean values \pm standard deviations of three separate experiments. **(C)** CFHR1 inhibits C5/C5b deposition on sheep erythrocytes (panel V) which were incubated in human CFH and C8 depleted plasma (HP $_{\Delta$ CFH- Δ C8}). CFHR1 does not effect C3b deposition (panel II). Factor H (CFH) inhibits both C3b and C5/C5b deposition (panels III and VI). Bars: 10 μ m **(D)** CFHR1 inhibits C5 convertase activity. Recombinant CFHR1 (column 3) as well as plasma purified CFHR1 (column 4) inhibit the C5 convertase and cleavage of C5. The C5 convertase was generated on the surface of sheep erythrocytes using purified C3, Factor B and Factor D in the presence of Ni²⁺ and properdin. Cleavage and C5a generation was determined by ELISA. CFH also affected C5a generation (column 5) in contrast to BSA (column 6). Data represent mean values of three separate experiments and standard deviations are indicated. *, P < 0.05, **P < 0.005 versus BSA.

Figure 4: CFHR1 but not CFH binds to C5

(A) CFHR1 was captured to the surface of a microtiter plate using the C-terminal-(C18) or the N-terminal binding mAbs (JHD10). C5 or C5b6 were applied with the fluid phase and bound proteins were detected with mAb to C5. Attachment of CFHR1 to C18 or JHD10 was verified with polyclonal CFHR1 antiserum (column 4 and 8). In addition binding of C5b6 to the mAB JHD10 in the absence of CFHR1 was assayed and was at background levels (column 7). Data represent mean values \pm standard deviation of three separate experiments. **P < 0.001 versus binding of C5 or C5b6 to C18 mediated CFHR1 immobilisation. **(B)** Formation of native CFHR1/C5 complexes in plasma were identified by immunoprecipitation. C5 antibodies were used to capture C5 complexed with CFHR1 from human plasma as shown by Western Blot

using polyclonal CFH antibodies (lower panel, lane 2, arrows) and monoclonal C5 antibodies (upper panel, lane 2, arrow). CFHR1 in human plasma (HP, lower panel, lane 3) or plasma purified CFHR1 (lower panel, lane 4) show similar mobilities. Also C5 in human plasma (upper panel, lane 3) or purified C5 (upper panel, lane 4) show similar mobilities compared to the immunoprecipitated C5 protein (upper panel, lane 2). Eluates derived from non coated columns incubated with HP contain neither CFHR1 (lower panel, lane 1) nor C5 (upper panel, lane 1). The extra band in the upper panel, lane 1 is considered unspecific. **(C)** Binding of C5 to immobilized CFHR1. CFHR1 (black line) or CFH/SCR10-13 (grey line) was immobilized to the surface of a sensor chip and C5 (50, 150, 200, 400 nM) was added in the fluid phase. **(D)** Binding of C5 to immobilized CFHR1 and CFH. CFHR1 (10 µg/ml) and CFH (30 µg/ml) were immobilized to a microtiter plate and incubated with increasing concentrations of C5 (1.5 to 50 µg/ml). Binding of C5 was detected with monoclonal C5 antibodies. Co represents reactivity of the mAb C5 to immobilized CFHR1 in the absence of C5. Representative data from two independent experiments are shown.

Figure 5: CFHR1 inhibits non enzymatic steps of the terminal complement pathway.

(A) Chicken erythrocytes were incubated with C5b6 complexes (5 ng/ml), increasing concentrations of CFHR1 and non lytic complement inactive defHP was added as a source for terminal complement components. MAC formation was assayed by following lyses of erythrocytes. Hemolysis was recorded after 30 min by measuring the absorbance at 415 nm. Increasing concentrations of CFHR1 (25 to 100 µg/ml) affected MAC activity and CFH or human serum albumin (HSA) showed no effect. Data represent mean values in % ± s.d. derived from three independent assays. **(B)**

