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Binding of Complement Factor H to Endothelial Cells Is Mediated by the Carboxy-Terminal Glycosaminoglycan Binding Site

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Factor H (FH), the major fluid phase regulator of the alternative complement pathway, mediates protection of plasma-exposed host structures. It has recently been shown that short consensus repeats 19 to 20 of FH are mutational hot spots associated with atypical hemolytic uremic syndrome (aHUS), a disease with endothelial cell damage. Domain 20 of FH contains binding sites for heparin, C3b, and the cleavage product C3d. To study the role of these binding sites in target recognition, we performed site-directed mutagenesis in domain 20 and assayed the resulting recombinant proteins. The mutant FH15-20A (substitutions R1203E, R1206E, and R1210S) bound neither heparin nor endothelial cells. Similarly, an aHUS-derived mutant FH protein (E1172Stop, lacking domain 20) failed to bind endothelial cells and showed impaired binding to heparin. Binding of FH to endothelial cells was inhibited by heparin and a specific monoclonal antibody that inhibited heparin but not C3d binding, demonstrating that the heparin site on domains 19 to 20 mediates interaction of FH to endothelial cells. Binding of FH15-20 to heparin was inhibited by several cell surface- and basement membrane-associated glycosaminoglycans, suggesting that binding site specificity is

not restricted to heparin. Thus, defective heparin/glycosaminoglycan-binding site on domains 19 to 20 of FH most probably mediates complement-induced endothelial cell damage in aHUS. (*Am J Pathol* 2005, 167:1173–1181)

The complement (C) system consists of a set of plasma and membrane-bound proteins and protects the organism against invading microbes, removes debris from plasma and tissues, and enhances cell-mediated immune responses. Complement can be activated through three pathways, of which the alternative pathway (AP) acts independently of antibodies. This pathway is initiated spontaneously in plasma leading to an attack against all particles, membranes, and cells that are plasma-exposed and not specifically protected.¹ The key molecule in AP activation is C3 and this protein is spontaneously hydrolyzed generating C3(H₂O), which is responsible for the formation of the fluid-phase C3 convertase of AP, and eventually leading to deposition of some C3b onto practically all surfaces in contact with plasma. In the absence of regulators the activation proceeds via an amplification cascade and leads to opsonization with C3b, generation of chemotactic and anaphylatoxic peptides, and formation of C membrane attack complexes resulting in cell lysis.

Regulation of AP activation occurs at the level of C3b and the AP C3 convertase C3bBb by the plasma proteins factor H (FH)² and FH-like protein 1 (FHL-1) and by three

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membrane-bound molecules (CD35, CD46, and CD55). FH is composed of 20 short consensus repeat domains (SCRs) each consisting of ~60 amino acids. It regulates AP activation by competing with factor B for binding to C3b, by acting as a co-factor for factor I leading to proteolytic inactivation of C3b, and by enhancing dissociation of the C3bBb complex.^{3–5} FH is the only known regulator involved in down-regulating AP activation on host structures that lack the membrane-bound regulators (eg, basement membranes in kidney glomeruli).^{6,7} Expression or binding of FH contributes to protection against complement of certain tumor cells⁸ and pathogenic microbes (eg, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Neisseria gonorrhoeae*, *Borrelia* sp., and *Candida albicans*).^{9–16}

The ability of the AP to discriminate between self non-activator structures and foreign activator structures depends on differential binding of FH to the complex consisting of the surface itself and the surface-bound C3b molecules.^{17,18} The nonactivator surface structures that have been reported or suggested to be involved include sialic acids and glycosaminoglycans such as heparin, heparan sulfate, and dextran sulfate. FH has three binding sites for C3b at SCR1-4, SCR8-15, and SCR20^{19–21} and three binding sites for heparin at SCR7, SCR20, and within SCRs 8 to 15.^{22–24} In addition, a binding site for sialic acids of *N. gonorrhoeae* has been suggested within SCRs 16 to 20.¹¹ It appears possible to convert an activator surface into a nonactivator surface by associating heparin onto the surface.²⁵ The physiological role of heparin-binding by FH and the role of the individual heparin- and the C3b-binding sites have not been characterized so far. Also, the exact mechanism how AP discriminates between activators and nonactivators is not yet known.

It has recently been shown that atypical hemolytic uremic syndrome (aHUS) is associated with mutations in the FH gene that cause either truncation of FH or point mutations.^{26–30} The hot spot for the point mutations is within SCRs 19 to 20 of FH. In addition, FH derived from plasma of some of these patients binds heparin with lower affinity than normal FH.²⁹ The aHUS syndrome is characterized by endothelial cell damage, microthrombosis, renal failure, and AP activation.³¹ Therefore, it seems that protection of endothelial cells from the AP attack is impaired in this condition. FH is known to bind to endothelial cells via the most carboxy-terminal domain.²⁹ So far the effect of the aHUS-associated mutations to binding of FH to both heparin and C3b/C3d in addition to endothelial cells has been measured in one study only²⁹ and both the analyzed single amino acid mutants (R1210C, R1215G) showed decreased binding to heparin, C3b/C3d, and endothelial cells. The observed combination of loss-of-function could be due to altered overall conformation of the domain SCR20, for example secondary to mispairing of the cysteine bridges (R1210C) or introduction of glycine to a stretch that in the previously reported tertiary structures of SCR domains is in the middle of a β -strand (R1215G). Therefore, the exact location of the endothelial cell-binding site and its relation

to the previously described heparin- and C3b/C3d-binding sites within SCRs 19 to 20 have not been defined.

