Functional properties of complement factor H-related proteins FHR-3 and FHR-4: binding to the C3d region of C3b and differential regulation by heparin

Jens Hellwage[a,b,*], T. Sakari Jokiranta[a,b], Vesa Koistinen[b], Outi Vaarala[c], Seppo Meri[b], Peter F. Zipfel[a,d]

[a] *Bernhard Nocht Institute for Tropical Medicine, Bernhard-Nocht-Strasse 74, D-20359 Hamburg, Germany*
[b] *Haartman Institute and HD-Diagnostics, Department of Bacteriology and Immunology, Haartmaninkatu 3, FIN-00290 Helsinki, Finland*
[c] *Department of Biochemistry, National Public Health Institute, Mannerheimintie 166, FIN-00300 Helsinki, Finland*
[d] *Clinic for Anaesthesiology and Intensive Care Medicine, Friedrich-Schiller-University Jena, Bachstr. 18, D-07740 Jena, Germany*

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Abstract The human factor H-related proteins FHR-3 and FHR-4 are members of a family of proteins related to the complement factor H. Here, we report that the two proteins bind to the C3d region of complement C3b. The apparent K_A values for the interactions of FHR-3 and FHR-4 with C3b are 7.5×10^6 M^{-1} and 2.9×10^6 M^{-1}, respectively. Binding studies performed with C3b-coated pneumococci confirmed the results obtained with the biosensor system. A C-terminal construct of factor H showed similar binding characteristics. The interaction of FHR-3, but not of FHR-4, with opsonised pneumococci was inhibited by heparin.

Key words: Complement regulation; Heparin binding; Surface plasmon resonance; Alternative pathway; *Streptococcus pneumoniae*

1. Introduction

The human factor H-related proteins 3 and 4 (FHR-3 and FHR-4) are members of a group of plasma proteins structurally and immunologically related to complement factor H (FH) [1,2]. The cDNAs encoding FHR-3 and FHR-4 were isolated from a human liver cDNA library and are 1269 and 1315 bp in size. The genes for FHR-3 and FHR-4 have recently been mapped to the 'regulators of complement activation' (RCA) gene cluster on human chromosome 1 [3]. The corresponding proteins are composed of 331 amino acids and display a striking similarity to each other. The FHR-3 protein exists in several glycosylated forms in human plasma with a molecular mass ranging from 45 to 56 kDa, while the FHR-4 protein forms a 86 kDa homodimer and is a constituent of plasma and triglyceride-rich lipoproteins [1,2].

The factor H protein family consists of factor H, the factor H-like protein 1 (FHL-1), which is derived from a differently processed transcript of the factor H gene [4–6], and four FHRs (FHR-1–FHR-4) [7]. All members of this family are exclusively arranged in short consensus repeat (SCR) domains, also called complement control protein modules. FHR-1, FHR-3 and FHR-4 are composed of five SCRs, while FHR-2 contains four, FHL-1 seven and factor H 20 SCRs [7]. SCR domains are also found in other complement and non-complement proteins [8].

The biological functions of factor H and FHL-1 in complement regulation are well established [9,10]. Both plasma proteins control alternative pathway complement activation in the fluid phase and factor H may function in the discrimination between self and non-self structures. Factor H and FHL-1 act as cofactors for the serine protease factor I in the proteolytic inactivation of C3b [9–13]. Both proteins also inhibit formation of the C3 convertase C3bBb by competing with factor B for binding to C3b and by accelerating the dissociation of the C3bBb complex [14,15]. Factor H allows for activation of the alternative pathway on foreign cells and particles, while it inhibits activation on host structures, probably by binding to C3b in combination with negatively charged glycosaminoglycans (e.g. heparan sulfate, chondroitin sulfate) or sialic acids [16–19]. Interaction of factor H with C3b is an important step in alternative pathway regulation and a total of three C3b binding sites have been localised on the factor H protein, implying a complex interaction of cooperative binding sites between factor H and C3b [20–22].

As the physiological functions of FHR-3 and FHR-4 are not yet known, our aim was to analyse whether the proteins interact with the central complement component C3b and possibly play a role in complement regulation. We used the surface plasmon resonance technique to analyse FHR-3 and FHR-4 binding to C3b and identified both proteins as ligands for C3b. The binding sites were mapped to the C3d region of C3b. Both FHR-3 and FHR-4 bound to C3b-opsonised pneumococci, suggesting that they could regulate complement-mediated processing of foreign antigens.