MAC formation on sheep erythrocytes was induced by incubation with C5b6, C7, C8 and C9 components and detected by hemolysis of cells (column 1). Preincubation of C5b6 with recombinant CFHR1 (50 $\mu\text{g/ml}$) or plasma purified CFHR1 (12.5 $\mu\text{g/ml}$) inhibited hemolysis (column 2, 3 respectively). CFH (12.5 $\mu\text{g/ml}$) showed no effect on MAC formation (column 4), but vitronectin (12.5 $\mu\text{g/ml}$) (column 5). BSA (12.5 $\mu\text{g/ml}$) did not induce hemolysis (column 6). Data represent mean values \pm standard deviation of three separate experiments (except for plasma purified CFHR1). **(C)** Inhibitory role of CFHR1 on MAC formation. CFHR1 purified from human plasma (0.1 to 0.3 μM) inhibited MAC formation on the surface of sheep erythrocytes (red triangles, red line). In addition the established MAC inhibitor vitronectin was assayed (blue diamond, blue line). CFHR1 and vitronectin had similar activity and BSA did not affect MAC formation (black squares, black line). A representative experiment out of two is shown.

Figure 1

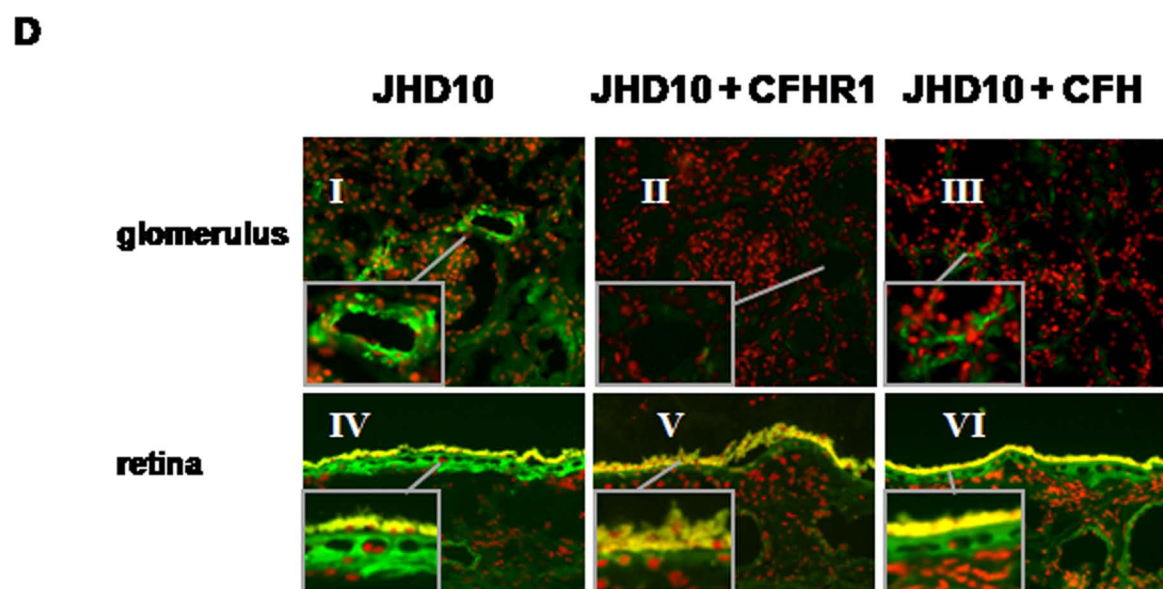
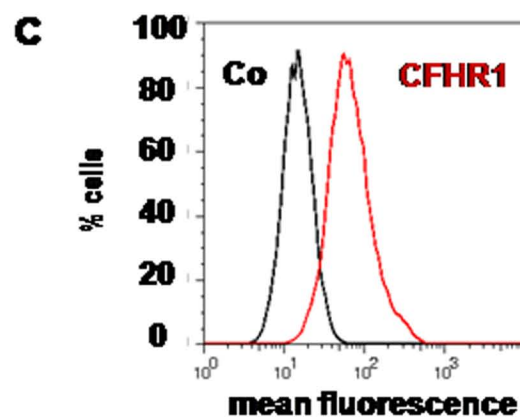
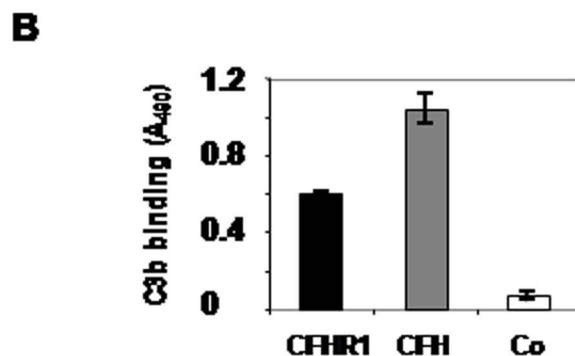
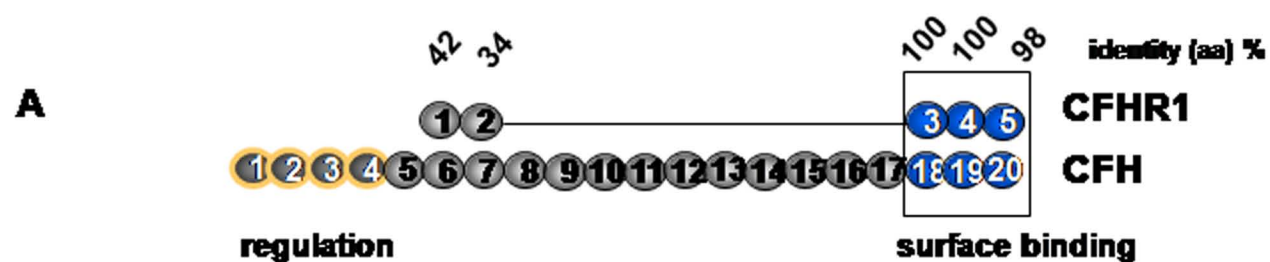
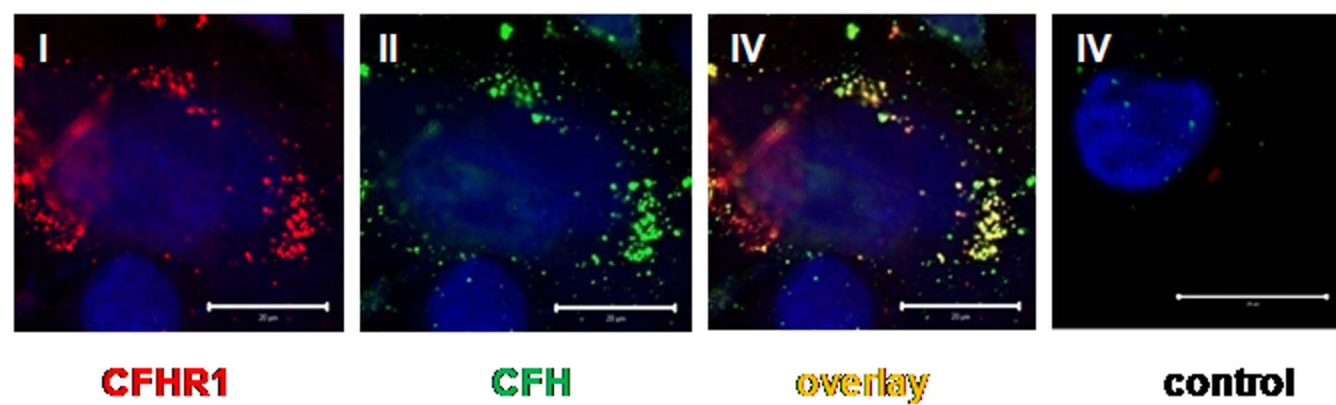