The goal of this study was to characterize the relationship between the binding sites of FH for heparin, C3d, and endothelial cells on SCRs 19 to 20. By site-directed mutagenesis of positively charged amino acids in SCR20 and the use of an aHUS patient-derived FH mutant (E1172Stop) that lacks SCR20 we show that binding of FH to endothelial cells is mediated by the heparin-, but not the C3d-binding site on SCRs 19 to 20. The heparin-binding site was indirectly shown to bind to several glycosaminoglycans (GAG) suggesting that FH is capable of binding to a variety of GAG-containing surfaces such as different cell types and basement membranes. Because endothelial cell damage is typical for aHUS it is suggested that the impaired binding of FH to endothelial cell-associated C3b via the carboxy-terminus of the mutant FH molecule causes impaired protection of endothelial cells and contributes to the pathogenesis of the disease.

Materials and Methods

Complement Components and Recombinant Proteins

FH and C3d were purified from human plasma obtained from consented healthy laboratory personnel as described previously³² or purchased from Calbiochem Corp. (La Jolla, CA). Generation and purification of FH15-20 and the mutant FH15-20AB (formerly called FH15-20mut) with five amino acid substitutions (R1203E, R1206E, R1210S, K1230S, R1231A) has been described earlier.²¹ Two new recombinant mutant constructs were generated by introducing two and three amino acid substitutions to the FH15-20 construct using the QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands). The mutant FH15-20A has three substitutions (R1203E, R1206E, and R1210S) and was generated in the vector pCR 2.1 (Invitrogen, Carlsbad, CA) using the following primers: forward 5'-GAG GGA TAT GAG CTT TCA TCA TCC TCT CAC ACA TTG CGA ACA AC and reverse 5'-GGA TGA TGA AAG CTC ATA TCC CTC TTT ACA CAC AAA TTC AAC TG. The mutant FH15-20B with two substitutions (K1230S and R1231A) was generated using primers: forward 5'-TCC GCC CCC GGG AAA AAG CCG AAT TCC AGC AC and reverse 5'-GGC GGA TGC ACA AGT TGG ATA CTC CA. The mutations were confirmed by DNA sequencing. Recombinant proteins FH15-20A and FH15-20B were expressed using pPICZ α expression vector (Invitrogen) in *Pichia pastoris* as described earlier.³³ The recombinant proteins were purified either by Ni²⁺-chelate chromatography (FH15-20 and FH15-20AB) or anti-FH19-20 affinity chromatography (FH15-20A and FH15-20B). The aHUS patient-derived FH mutant that is truncated at residue 1172 due to a missense mutation (FH-E1172Stop) was obtained from a consented Caucasian male patient according to the protocol H1.2000.0046746 of the Mario Negri Institute for Pharmacological Research, approved by the

ethical Committee of Regione Lombardia (Italy) and purified as described elsewhere.²⁹ Briefly, the patient serum containing the FH-E1172Stop mutant was precipitated stepwise with 7 and 13% polyethyleneglycol 6000 and the FH-containing precipitate was dissolved to phosphate-buffered saline (PBS) and subjected to further purification with heparin affinity chromatography. Purity of all of the self-purified and purchased proteins was >95% as confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining and the functional integrity of the FH and C3b prepartes was verified using surface plasmon resonance technique.

SDS-PAGE and Western Blotting

The purified recombinant FH constructs were separated by SDS-PAGE (10% gel under nonreducing conditions) and analyzed using Western blotting. Western blotting was performed after electrotransfer of proteins from SDS-PAGE gels to nitrocellulose (0.45 μ m; Schleicher & Schuell, Dassel, Germany). After blocking with RotiBloc (Roth, Karlsruhe, Germany) in PBS, polyclonal goat anti-FH IgG (1:1000 in PBS containing 0.05% Tween 20; Calbiochem, Schwalbach, Germany) was used as a primary antibody (17 hours at 4°C). Peroxidase-conjugated rabbit anti-goat IgG antibody (1:2000 in PBS containing 0.05% Tween 20) was used as the secondary antibody. After three washing steps the bound secondary antibody was detected by standard enhanced chemiluminescence assay.

Heparin Affinity Chromatography

For heparin affinity chromatography, culture supernatants (10 ml) containing FH15-20, FH15-20A, FH15-20B, or FH15-20AB were diluted fourfold with 1/3 PBS (5 mmol/L phosphate and 45 mmol/L NaCl) and serum of the patient with the FH mutation E1172Stop was diluted fivefold with PBS. The diluted samples were applied to 1-ml heparin affinity columns (HiTrap Heparin; Amersham Pharmacia Biotech, Uppsala, Sweden) at 22°C. After washing with 30 ml of 1/3 PBS, bound proteins were eluted with a linear NaCl gradient (50 to 600 mmol/L). Aliquots of the wash and elute fractions (200 μ l for culture supernatants and 400 μ l for FH-E1172Stop serum) were subjected to SDS-PAGE and Western blotting and salt-concentration was measured using CDM210 conductivity meter (Radiometer Analytical SAS, France).

Cell Culture and Cell Harvesting

Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum, 300 U/ml penicillin (BioWhittaker), 300 μ g/ml streptomycin (BioWhittaker), and 6 mmol/L L-glutamine (BioWhittaker) at 37°C in the presence of 5% CO₂. Adherent cells were harvested by incubation for 10 minutes at 37°C with PBS containing 0.2

mg/ml ethylenediamine tetraacetic acid. The cells were washed twice with PBS and resuspended in fluorescence-activated cell sorting buffer (1/2 PBS with 1% bovine serum albumin; 7.5 mmol/L phosphate and 67.5 mmol/L NaCl). Viability of the cells after detaching with ethylenediamine tetraacetic acid was checked by propidium iodide staining and less than 5% of the cells loaded the stain and were therefore considered to be damaged.