2. Materials and methods

2.1. Expression and purification of FHR-3 and FHR-4

Subcloning of FHR-3 cDNA DOWN16 into the baculovirus expression vector pBSV-8His [1,23], recombinant expression and purification of FHR-3 and FHR-4 was performed as described recently [24]. *Spodoptera frugiperda* cells (Sf9) were grown at 28°C in monolayer culture in protein-free Express medium (BioWhittaker, Verviers, Belgium) in the presence of streptomycin (100 μg/ml), penicillin (100 U/ml) and amphotericin B (250 ng/ml) (Life Technologies, Eggenstein,

*Corresponding author. Fax: (358)-9-1912 6382.
E-mail: jens.hellwage@helsinki.fi

Abbreviations: FH, complement factor H; FHDS, factor H-deficient serum; FHR-3, factor H-related protein 3; FHR-4, factor H-related protein 4; SCR, short consensus repeat

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Germany). Sf9 cells were infected with recombinant virus using a multiplicity of infection of five. The culture supernatant was harvested after 9 days and recombinant proteins were purified by nickel chelate chromatography as described [23]. Purified recombinant proteins were dialysed against 1/3 VBS (50 mM NaCl, 3.3 mM barbiturate, pH 7.5) overnight and concentrated using an Ultrafree-15 Centrifugal Filter Device Biomax-5K (Millipore, Bedford, MA, USA). The protein concentration was measured with the BCA Protein Assay reagent (Pierce Chemical, Rockford, IL, USA).

2.2. Purification of complement components

Plasma proteins C3, factors B, D, H and I were purified from human plasma and C3b was generated as described previously [25,26]. C3d was prepared from purified human C3 by a modification of the method of Eggertsen et al. [27]. Briefly, C3 at a concentration of 4 mg/ml in VBS was incubated with TPCK-treated trypsin (Sigma, St. Louis, MO, USA) for 2 h at 37°C at an enzyme/substrate ratio of 1:60. The reaction was stopped by addition of soybean trypsin inhibitor (Sigma) at a ratio of 1:3 trypsin to inhibitor (w/w). C3d was isolated using a Mono Q column (Pharmacia, Uppsala, Sweden) with a 0.1–0.4 M NaCl gradient in 0.025 M Tris-HCl, pH 8.3. In accordance with the findings of Eggertsen et al. [27], C3d was eluted in an asymmetrical peak shortly before the major peak consisting of the higher molecular weight products and appeared under reducing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis as a triplet of bands with apparent molecular masses of 30–35 kDa. The identity of all three bands as C3d was confirmed by their reactivity with the previously described C3d-specific monoclonal antibody 4C2 [25].

2.3. SDS-PAGE and Western blot analyses

Recombinant FHR-3 and FHR-4 purified by nickel affinity chromatography and human plasma were subjected to SDS-PAGE using 10% or 12% gels and detected by Western blotting using polyclonal antisera raised against recombinant FHR-3 or FHR-4 [24,28].

2.4. Heparin affinity chromatography

Five ml of normal human serum diluted in equilibration buffer (1/3×VBS, 50 mM NaCl, 0.05% azide) was applied on a 1 ml agarose column coupled with 10 mg porcine heparin (HiTrap Heparin, Pharmacia). Samples were passed over the column five times at a flow rate of 1 ml/min at room temperature and the fall-through was collected. After washing with 5 ml of equilibration buffer, elution was performed by a linear NaCl gradient from 50 mM to 1 M NaCl at a flow rate of 1 ml/min in 20 min. Fractions of 0.5 ml were collected and the column was regenerated with 5 ml of 2 M NaCl followed by 15 ml of equilibration buffer. The elution fractions were subjected to SDS-PAGE and Western blotting.

2.5. Analysis of interaction with C3b and C3d using surface plasmon resonance

Surface plasmon resonance measurements were performed using a Biacore 2000 instrument and BIAevaluation software V 3.0 (Biacore AB, Uppsala, Sweden). C3b was immobilised on a carboxylated dextran CM5 sensor chip (Biacore AB, Uppsala, Sweden) either by using the amine coupling procedure according to the protocol of the manufacturer or by enzymatic cascade formation of C3b in the presence of 1 mM Ni^{2+} by sequential addition of purified C3, factor B and factor D ([22] and Jokiranta et al., manuscript in preparation). Briefly, 20 μg of C3, 5 μg of factor B and 0.05 μg of factor D were first injected together, followed by 3–5 cycles of injecting factors B and D or C3 sequentially to generate alternative pathway C3 convertases and subsequently C3b molecules bound to the surface via the physiological ester or amide bond. Binding analyses with recombinant FHR-3 and FHR-4 were performed using 1/3 VBS buffer at a flow rate of 5 μl/min and at 30 μl/min for kinetic analyses. After each experiment, the surface was regenerated by passing 30–50 μl of regeneration buffer (2 M NaCl in acetate buffer, pH 4.6) through the flow cell. For analysis of interaction with C3d, the protein was bound to the pre-activated CM5 chip with the standard amine coupling method provided by the manufacturer.

2.6. Radiolabelling of C3b and cofactor analysis

Human C3b (200 μg; from Advanced Research Technologies, San Diego, CA, USA) was labelled with ^{125}I using the Iodogen technique. The cofactor assay was performed as described using either purified factor I or factor H-deficient serum (FHDS) as a source for factor I [29]. The FHDS (kindly provided by Drs. Marina Noris and Silvia Orisio, Mario Negri Institute, Bergamo, Italy) was diluted 1:200 in VBS. The radiolabelled C3b was added to factor I or FHDS in the absence or presence of a cofactor. Factor H was added to a final concentration of 40, 4, 0.4 and 0 μg/ml for titrating its cofactor activity in this assay system. A concentration of 4 μg/ml was then used as a basic level to measure enhancement of cofactor activity by FHRs. Recombinant FHR-3 and FHR-4 were added to final concentrations of 100 and 200 μg/ml. The final reaction volume was 40 μl. C3b incubated with VBS or factor H-deficient plasma in the same dilution served as negative controls. The mixture was incubated at 37°C for 4 h, reduced and analysed by SDS-PAGE. Gels were fixed for 10 min in 10% acetic acid, dried and autoradiographed.