Figure 1

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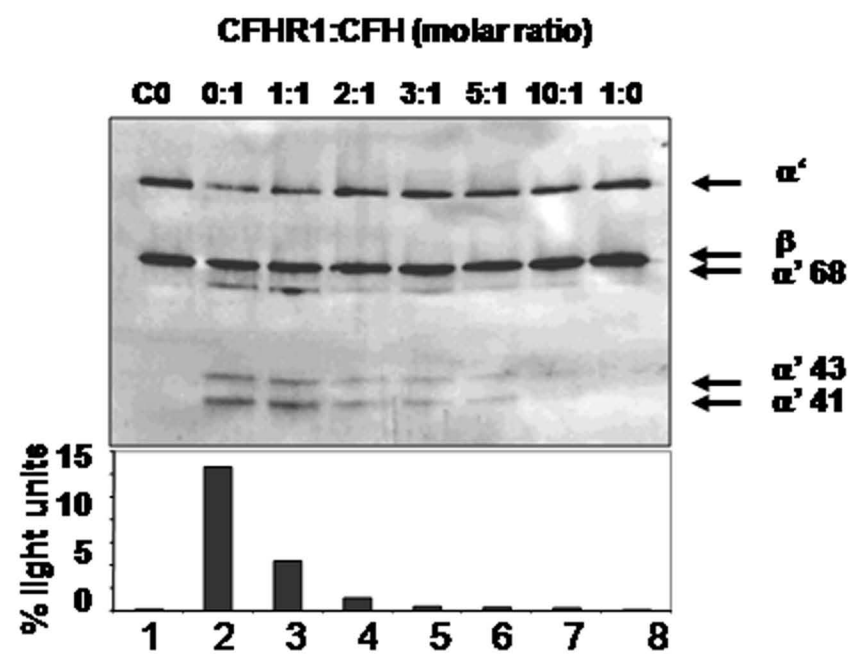