Flow Cytometry

HUVECs (1×10^6 in 100 μ l) were incubated with 5 μ g of FH, wild-type FH15-20, mutant FH15-20, FH-E1172Stop, or buffer (negative control) for 1 hour at 37°C with shaking (300 rpm). For inhibition studies, FH or FH15-20 was first preincubated with heparin or dextran sulfate (molecular weight, 8000 d; both from Sigma Chemical Co., St. Louis, MO) at final concentrations of 3, 30, and 300 μ g/ml and thereafter added to the cell suspension. The Fab fragment of the 3D11 monoclonal antibody (mAb)³⁴ was prepared using standard procedures and used in inhibition studies at a final concentration of 1.25, 2.5, and 5 μ g/ml. C3d was also used in this inhibition study at final concentration of 0.14, 1.4, and 14 μ g/ml. Cells were washed with 900 μ l of buffer to remove unbound protein, centrifuged, resuspended in 100 μ l, and incubated for 60 minutes on ice with 6 μ g of monoclonal antibody IXF9, recognizing SCR18 of FH.³⁵ When the Fab fragment of 3D11 mAb was used in the inhibition experiment we used polyclonal goat anti-FH antibody (1:1000 dilution, Calbiochem). Binding of IXF9 to the recombinant FH15-20 constructs and the FH-E1172Stop mutant was confirmed using Western blotting (data not shown). After washing, 1 μ g of fluorescein isothiocyanate-labeled goat anti-mouse F(ab')₂ fragment (DAKO A/S, Glostrup, Denmark) or fluorescein isothiocyanate-labeled rabbit anti-goat IgG (DAKO) (for IXF9 and goat anti-FH, respectively) was added and the mixture was incubated for 30 minutes on ice. Cells were washed, resuspended in 300 μ l of buffer, and analyzed with FACS Calibur equipment (Becton Dickinson, Heidelberg, Germany) using CellQuest software (Becton Dickinson). The results are shown after gating the cells based on forward and side scatter characteristics for single cells, excluding cell clusters or fragments.

Surface Plasmon Resonance Technique

Real-time monitored surface plasmon resonance assays were performed using Biacore 2000 or 3000 instruments and the data were analyzed using BiaEvaluation 3.0 software (Biacore AB, Uppsala, Sweden). For coupling onto carboxylated dextran chips (Sensor Chip CM5, Biacore AB) heparin-albumin (code H0403; 3 to 6 mol heparin per mol albumin; Sigma) and C3d were dialyzed against 20 mmol/L acetate buffer (pH 4.5) and 20 to 50 μ l of a 50 to 200 μ g/ml solution was used for coupling, essentially as described earlier for other proteins.³⁶ The coupling efficiency was 980 to 5300 resonance units for C3d and 570 to 1500 resonance units for heparin-albumin. The used flow cells contained ~5.2 ng/mm² of C3d or 0.8 ng/mm² of heparin-albumin. For

the binding assays, all of the reagents were dialyzed against 0.5-fold veronal-buffered saline (7.5 mmol/L barbitalurate and 67.5 mmol/L NaCl), diluted with 0.5-fold veronal-buffered saline and centrifuged (10 minutes at 14,000 × g). The protein concentrations of the dialyzed reagents were measured using the BCA protein assay (Pierce Chemical Co., Rockford, IL).

The effect of glycosaminoglycans and other polyanionic molecules on FH-heparin or FH-C3d interactions was analyzed by incubating FH (100 μg/ml) or recombinant FH15-20 construct (150 μg/ml) with the polyanions or buffer (30 minutes at 22°C) before injecting the mixtures to the heparin-albumin- or C3d-coated flow cells or a control flow cell (activated and deactivated flow cell without any coupled proteins, blank channel). The tested polyanions (heparin type I, dextran sulfate 8000, chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, fucoidan, keratan sulfate, colominic acid, and dextran) were purchased from Sigma and used at final concentrations of 500 μg/ml. Each sample was injected separately through the control and the heparin-albumin- or C3d-coupled flow cell using a flow rate of 5 μl/min and 0.5-fold veronal-buffered saline as the running buffer at 25°C or 37°C. The binding assays were performed at least in duplicate using independently prepared sensor chips. The effect of monoclonal anti-FH antibody 3D11 on FH binding to heparin and C3d was determined by incubating FH (90 μg/ml) with three different concentrations on the antibody (4, 13, and 61 μg/ml) (30 minutes at 22°C) before injecting the mixtures onto the flow cells coated with heparin-albumin or C3d at 25°C.

Results

Generation of Mutations in SCR20 of FH

Site-directed mutagenesis was used to generate two novel mutant constructs of FH15-20, FH15-20A (containing substitutions R1203E, R1206E, and R1210S), and FH15-20B (substitutions K1230S and R1231A). Mutant proteins were expressed in *P. pastoris* without any C-terminal tags to exclude the possible role of the his-tag in interfering with the binding characteristics. The wild-type FH15-20 and previously generated mutant FH15-20AB (mutations R1203E, R1206E, R1210S, K1230S, and R1231A) were expressed in insect cells with a his-tag. The apparent molecular mass of the constructs were ~36 kd for the yeast-expressed FH15-20A and FH15-20B mutants (Figure 1A) and 43 kd for the insect cell-expressed wild-type FH15-20 and the FH15-20AB mutant. Schematic representation of the recombinant FH15-20 constructs and the patient plasma-derived FH-E1172Stop mutant used is shown in Figure 1B.