2.7. Binding of FHR-3 and FHR-4 to C3b-opsonised pneumococci

Heat-inactivated pneumococci (isolated from a case of acute otitis media) were coated with C3b by the following procedure: particles were incubated with 1 mg C3, 0.1 mg factor B and 2 μg factor D for 20 min at 30°C in VBS containing 0.1% gelatin and 2.5 mM $NiCl_2$ (GVB-Ni). After washing twice with GVB-Ni, the cells were treated with factors B and D for 4 min at 30°C, washed with GVB-EDTA (2 mM EDTA in GVB) and incubated with 1 mg C3 (20 min at 30°C). After repetition of the last cycle with factors B, D and C3, the particles were washed and used as a 10% solution. In the binding assay, the indicated amounts of opsonised pneumococci were incubated (15 min at 37°C) with the radiolabelled FHRs (60 000 cpm) or a C-terminal recombinant fragment of factor H (SCRs 15–20) in a total volume of 100 μl GVB. Particle-associated factor H was separated from unbound ligand by centrifuging the mixtures through 20% sucrose. After cutting off the pellets, the binding of the FHR/factor H proteins was calculated as a percentage of the total radioactivity input. All experiments were performed a least in duplicate. For the heparin inhibition assay, the indicated amounts of heparin (Sigma, St. Louis, MO, USA) were added to the reaction mixture.

3. Results

3.1. Structure of FHR-3 and FHR-4

As FHR-3 and FHR-4 are related in structure and sequence to each other and to FH, we were interested in studying the function of these proteins and asked whether the two proteins display complement regulatory activities. Both FHR-3 and FHR-4 are composed of five SCR units, a structural comparison of the two proteins is shown in Fig. 1A. SCRs 1–3 of FHR-3 and FHR-4 show similarity with SCRs 6, 7, 8 and 6, 8, 9 of FH, respectively. SCRs 4–5 of FHR-3 and FHR-4 are almost identical to each other and display a high degree of similarity with the C-terminal SCRs 19 and 20 of FH [7].

3.2. Heparin affinity purification of FHR-3 from human serum

Binding of recombinant FHR-3 to heparin has been described recently [19]. To exploit this property, we used heparin affinity chromatography to enrich the native FHR-3 protein from human plasma. FHR-4 did not bind to heparin and its homodimeric form of 86 kDa was found in the fall-through and wash fractions (Fig. 1B) [2,24]. FHR-3 and FH bound to the heparin matrix and were eluted with a linear NaCl gradient. Both proteins were detected in the elute fractions by Western blotting (Fig. 1C). The relative mass of the recombinant FHR-3 protein ranges from 45 to 56 kDa, as it is expressed in differently glycosylated forms (Fig. 1C). These results showed a difference in the heparin binding properties of FHR-3 and FHR-4.