Figure 2

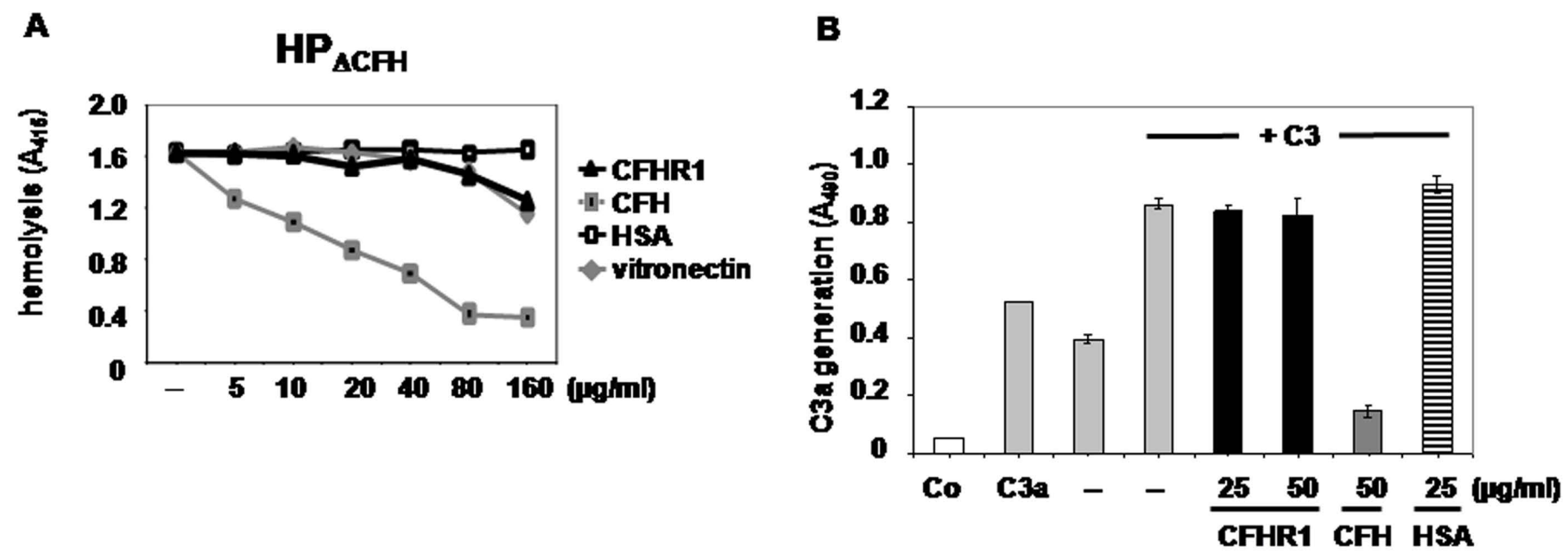


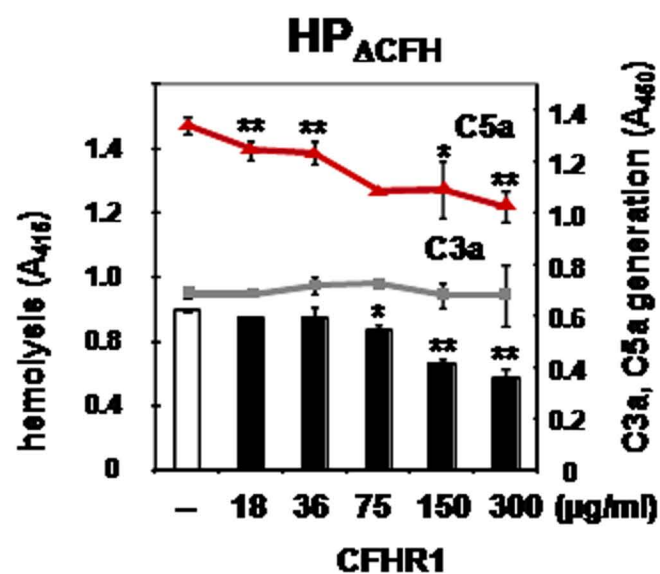
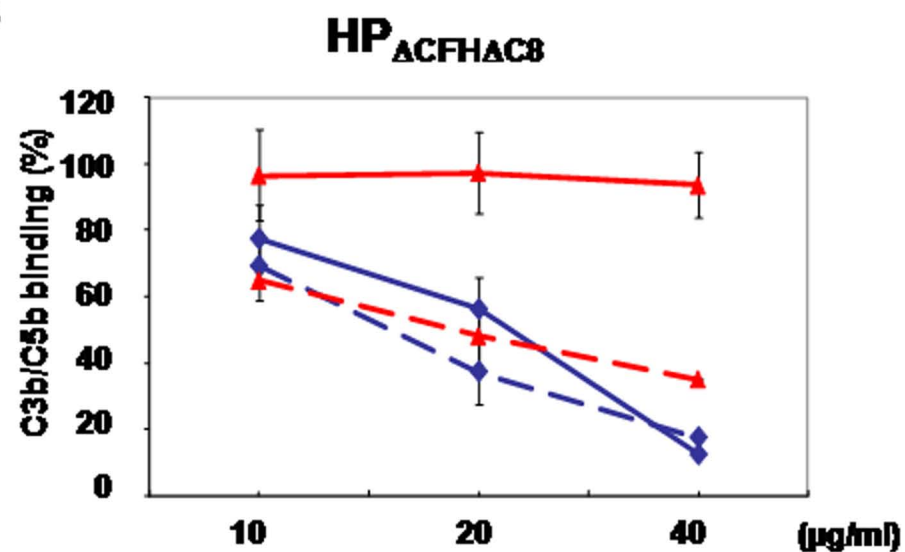