Binding of FH C-Terminus to Heparin

FH has three binding sites for heparin, located at SCRs 7, 8 to 15, and 20. To analyze the role of SCR20 in binding of FH to heparin, serum of an aHUS patient being heterozygous for a FH mutation that results in a lack of

Figure 1. Recombinant FH constructs and patient-derived FH mutant used. **A:** SDS-PAGE analysis of recombinant FH constructs. The recombinant constructs were expressed either in the baculovirus or *Pichia pastoris* systems and purified by Ni²⁺-chelate or anti-FH affinity chromatography. Each fragment was analyzed by SDS-PAGE using a 10% gel under nonreducing conditions and silver staining. Mobility of the size markers is indicated. **B:** Schematic representation of the recombinant FH15-20 constructs and the patient-derived FH mutant (FH-E1172Stop) used.

SCR20 (FH-E1172Stop) was subjected to heparin affinity chromatography. The FH-E1172Stop mutant bound to heparin with lower affinity than wild-type FH, eluting from the heparin column at ~220 mmol/L NaCl whereas wild-type FH eluted at ~290 mmol/L NaCl (Figure 2A).

Because SCR20 appeared to be essential for full avidity between FH and heparin under physiological ionic strength, we analyzed binding of the mutants and the wild-type protein to heparin. Mutant constructs FH15-20A and FH15-20AB did not bind whereas the FH15-20B mutant and FH15-20 bound to heparin. The first two

Figure 2. Binding of patient-derived and recombinant FH mutants to heparin. Serum of the patient with the heterozygous FH-E1172Stop mutation, or supernatant containing recombinant constructs FH15-20A, FH15-20B, FH15-20AB, or FH15-20 were applied to heparin columns and eluted by a linear salt gradient. **A:** Western blot of elution fractions after application of patient serum containing both FH-E1172Stop mutant and normal FH to heparin column. Mobility of the normal and mutant FH are indicated and the average salt concentration corresponding to each fraction is marked above the lanes. **B:** Western blot of starting supernatant, washing, and elution fractions after application of FH15-20A, FH15-20B, FH15-20AB, or FH15-20 to the heparin column. The indicated salt concentration of each fraction is based on conductivity measurement.

Figure 3. Binding of FH and the mutant FH proteins to endothelial cells. Mutant proteins FH15-20A, FH15-20B, and FH15-20AB (**A–C**), patient-derived mutant FH-E1172Stop (**D**), recombinant construct FH15-20 (**E**), and FH (**F**), were incubated with HUVECs and binding of the proteins was analyzed using anti-FH mAb and fluorescein isothiocyanate-conjugated rabbit anti-mouse Ig in flow cytometry, as described in Materials and Methods.

mutants were detected in the flow through and early washing fractions and the latter two proteins were eluted starting at ~260 mmol/L (Figure 2B).

Binding of Mutant Proteins to Endothelial Cells

Flow cytometry was used to test binding of FH15-20 and FH15-20 mutants to HUVECs. The patient-derived FH-E1172Stop mutant was used as a control because it has previously been shown not to bind to endothelial cells.²⁹ The mutant proteins FH15-20A, FH15-20AB, and FH-E1172Stop, did not bind to HUVECs at all, FH15-20B bound weakly and the FH15-20 construct bound to the cells comparably to FH (Figure 3). This reduced binding indicates that the endothelial cell-binding site on SCR20 of FH is dependent on one or more of the residues mutated in FH15-20A. We could not detect any functional interference with the his-tag on the recombinant constructs because the tagless FH15-20B and his-tagged FH15-20 bound to heparin and HUVECs whereas the tagless FH15-20A and his-tagged FH15-20AB failed to bind to both heparin and the endothelial cells.

Effect of Polyanions on the Interaction between FH15-20 and Heparin

Because different polyanions are found on plasma-exposed host surfaces such as endothelial cells and glomerular basement membranes, we tested which polyanions compete with heparin for binding to the FH15-20 construct using the surface plasmon reso-

Figure 4. Effects of polyanions on the interactions of FH15-20. Surface plasmon resonance technique was used to test binding of recombinant FH15-20 to heparin and C3d in the presence of glycosaminoglycans and other polyanionic molecules. Before the analysis the construct was preincubated with either buffer or one of the polyanionic molecules (heparin, dextran sulfate 8000, chondroitin sulfate A, keratan sulfate, colominic acid, or fucoidan). The mixtures were injected separately into the control flow cell (blank channel) and into a flow cell coupled with heparin-albumin (**A**) or C3d (**B, C**). The experiments were conducted at 25°C and 37°C but the temperature did not have a clear effect on the interactions or the inhibitory effects of the glycosaminoglycans (data obtained at 25°C is shown).

nance technique. Heparin, dextran sulfate, chondroitin sulfate A, and fucoidan efficiently inhibited binding of FH15-20 to heparin-albumin whereas colominic acid had no effect, and keratan sulfate had a partially inhibiting effect (Figure 4A). Direct binding of FH15-20 to

Table 1. Inhibitory Effects of Polyanionic Molecules, Anti-FH mAb 3D11, and C3d on the Interactions of Factor H or Its SCRs 15 to 20

	Inhibition of		
	Heparin binding	C3d binding	HUVEC binding
Heparin	+++*	+++	+++
Dextran sulfate 8000	+++	+++	+++
Chondroitin sulfate A	+++	++	n.t. [†]
Chondroitin sulfate B	n.t.	+++	n.t.
Chondroitin sulfate C	n.t.	+++	n.t.
Keratan sulfate	+	++	n.t.
Colominic acid	—	+	n.t.
Fucoidan	+++	+++	n.t.
Anti-FH mAb 3D11	+++	—	++ [‡]
Fluid phase C3d	—	+++ [§]	—

*+++ , strong inhibition; ++ , clear inhibition; + , some inhibition; — , no inhibition; based on results shown in Figures 4, 5, and 6.