3.3. Binding analysis of FHR-3 to C3b and C3d

We used the surface plasmon resonance technique to ana-

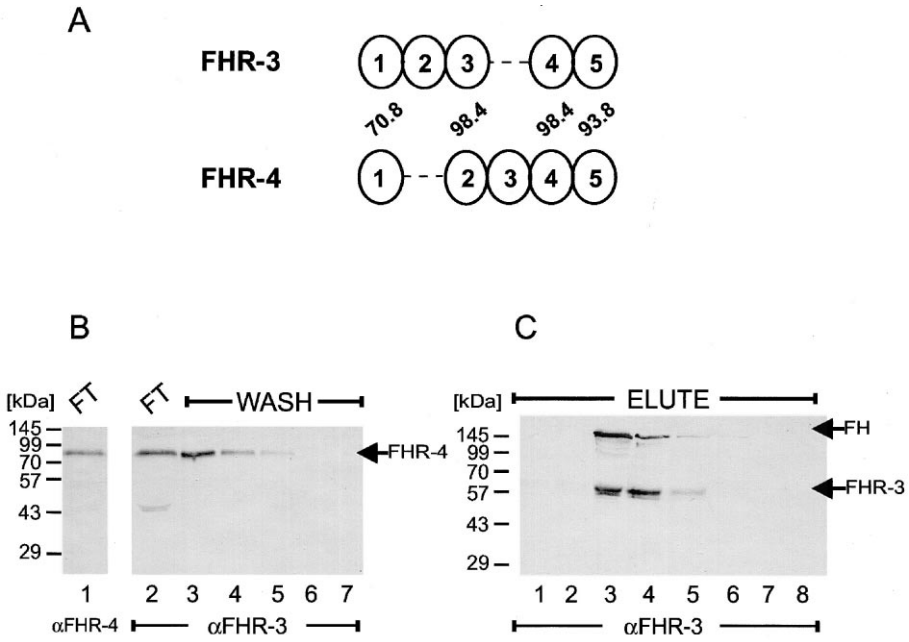

Fig. 1. Structural comparison and heparin binding characteristics of native FHR-3 and FHR-4 proteins. A: The SCR domain structure of FHR-3 and FHR-4 is shown. SCRs are numbered consecutively and the percentage of amino acid similarity of single SCRs is indicated. B: Five ml of diluted human plasma was applied onto a heparin agarose column and after washing, bound proteins were eluted with a linear salt gradient. Analysis of the fall-through (FT) and wash fractions. FHR-4 protein did not bind heparin and was detected with anti-FHR-4 (lane 1) antiserum. For the detection of FHR-3, anti-FHR-3 (lanes 2–7) antiserum was used for the fall-through (FT) and wash fractions. The doublet band of about 43 kDa (lane 2) was not further analysed. C: Detection of FHR-3 in the elute fractions using anti-FHR-3 antiserum. Due to cross-reactivity, FH was also detected in the eluted fractions. The mobility of the size markers is indicated in kDa.

lyse the interaction of FHR-3 and FHR-4 with C3b. C3b was bound to the sensor chip surface either by standard amine coupling or by a new enzymatic method [22]. To further localise the binding site within the C3b molecule, the fragments C3c and C3d were prepared and immobilised onto the sensor chip by amine coupling. We first analysed the interaction of fluid phase FHR-3 with C3b bound to the solid phase. A representative experiment is shown in Fig. 2. Binding of FHR-3 to C3b corresponded to an increase of approximately 3000 resonance units (Fig. 2A, left panel). After 240 s, the FHR-3 solution was replaced with buffer and the dissociation of the FHR-3/C3b complex was followed. Addition of FHR-3 to a non-coated flow cell showed only a bulk effect (control). A specific binding curve, as shown in the right part of Fig. 2A, was obtained by subtraction of the background values. For a more detailed localisation of the interacting region within the C3 molecule, C3c and C3d were used as ligands instead of C3b. No binding to C3c was detected (data not shown), but the interaction of FHR-3 with C3d was similar to the interaction with C3b (Fig. 2B).

3.4. Binding of C3b/C3d to immobilised FHR-3

In order to confirm the interaction of FHR-3 with C3b and C3d, a reverse setting was used, where FHR-3 was immobilised on the chip surface and C3b or C3d was added as fluid phase ligands. With this approach, similar interactions as described above were observed for both C3b (Fig. 2C) and C3d (Fig. 2D). These results show that FHR-3 binds to C3b and C3d and suggest a single interaction site located within the C3d region.

3.5. Binding of FHR-4 to C3b and C3d

We next analysed the interaction of FHR-4 with C3b and C3d immobilised to the chip surface. Similar to FHR-3, FHR-4 was found to bind to C3b (Fig. 3A) and C3d (Fig. 3B) but not to C3c (data not shown). In the reverse setting, binding was observed when FHR-4 was coupled to the chip surface and C3b and C3d were added in the fluid phase (Fig. 3C and 3D).

The apparent overall association (on-rate) and dissociation (off-rate) rates of FHR-3 and FHR-4 were measured for interaction with both C3b and C3d. For both proteins, the kinetics were analysed using a 1:1 binding model with mass transfer. This model was used according to the observation that recombinant FHR-4 exists predominantly in a monomeric form [2,24]. The apparent affinity constants were calculated from the measured rates and are shown in Table 1. The apparent association and dissociation rates of the FHR-3/C3b complex obtained either using C3b with amine coupling or after coupling with the enzymatic method were within the same range (Table 1). The values measured for the on-rate and off-rate of FHR-4/C3b were similar to the results obtained with FHR-3 (Table 1). In all cases, similar results were obtained with both coupling methods and independently of which of the ligands was immobilised to the chip surface.

3.6. Effect of FHRs on the factor I-mediated degradation of C3b

We next assessed whether FHR-3 and FHR-4 act as cofactors for the factor I-mediated C3b inactivation. To this end, recombinant FHR-3, FHR-4 or purified factor H together with [^{125}I]C3b were incubated with factor I. The direct cofactor activity of the FHRs was very low, being detectable only at a high and non-physiological concentration of 400 μg/ml (data not shown). We then used FHDS as a source for factor I. In this situation, the cofactor activity was considerably