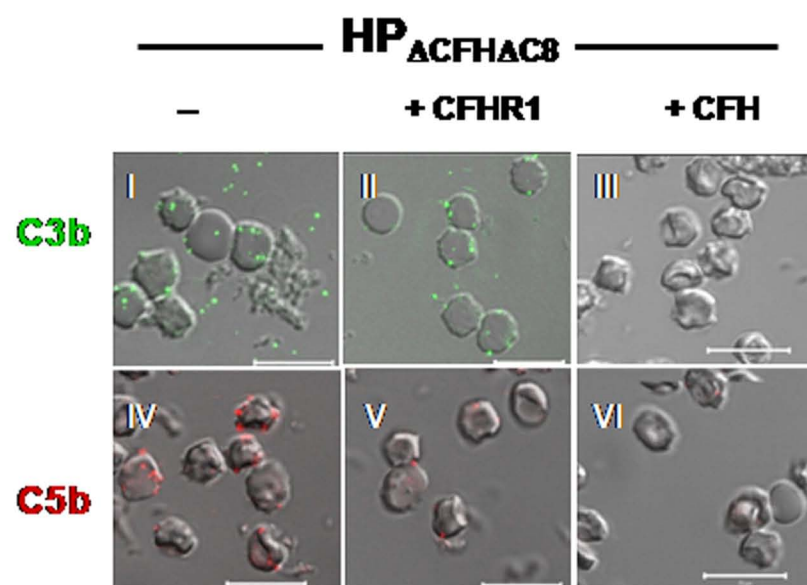
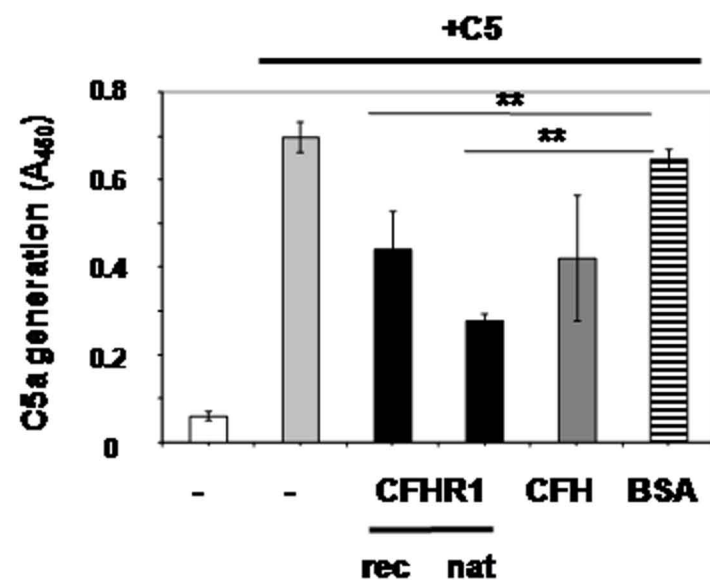
Figure 3**A****B****C****D**