[†]n.t., not tested.

[‡]Fab fragment used.

[§]Data not shown.

heparin was shown and the data implies indirectly that also dextran sulfate, chondroitin sulfate A, and fucoidan interact with the heparin/glycosaminoglycan-binding site within SCR20.

Effect of Polyanions on the Interaction between FH15-20 and C3d

Heparin- and C3b/C3d-binding sites are adjacent within SCR20 of FH and probably partially overlapping.²¹ Therefore we tested which polyanions interfere with the FH-C3d interaction. Binding of FH15-20 to solid phase-coupled C3d was analyzed in the presence of heparin, dextran sulfate, chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, keratan sulfate, colominic acid, or fucoidan using surface plasmon resonance technique. All tested polyanions had an inhibitory effect but only partial inhibition was observed with chondroitin sulfate A and keratan sulfate and minimal inhibition was observed with colominic acid (Figure 4, B and C). These results indicate that several glycosaminoglycans in addition to heparin interfere with FH binding to C3d, and provide additional indirect evidence for overlapping heparin- and C3d-binding sites within SCR20 of FH (Table 1).

Binding Sites on FH SCR20 for Heparin and C3d Are Distinct

To further analyze the close proximity of the heparin- and C3d-binding sites, we tested if C3d could inhibit FH15-20 binding to heparin-albumin but no inhibition was detected (Figure 5A). In contrast, the signal was higher in the presence of C3d, indicating that C3d is not only incapable of inhibiting FH15-20 binding to heparin but that FH15-20, C3d, and heparin may form a ternary complex and that heparin and C3d can simultaneously bind to FH.

Next, binding of full-length FH to heparin and C3d was investigated in the presence of a monoclonal antibody 3D11 that binds to SCR18 of FH. This mAb inhibited binding of FH to heparin-albumin in a dose-dependent

manner (Figure 5B). However, 3D11 mAb did not inhibit binding of FH to C3d. In contrast, it facilitated the association of FH with C3d (Figure 5C) probably due to an increased avidity of the formed FH-3D11-FH complexes to C3d. These results show that heparin and C3d sites on SCR20 of FH are distinct and provide further support for the conclusion that the heparin-binding site on SCR20 is responsible for a significant part of heparin binding of full-length FH.

Binding of FH to Endothelial Cells Is Mediated by the Heparin-Binding Site within SCR20

After demonstrating that the heparin- and C3d-binding sites within FH SCR20 are distinct we tested which of these sites is involved in binding of FH to endothelial cells. Flow cytometry revealed binding of full-length FH and FH15-20 to HUVECs in the presence of increasing amounts of heparin, dextran sulfate (molecular weight, 8000 d), Fab fragment of the 3D11 mAb, or C3d. Soluble heparin inhibited dose-dependent binding of FH to the cells (Figure 6A). Heparin and dextran sulfate completely blocked binding of FH15-20 to HUVECs at similar concentrations (Figure 6, B and C). The Fab fragment of 3D11 mAb, that inhibits heparin but not C3d binding, reduced dose-dependent binding of FH to the endothelial cells (Figure 6, D and E), whereas C3d did not show any clear effects on the binding (Figure 6F). The results indicate that FH uses the heparin-binding site within SCR20 for interaction with endothelial cells.

Discussion

In this study we used site-directed mutagenesis of several positively charged amino acids within SCR20 and the aHUS patient-derived FH-E1172Stop mutant to show that the major binding site on FH for heparin and endothelial cells is located on SCR20. This heparin-binding site is distinct from the C3d-binding site and mediates binding of FH to endothelial cells.

Figure 5. Effects of C3d or anti-FH mAb on the interactions of FH15-20 and FH with heparin. **A:** The possible effect of C3d on FH15-20-heparin interaction was tested using surface plasmon resonance analysis. The FH15-20 construct was preincubated with C3d (or buffer as a control) and the mixtures were injected to a flow cell coupled with heparin-albumin. The effects that anti-FH mAb 3D11 has on binding of FH to solid phase-bound heparin (**B**) and C3d (**C**) were analyzed by preincubating the FH with the 3D11 mAb (or buffer as a control) before analysis with surface plasmon resonance.

This is the first report to show that under physiological conditions the carboxy-terminal heparin-binding site on SCR20 is essential for full heparin binding of FH. It has earlier been shown that FH has three heparin-binding sites, one within SCRs 6 to 7,²² one in the middle part of FH²⁴ (within SCR9 according to R. Ormsby et al, submitted), and one requiring SCR20.²³ So far no systematic

Figure 6. Inhibition of FH and FH15-20 binding to HUVECs by heparin or mAb. To analyze the role of heparin-binding sites in binding of FH and FH15-20 to HUVECs we preincubated FH (**A**) or FH15-20 (**B**) with heparin (3, 30, and 300 $\mu\text{g/ml}$), or FH15-20 with dextran sulfate (DS; 3, 30, and 300 $\mu\text{g/ml}$) (**C**) before adding the mixture to the cells and the subsequent flow cytometric analysis. In each test a cell preparation treated without FH or FH15-20 was used as a negative control and is indicated as control. **D:** To analyze the effect of the carboxy-terminally binding anti-FH mAb 3D11, FH15-20 was preincubated with the Fab fragment before adding the mixture onto HUVECs and subsequent flow cytometric analysis. **E** and **F:** To further study the effects of 3D11 and C3d on the binding of FH15-20 to HUVECs, a range of concentration of 3D11 and C3d was used and the percentage of specific mean fluorescence intensity (MFI) is shown. The bound proteins were detected using monoclonal mouse anti-FH antibody (IXF9) and a fluorescein isothiocyanate-labeled secondary antibody. For the experiment in the presence of Fab fragment of 3D11 and C3d we used polyclonal anti-FH antibody instead of IXF9.

comparison of the affinities or the accessibility of the different heparin-binding sites has been performed.