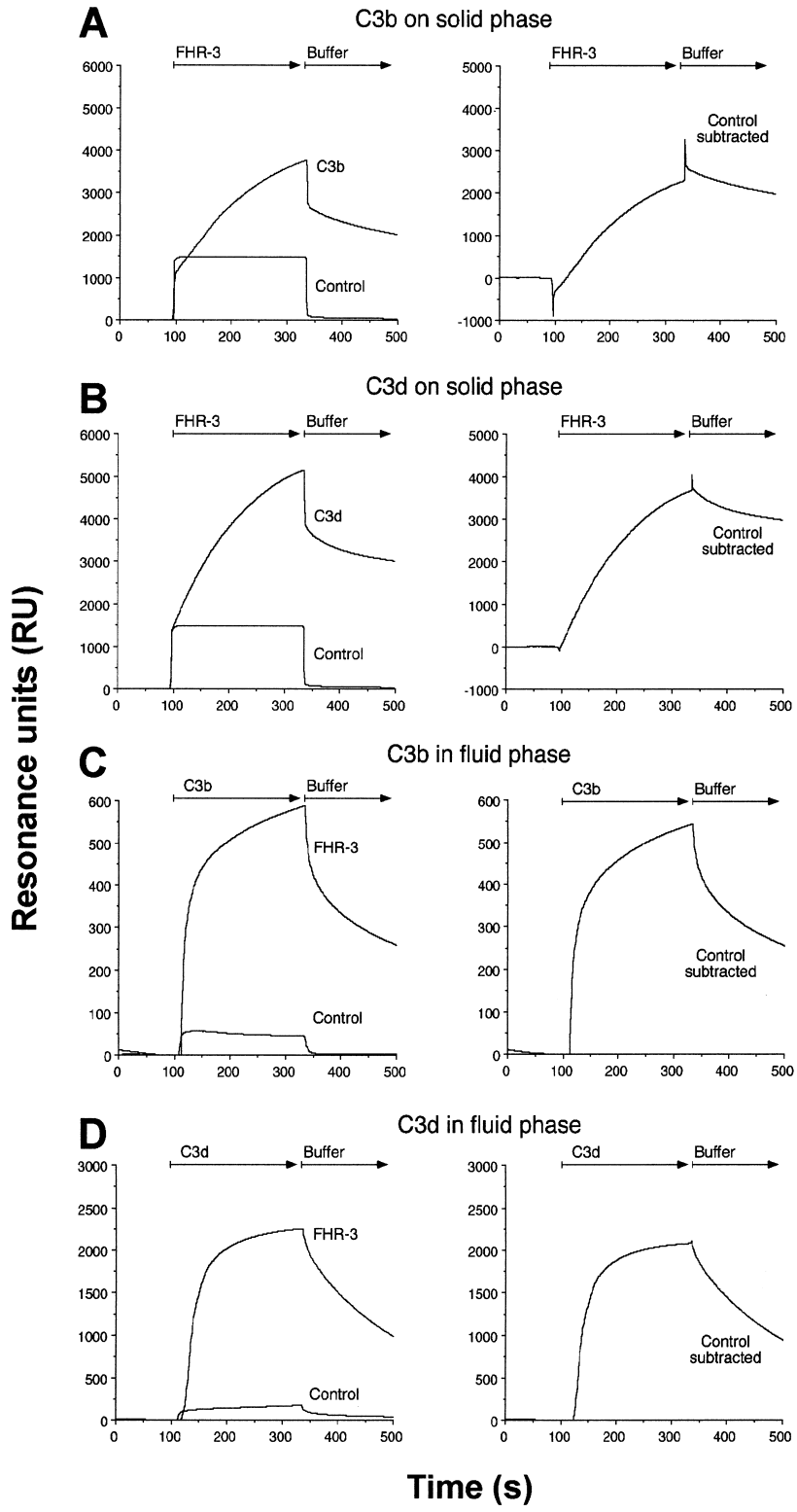

Fig. 2. Determination of interactions of FHR-3 with C3b and C3d by surface plasmon resonance. Interactions of recombinant FHR-3 with C3b either bound to the solid phase (A) or in the fluid phase (C) and with C3d bound to the solid phase (B) or in the fluid phase (D) were analysed with the Biacore equipment. Resonance units are shown as a function of time. The left part displays the binding curves in comparison with the control flow cell (where no coating was performed), while in the right part, the control was subtracted. The arrow indicates the time of injection of the ligand.