Figure 4

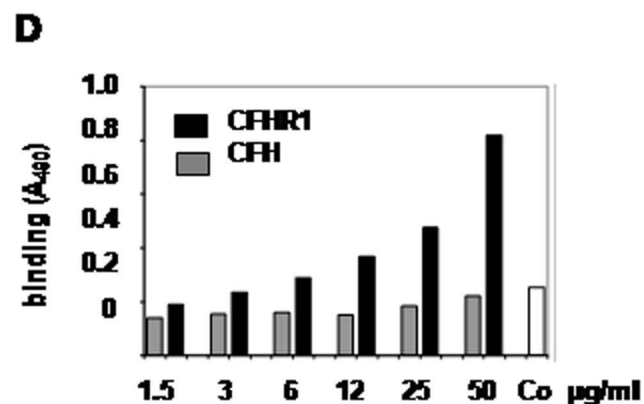
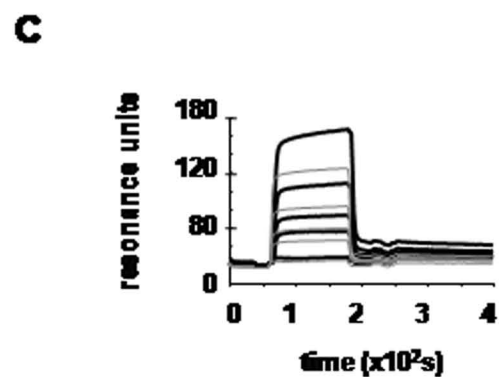
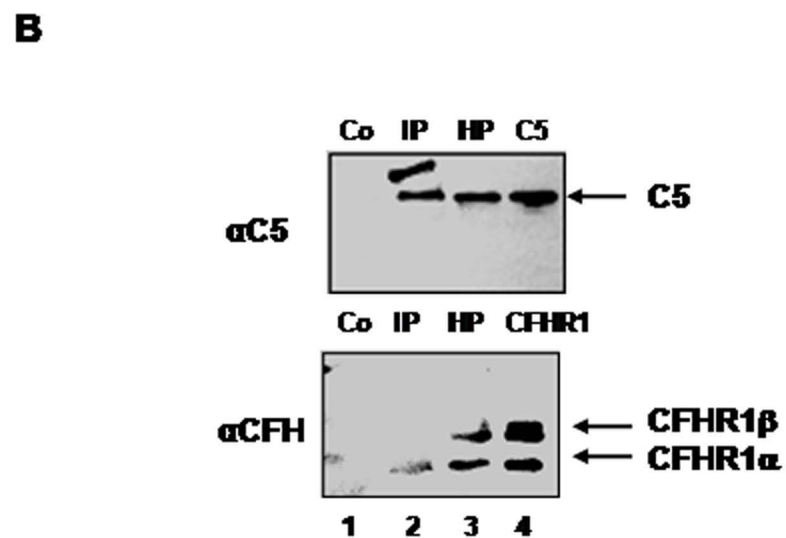
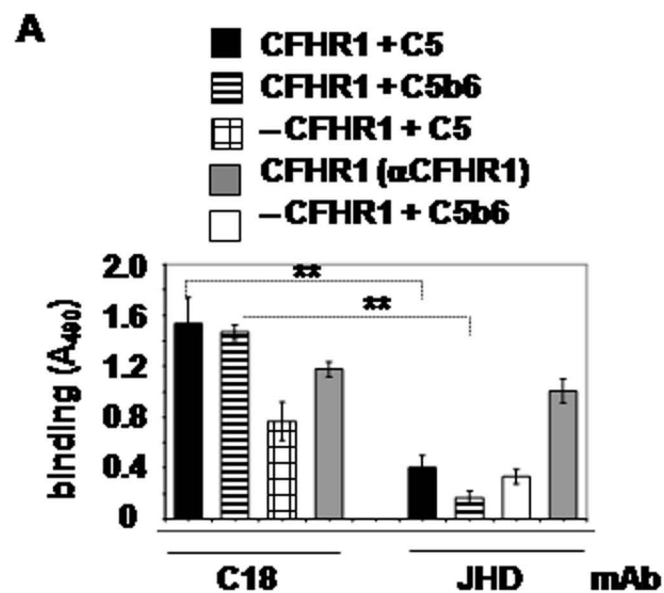
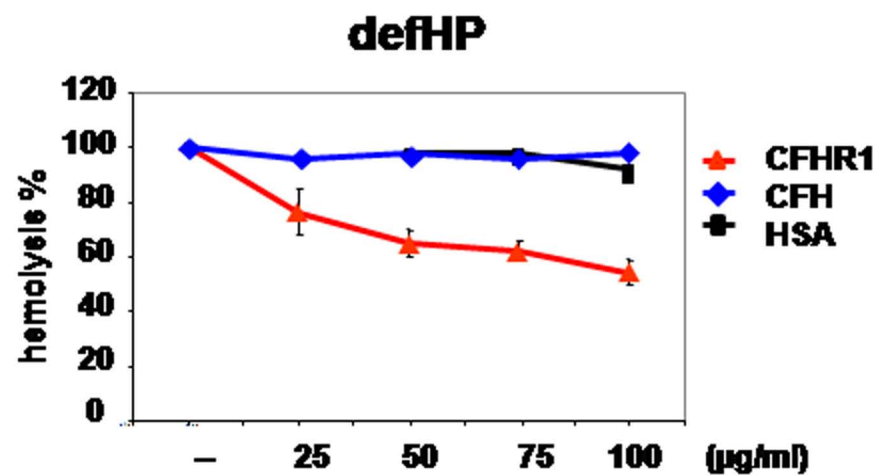
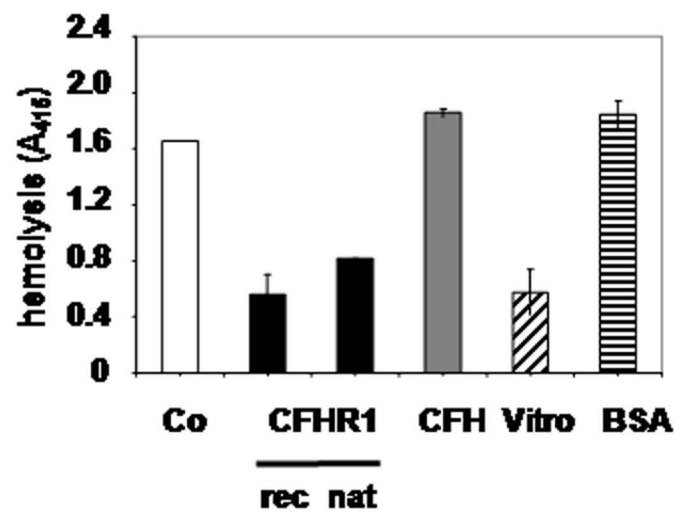


Figure 5

A



B



C