FH utilizes the heparin/glycosaminoglycan site on SCR20 for endothelial cell binding. FH can bind to malignant glioma cells,⁸ synovial fibroblasts,³⁷ and endothelial cells.²⁹ Despite intensive searching,^{38,39} no receptor protein for FH on cells has been identified. On the basis of this report it is likely that FH binding to endothelial cells, and possibly other cells, is mediated by cell-surface glycosaminoglycans or other polyanionic carbohydrates rather than a receptor protein. Similarly binding of FH to sheep RBC-C3b complexes is inhibited by fluid-phase glycosaminoglycans⁴⁰ and prevented by chemical modification of the RBC-bound sialic acids.^{41,42} In addition, FH binds to sialic acid of *N. gonorrhoeae* via SCRs 16 to 20.¹¹ Therefore, it is possible that FH binds to some human cells by association with sialic acids. However, in the assays presented here we did not detect any inhibition of FH binding to heparin by bacterial polysialic acid (colominic acid) or the highly sialylated protein fetuin (data not shown). Because endothelial cells have heparan sulfate on their surfaces it is more likely that the ligand

for FH on endothelial cells is this glycosaminoglycan rather than sialic acids.

Heparin and several other polyanions block the FH-C3d interaction, but C3d does not interfere with the FH-heparin interaction (Figures 4 and 5, Table 1). Similarly anti-FH mAb 3D11 blocked heparin but not C3d binding of the FH15-20 construct (Figure 5). The current results show that the heparin- and C3d-binding sites are distinct and only partially overlapping. This is consistent with our previous binding site mapping results.²¹ The results confirm and extend the existing data about the mechanism how FH discriminates between activator and nonactivator surfaces. The avidity of FH for C3b in the fluid phase and bound onto certain surfaces (nonactivators) is ~10 times higher than that for C3b bound onto activator surfaces (eg, zymosan yeast particles).^{43,44} It is possible that the C3d-binding site on C3b is hidden at least on some surfaces, and that FH binding to nonactivator cell or basement membrane glycosaminoglycans can compensate for this. Alternatively, FH that has first bound to C3d could also bind to a glycosaminoglycan, thereby increasing the overall avidity of FH for the C3b-nonactivator surface complex. In either case the local and possibly tissue-specific microenvironment determines the physiological situation in which association of FH with certain glycosaminoglycans is required. Based on pathological changes in aHUS and experimental evidence in rheumatoid arthritis this additional complement inhibition is most likely required and provided in kidney glomeruli (probably by heparan sulfate and chondroitin sulfate),⁷ synovial tissues (probably by keratan sulfate and chondroitin sulfate),³⁷ and on endothelial cells (probably by heparan sulfate).²⁹

Site-directed mutagenesis of positively charged residues on SCR20 of FH resulted in impaired heparin and endothelial cell binding. These results provide new insights into pathogenesis of FH gene mutation-associated aHUS. The aHUS-associated FH mutations lead to formation of either truncated FH where the carboxy-terminus is lacking or to FH where point mutations are introduced and in rare cases to defective secretion of FH.^{26–30,45–47} The hot spot of the point mutations is within SCRs 19 to 20, where both heparin- and C3d-binding sites have been localized. Currently it is not known which of these interactions are impaired for the development of clinical disease or indeed if both of them need to be impaired. It has recently been shown that cell binding of recombinant FH constructs containing aHUS-associated mutations is impaired.^{28,29} Our results suggest that in at least some of the aHUS patients who have a carboxy-terminal FH mutation the endothelial cell damage follows defective cell binding by the heparin/glycosaminoglycan-binding site on SCR20.

In conclusion, this study shows that FH binds to endothelial cells via the heparin/glycosaminoglycan-binding site within SCR20. The results suggest that the AP discrimination between activator and nonactivator surfaces is mediated by FH binding to glycosaminoglycans or anionic carbohydrate structures via SCR20. In addition the results suggest that damage to endothelial cells in aHUS is caused by impaired protection of the cells by a defect in glycosaminoglycan binding of the mutated patient FH.