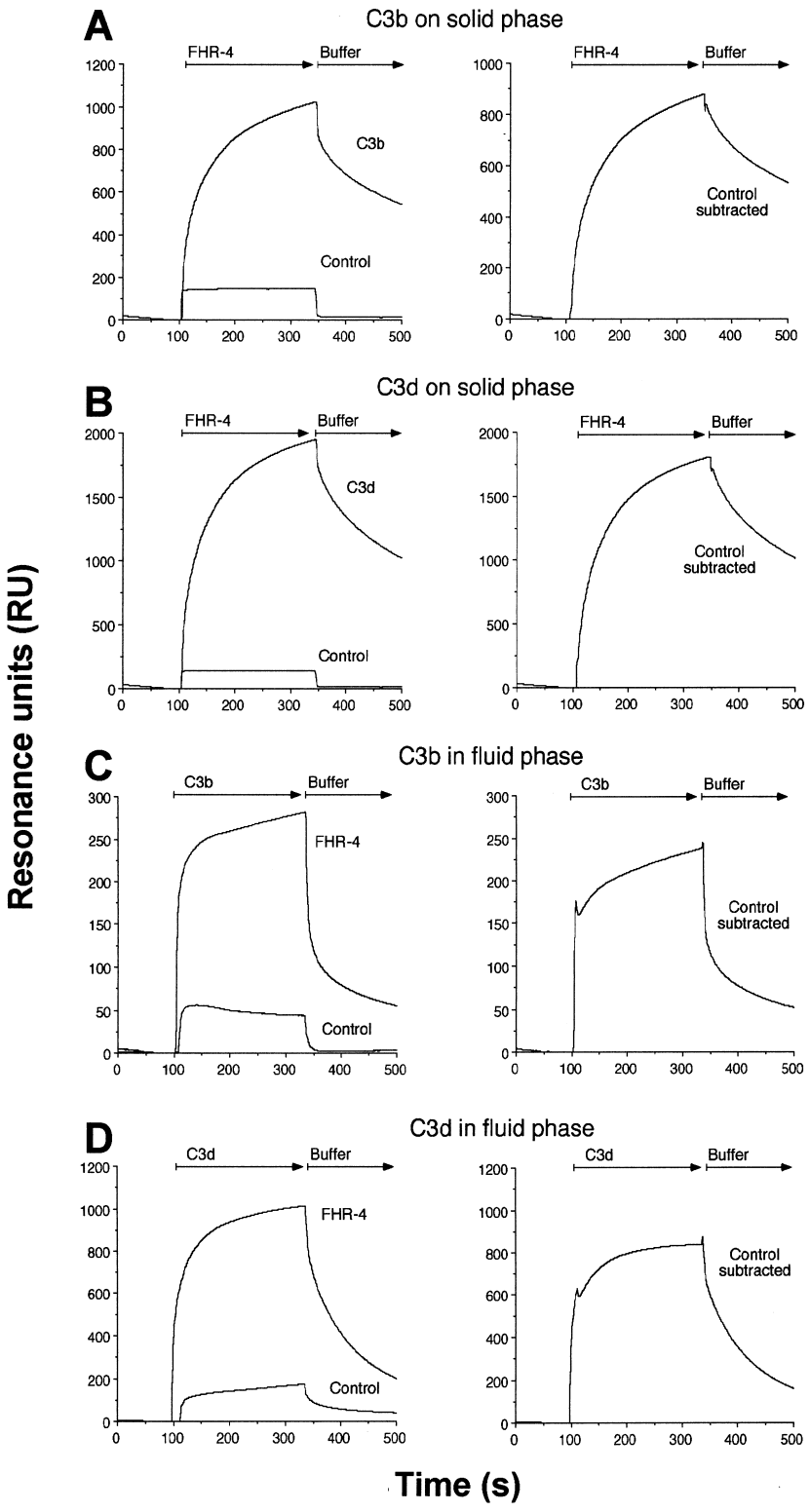

Fig. 3. Interactions of FHR-4 with C3b and C3d determined by surface plasmon resonance. Interactions of recombinant FHR-4 with C3b either on the solid phase (A) or in the fluid phase (C) and with C3d on the solid phase (B) or in the fluid phase (D) were analysed similarly as described for Fig. 2.

higher when FHR-3 and FHR-4 were added (Fig. 4). Thus, it seemed that a component in the FHDS was needed for the cofactor activity. As the FHDS contains trace amounts of FHL-1, which is also a cofactor for factor I in the cleavage of C3b [11,13], we considered the possibility that FHR-3 and FHR-4 promoted the cofactor activity of FHL-1 in the assay. This was confirmed when purified factor H was added to the reaction mixture. We observed that both FHR-3 and FHR-4 enhanced the cofactor activity of factor H. The addition of recombinant proteins FHR-3 and FHR-4 promoted the cleav-

Table 1
Quantitative analysis of the interactions between FHR-3/FHR-4 and fragments of C3

Solid phase	Fluid phase	Apparent k_a (1/Ms)	Apparent k_d (1/s)	Apparent K_A (1/M)	Apparent K_D (M)
C3b (Enz)[a]	FHR-3	2.0×10^{4}	2.7×10^{-3}	7.5×10^{6}	1.3×10^{-7}
C3b (Amine)[b]	FHR-3	1.7×10^{4}	1.4×10^{-3}	1.3×10^{7}	8.0×10^{-8}
C3d	FHR-3	1.8×10^{4}	1.6×10^{-3}	1.2×10^{7}	8.7×10^{-8}
C3b (Enz)[a]	FHR-4	1.6×10^{4}	5.4×10^{-3}	2.9×10^{6}	3.4×10^{-7}
C3b (Amine)[b]	FHR-4	2.3×10^{4}	5.6×10^{-3}	4.2×10^{6}	2.4×10^{-7}
C3d	FHR-4	2.0×10^{4}	5.1×10^{-3}	3.9×10^{6}	2.6×10^{-7}

Association and dissociation rates were determined by surface plasmon resonance analysis. The equilibrium constants were calculated from the rate constants.
[a]Enz: Protein coupling of C3b onto the chip surface was performed by an enzymatic procedure.
[b]Amine: C3b was immobilised using the amine coupling method.

age of the 112 kDa α′-chain of C3b into two fragments of 68 and 41/43 kDa (Fig. 4).