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References

1. Fearon DT, Austen KF: Activation of the alternative complement pathway with rabbit erythrocytes by circumvention of the regulatory action of endogenous control proteins. *J Exp Med* 1977, 146:22–33
2. Nilsson UR, Müller-Eberhard HJ: Isolation of β 1H globulin from human serum and its characterization as the fifth component of complement. *J Exp Med* 1965, 122:277–298
3. Whaley K, Ruddy S: Modulation of the alternative complement pathway by β 1H globulin. *J Exp Med* 1976, 144:1147–1163
4. Weiler JM, Daha MR, Austen KF, Fearon DT: Control of the amplification convertase of complement by the plasma protein β 1H. *Proc Natl Acad Sci USA* 1976, 73:3268–3272
5. Pangburn MK, Schreiber RD, Müller-Eberhard HJ: Human complement C3b inactivator: isolation, characterization, and demonstration of an absolute requirement for the serum protein β 1H for cleavage of C3b and C4b in solution. *J Exp Med* 1977, 146:257–270
6. Meri S, Koistinen V, Miettinen A, Törnroth T, Seppälä IJ: Activation of the alternative pathway of complement by monoclonal λ light chains in membranoproliferative glomerulonephritis. *J Exp Med* 1992, 175:939–950
7. Pickering MC, Cook HT, Warren J, Bygrave AE, Moss J, Walport MJ, Botto M: Uncontrolled C3 activation causes membranoproliferative glomerulonephritis in mice deficient in complement factor H. *Nat Genet* 2002, 31:424–428
8. Junnikkala S, Jokiranta TS, Friese MA, Jarva H, Zipfel PF, Meri S: Exceptional resistance of human H2 glioblastoma cells to complement-mediated killing by expression and utilization of factor H and factor H-like protein 1. *J Immunol* 2000, 164:6075–6081
9. Horstmann RD, Sievertsen HJ, Knobloch J, Fischetti VA: Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H. *Proc Natl Acad Sci USA* 1988, 85:1657–1661
10. Jarva H, Jokiranta TS, Wurzner R, Meri S: Complement resistance mechanisms of streptococci. *Mol Immunol* 2003, 40:95–107
11. Ram S, Sharma AK, Simpson SD, Gulati S, McQuillen DP, Pangburn MK, Rice PA: A novel sialic acid binding site on factor H mediates serum resistance of sialylated *Neisseria gonorrhoeae*. *J Exp Med* 1998, 187:743–752
12. Hellwage J, Meri T, Heikkilä T, Alitalo A, Panelius J, Lahdenne P, Seppälä IJ, Meri S: The complement regulator factor H binds to the surface protein OspE of *Borrelia burgdorferi*. *J Biol Chem* 2001, 276:8427–8435
13. Kraiczy P, Hellwage J, Skerka C, Becker H, Kirschfink M, Simon MM, Brade V, Zipfel PF, Wallich R: Complement resistance of *Borrelia burgdorferi* correlates with the expression of BbCRASP-1, a novel linear plasmid-encoded surface protein that interacts with human factor H and FHL-1 and is unrelated to Erp proteins. *J Biol Chem* 2004, 279:2421–2429
14. Meri T, Hartmann A, Lenk D, Eck R, Wurzner R, Hellwage J, Meri S, Zipfel PF: The yeast *Candida albicans* binds complement regulators factor H and FHL-1. *Infect Immun* 2002, 70:5185–5192
15. McDowell JV, Wolfgang J, Tran E, Metts MS, Hamilton D, Marconi RT: Comprehensive analysis of the factor H binding capabilities of *Borrelia* species associated with Lyme disease: delineation of two distinct classes of factor H binding proteins. *Infect Immun* 2003, 71:3597–3602
16. McDowell JV, Tran E, Hamilton D, Wolfgang J, Miller K, Marconi RT: Analysis of the ability of spirochete species associated with relapsing fever, avian borreliosis, and epizootic bovine abortion to bind factor H and cleave C3b. *J Clin Microbiol* 2003, 41:3905–3910
17. Pangburn MK, Schreiber RD, Müller-Eberhard HJ: C3b deposition during activation of the alternative complement pathway and the effect of deposition on the activating surface. *J Immunol* 1983, 131:1930–1935

18. Meri S, Pangburn MK: Discrimination between activators and nonactivators of the alternative pathway of complement: regulation via a sialic acid/polyanion binding site on factor H. *Proc Natl Acad Sci USA* 1990, 87:3982-3986
19. Sharma AK, Pangburn MK: Identification of three physically and functionally distinct binding sites for C3b in human complement factor H by deletion mutagenesis. *Proc Natl Acad Sci USA* 1996, 93:10996-11001
20. Jokiranta TS, Hellwage J, Koistinen V, Zipfel PF, Meri S: Each of the three binding sites on complement factor H interacts with a distinct site on C3b. *J Biol Chem* 2000, 275:27657-27662
21. Hellwage J, Jokiranta TS, Friese MA, Wolk TU, Kampen E, Zipfel PF, Meri S: Complement C3b/C3d and cell surface polyanions are recognized by overlapping binding sites on the most carboxyl-terminal domain of complement factor H. *J Immunol* 2002, 169:6935-6944
22. Blackmore TK, Sadlon TA, Ward HM, Lublin DM, Gordon DL: Identification of a heparin binding domain in the seventh short consensus repeat of complement factor H. *J Immunol* 1996, 157:5422-5427
23. Blackmore TK, Hellwage J, Sadlon TA, Higgs N, Zipfel PF, Ward HM, Gordon DL: Identification of the second heparin-binding domain in human complement factor H. *J Immunol* 1998, 160:3342-3348
24. Pangburn MK, Atkinson MA, Meri S: Localization of the heparin-binding site on complement factor H. *J Biol Chem* 1991, 266:16847-16853
25. Kazatchkine MD, Fearon DT, Silbert JE, Austen KF: Surface-associated heparin inhibits zymosan-induced activation of the human alternative complement pathway by augmenting the regulatory action of the control proteins on particle-bound C3b. *J Exp Med* 1979, 150:1202-1215
26. Warwicker P, Goodship T, Donne R, Pirson Y, Nicholls A, Ward R, Turnpenny P, Goodship J: Genetic studies into inherited and sporadic hemolytic uremic syndrome. *Kidney Int* 1998, 53:836-844
27. Caprioli J, Bettinaglio P, Zipfel PF, Amadei B, Daina E, Gamba S, Skerka C, Marziliano N, Remuzzi G, Noris M: The molecular basis of familial hemolytic uremic syndrome: mutation analysis of factor H gene reveals a hot spot in short consensus repeat 20. *J Am Soc Nephrol* 2001, 12:297-307
28. Sanchez-Corral P, Perez-Caballero D, Huarte O, Simckes AM, Goicoechea E, Lopez-Trascasa M, Rodriguez de Cordoba S: Structural and functional characterization of factor H mutations associated with atypical hemolytic uremic syndrome. *Am J Hum Genet* 2002, 71:1285-1295
29. Manuelian T, Hellwage J, Meri S, Caprioli J, Noris M, Heinen S, Jozsi M, Neumann HP, Remuzzi G, Zipfel PF: Mutations in factor H reduce binding affinity to C3b and heparin and surface attachment to endothelial cells in hemolytic uremic syndrome. *J Clin Invest* 2003, 111:1181-1190
30. Dragon-Durey MA, Fremeaux-Bacchi V, Loirat C, Blouin J, Niaudet P, Deschenes G, Coppo P, Herman Fridman W, Weiss L: Heterozygous and homozygous factor H deficiencies associated with hemolytic uremic syndrome or membranoproliferative glomerulonephritis: report and genetic analysis of 16 cases. *J Am Soc Nephrol* 2004, 15:787-795
31. Ruggerenti P, Noris M, Remuzzi G: Thrombotic microangiopathy, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura. *Kidney Int* 2001, 60:831-846
32. Sim RB, Twose TM, Paterson DS, Sim E: The covalent-binding reaction of complement component C3. *Biochem J* 1981, 193:115-127
33. Giannakis E, Jokiranta TS, Ormsby RJ, Duthy TG, Male DA, Christiansen D, Fischetti VA, Bagley C, Loveland BE, Gordon DL: Identification of the streptococcal M protein binding site on membrane cofactor protein (CD46). *J Immunol* 2002, 168:4585-4592
34. Fontaine M, Demares MJ, Koistinen V, Day AJ, Davrinche C, Sim RB, Ripoch J: Truncated forms of human complement factor H. *Biochem J* 1989, 258:927-930
35. Proding WM, Hellwage J, Spruth M, Dierich MP, Zipfel PF: The C-terminus of factor H: monoclonal antibodies inhibit heparin binding and identify epitopes common to factor H and factor H-related proteins. *Biochem J* 1998, 331:41-47
36. Jokiranta TS, Westin J, Nilsson UR, Nilsson B, Hellwage J, Lofas S, Gordon DL, Ekdahl KN, Meri S: Complement C3b interactions studied with surface plasmon resonance technique. *Int Immunopharmacol* 2001, 1:495-506
37. Friese MA, Manuelian T, Junnikkala S, Hellwage J, Meri S, Peter HH, Gordon DL, Eibel H, Zipfel PF: Release of endogenous anti-inflammatory complement regulators FHL-1 and factor H protects synovial fibroblasts during rheumatoid arthritis. *Clin Exp Immunol* 2003, 132:485-495
38. Erdei A, Sim RB: Complement factor H-binding protein of Raji cells and tonsil B lymphocytes. *Biochem J* 1987, 246:149-156
39. DiScipio RG, Daffern PJ, Schraufstatter IU, Sriramara P: Human polymorphonuclear leukocytes adhere to complement factor H through an interaction that involves α M β 2 (CD11b/CD18). *J Immunol* 1998, 160:4057-4066
40. Meri S, Pangburn MK: Regulation of alternative pathway complement activation by glycosaminoglycans: specificity of the polyanion binding site on factor H. *Biochem Biophys Res Commun* 1994, 198:52-59
41. Fearon DT: Regulation by membrane sialic acid of β 1H-dependent decay-dissociation of amplification C3 convertase of the alternative complement pathway. *Proc Natl Acad Sci USA* 1978, 75:1971-1975
42. Michalek MT, Mold C, Bremer EG: Inhibition of the alternative pathway of human complement by structural analogues of sialic acid. *J Immunol* 1988, 140:1588-1594
43. Pangburn MK: Analysis of the mechanism of recognition in the complement alternative pathway using C3b-bound low molecular weight polysaccharides. *J Immunol* 1989, 142:2759-2765
44. Pangburn MK, Morrison DC, Schreiber RD, Müller-Eberhard HJ: Activation of the alternative complement pathway: recognition of surface structures on activators by bound C3b. *J Immunol* 1980, 124:977-982
45. Richards A, Buddles MR, Donne RL, Kaplan BS, Kirk E, Venning MC, Tieleman CL, Goodship JA, Goodship TH: Factor H mutations in hemolytic uremic syndrome cluster in exons 18-20, a domain important for host cell recognition. *Am J Hum Genet* 2001, 68:485-490
46. Landau D, Shalev H, Levy-Finer G, Polonsky A, Segev Y, Katchko L: Familial hemolytic uremic syndrome associated with complement factor H deficiency. *J Pediatr* 2001, 138:412-417
47. Neumann HP, Salzmann M, Bohnert-Iwan B, Mannuelian T, Skerka C, Lenk D, Bender BU, Cybulla M, Riegler P, Konigsrainer A, Neyer U, Bock A, Widmer U, Male DA, Franke G, Zipfel PF: Haemolytic uraemic syndrome and mutations of the factor H gene: a registry-based study of German speaking countries. *J Med Genet* 2003, 40:676-681