3.7. Binding of FHR-3 and FHR-4 to C3b-opsonised pneumococci

To confirm the results obtained with the biosensor system and to assess binding of FHRs to C3b in a more physiological situation, pneumococci were opsonised with C3b and binding of FHR-3 and FHR-4 were examined. As shown in Fig. 5A, both FHR-3 and FHR-4 bound to C3b on pneumococci. A similar binding was seen with a C-terminal construct SCR 15-20 of factor H, which is known to contain one binding site for C3b/C3d. FHR-3 showed a stronger binding to the C3b pneumococci surface (up to 24.3% binding) than the FH construct SCR 15-20 (9.6%) or FHR-4 (7.5%). The negative control [^{125}I]BSA showed no binding. To test if the enhanced binding of FHR-3 to the pathogen surface was due to an interaction with its heparin binding site, the effect of heparin on the interaction was investigated. As shown in Fig. 5B, the addition of heparin reduced the binding of FHR-3 to the C3b-coated pneumococcal surface to the level of FHR-4. This result suggests that FHR-3 uses both its C3b/C3d and heparin binding sites for the interaction with the surface of the opsonised pneumococci.

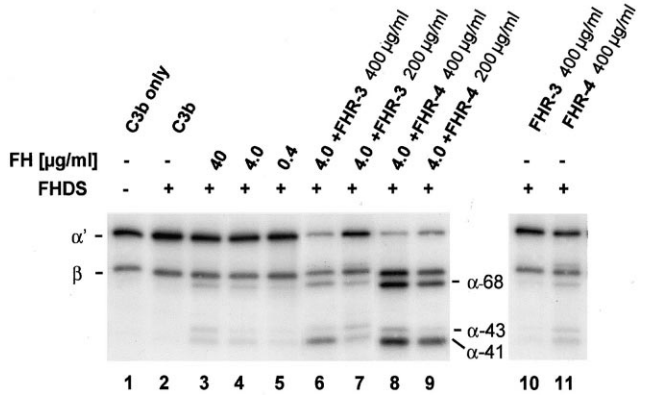

Fig. 4. Factor I cofactor assay. The ability of recombinant FHR-3 and FHR-4 proteins to promote factor I-mediated cleavage of C3b was assayed in human factor H-deficient plasma. Recombinant FHR-3 or FHR-4 and purified factor H were added together with radiolabelled [^{125}I]C3b to diluted human factor H-deficient plasma. After incubation for 4 h, the samples were separated by SDS-PAGE and the gels were analysed by autoradiography. C3b (incubated in VBS, lane 1) and C3b in the diluted FHDS (lane 2) served as negative controls. With the positive control factor H (lane 3), the C3b α-chain was cleaved and products of 41, 43 and 68 kDa were detected. A titration of factor H activity is shown in lanes 3–5. A similar cleavage pattern was seen using FHR-3 (lane 10) and FHR-4 (lane 11) as cofactors, although the activity was weak. The activity of factor H (4 μg/ml) was clearly enhanced by the addition of FHR-3 (200 μg/ml in lane 6 and 100 μg/ml in lane 7) or FHR-4 (200 μg/ml in lane 8 and 100 μg/ml in lane 9).

4. Discussion

Although four human factor H-related molecules have been identified and cloned, little is known about the biological functions of these proteins. Both FHR-3 and FHR-4 are present in the free form in human plasma and in addition, FHR-4 is a constituent of human lipoproteins [2]. Due to their

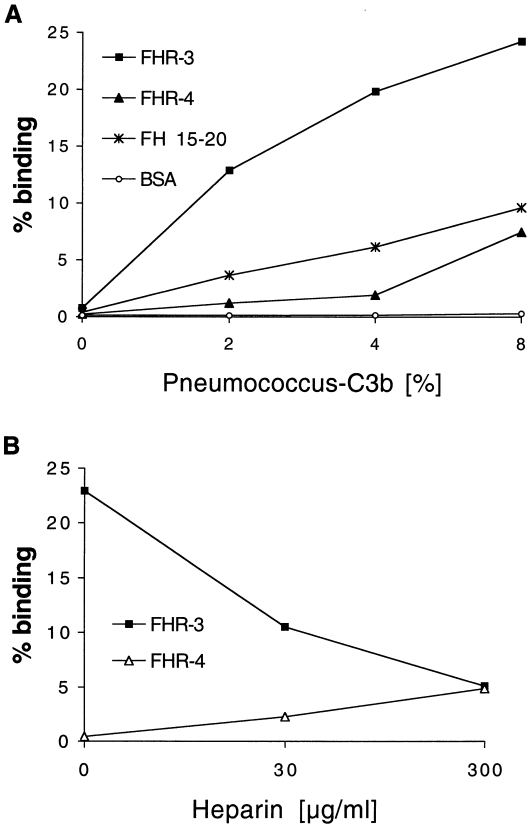

Fig. 5. Interaction of FHR-3, FHR-4 and a C-terminal fragment of factor H with C3b-coated pneumococci. A: Opsonised pneumococci were incubated with radiolabelled FHR-3, FHR-4 or a C-terminal construct SCR 15-20 of factor H for 15 min at 37°C. BSA was used as a control. B: The influence of increasing amounts of heparin (0–300 μg/ml) on the interaction between FHR-3 and FHR-4 and opsonised pneumococci (5%) was measured.

structural similarity with other C3b binding proteins such as factor H, CR1 (CD35), CR2 (CD21) and MCP (CD46), we have analysed whether FHR-3 and FHR-4 share properties with these complement regulators or receptors. Using biosensor real time measurements, we observed that recombinant FHR-3 and FHR-4 bound to the complement component C3b and to its proteolytic cleavage fragment C3d. As binding to C3c was excluded and as both FHR-3 and FHR-4 bound to C3b and C3d with similar affinities, we propose the presence of a single binding site within the C3d region.

In experiments performed to test functional similarity of the FHRs to factor H, neither FHR-3 nor FHR-4 showed decay accelerating activity for C3bBb convertases assembled on sheep red blood cells (data not shown). We also tested the cofactor activity for C3b inactivation by factor I. The proteins alone displayed no significant activity. However, when limited amounts of factor H/FHL-1 were present, the FHRs had a promoting effect on the inactivation of C3b (Fig. 4). This suggests that binding of FHR-3 and FHR-4 to C3b affects its conformation and sensitivity to inactivation by factor I. Semiquantitative analysis with the phosphorimaging software showed that FHR-3 had about 54% of the activity of FHR-4 in this fluid phase assay (Fig. 4). Subsequently, we tested binding of FHRs to C3b on a pathogen surface as this represents a more physiological situation. Both FHRs bound to C3b deposited on pneumococci (Fig. 5A). In this situation, FHR-3 bound more strongly than FHR-4. However, the interaction of FHR-3, but not of FHR-4, with the C3b-coated pathogen surface could be inhibited by the addition of heparin (Fig. 5B). This indicates that the two proteins have different binding characteristics under physiological conditions.

The high degree of relatedness of FHR-3 and FHR-4 is in agreement with the functional similarity as both proteins bind to C3b and C3d. In human plasma, the concentration of FHR-3 is low and native FHR-3 protein can only be detected by means of immunopurification [24] or by heparin affinity chromatography (Fig. 1). In contrast, neither the recombinant [19] nor the native form of FHR-4 interacts with heparin (Fig. 1). The difference in the ability to bind heparin represents one clear functional difference between FHR-3 and FHR-4 and suggests a different distribution of these proteins in vivo. The different localisation is also supported by the association of native FHR-4 [2] but not of FHR-3 with chylomicrons and triglyceride-rich lipoproteins. An interaction with lipids has also been shown for complement regulators, e.g. C4b binding protein and clusterin [30,31], as well as for the FHRs FHR-1 and FHR-2 [32].

We have demonstrated binding of FHR-3 and FHR-4 to the central complement component C3b. Proteolytic cleavage of C3b results first in iC3b and subsequently in the large C3c and the small C3dg fragment. FHR-3 and FHR-4 bound to the C3d (Fig. 2 and 3) but not to the C3c fragment of C3 (data not shown). An interaction with the C3d region has also been shown for the complement receptors CR1 and CR2 and for the regulator factor H [33]. For factor H, two interaction sites on the α'-chain of human C3 have been described in detail. One site is represented by residues 727–768 of the C3c fragment, while a second site was mapped to the C3d region of residues 1199–1274 [34]. The apparent overall association and dissociation rates for FHR-3 and FHR-4 with both C3b and C3d are in the same order of magnitude (Table 1). The apparent K_A values for the interactions of FHR-3 and FHR-4 with C3b are 7.5×10^6 M^{-1} and 2.9×10^6 M^{-1}, respectively. We propose a single point interaction and the location of the interacting domain within the C3d region of C3b. These affinity constants are comparable to that obtained for the interaction of factor H and C3d in the same system (2.8×10^6 M^{-1}, [22] and Jokiranta et al., manuscript in preparation).

The conservation of a C3b/C3d binding site suggests a C3 regulatory function for FHR-3 and FHR-4. Although all three proteins exhibit overlapping functions in C3d binding, differences exist between the two FHR proteins and factor H: (i) The homology of FHR-3 and FHR-4 with factor H excludes the complement regulatory domains of factor H which are located within the N-terminal four SCRs of the protein [11,12,15]. (ii) The data presented in Fig. 4 suggest that the two FHR proteins can 'prime' C3b to inactivation by factor I in the presence of a cofactor (e.g. factor H/FHL-1), although (iii) neither FHR-3 nor FHR-4 acts as efficiently as a cofactor on their own and both proteins lack decay accelerating activity for the C3 convertase. FHR-3 and FHR-4 interact with C3b/C3d, but they might possess additional, yet unidentified functions. These may be dependent on their C3d binding activity.

In conclusion, we have identified FHR-3 and FHR-4 as new ligands for the complement component C3b and localised their binding site to the C3d region. Their ability to bind C3b and C3d suggests specific functions in modulating the conformation and stability of C3b or in the processing of C3b or C3dg-coated particles. As C3dg has been proposed to act as an endogenous adjuvant, it will be of interest to define a role of these two FHR proteins at the interface between the innate and the acquired immunity [35]. Binding to opsonised pneumococci suggests that the FHRs could have a role in regulating processing of C3d-coated antigens derived from exogenous or endogenous sources.

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