Structure of complement factor H carboxyl-terminus reveals molecular basis of atypical haemolytic uremic syndrome

T Sakari Jokiranta^{1,*}, Veli-Pekka Jaakola^{2,3}, Markus J Lehtinen¹, Maria Pärepallo¹, Seppo Meri¹ and Adrian Goldman²

¹Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki and HUSLAB, Helsinki University Central Hospital, Helsinki, Finland and ²Structural Biology and Biophysics, Institute of Biotechnology, University of Helsinki, Helsinki, Finland

Factor H (FH) is the key regulator of the alternative pathway of complement. The carboxyl-terminal domains 19–20 of FH interact with the major opsonin C3b, glycosaminoglycans, and endothelial cells. Mutations within this area are associated with atypical haemolytic uremic syndrome (aHUS), a disease characterized by damage to endothelial cells, erythrocytes, and kidney glomeruli. The structure of recombinant FH19–20, solved at 1.8 Å by X-ray crystallography, reveals that the short consensus repeat domain 20 contains, unusually, a short α -helix, and a patch of basic residues at its base. Most aHUS-associated mutations either destabilize the structure or cluster in a unique region on the surface of FH20. This region is close to, but distinct from, the primary heparin-binding patch of basic residues. By mutating five residues in this region, we show that it is involved, not in heparin, but in C3b binding. Therefore, the majority of the aHUS-associated mutations on the surface of FH19–20 interfere with the interaction between FH and C3b. This obviously leads to impaired control of complement attack on plasma-exposed cell surfaces in aHUS.

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Introduction

The complement (C) system consists of a set of plasma and membrane-bound proteins, which protect the human body against invading organisms, remove debris from plasma and tissues, and enhance cell-mediated immune responses.

*Corresponding author. Department of Bacteriology and Immunology, Haartman Institute, PO Box 21 (Haartmaninkatu 3), University of Helsinki, Helsinki 00014 Finland. Tel.: +358 9 1911; Fax: +358 9 1912 6382; E-mail: sakari.jokiranta@helsinki.fi

³Present address: The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

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Complement can be activated through three pathways, of which only the alternative pathway (AP) does not need a pattern recognition molecule or antibodies for activation. Activation occurs spontaneously in plasma leading to attack against all plasma-exposed surfaces that are not specifically protected. The key molecule in the AP activation is C3. It spontaneously hydrolyses at a low rate leading to deposition of some C3b molecules onto practically all surfaces in contact with plasma. In the absence of regulators, the activation is amplified and leads to efficient opsonization of foreign structures with C3b. Subsequently, C membrane attack complexes form and lyse the target cells. In addition, surface-bound C3b is cleaved to C3d that acts as a molecular adjuvant in enhancing humoral immunity via promoting binding of antigen–C3d complexes to CD21 on B cells (Dempsey *et al*, 1996).

Factor H (FH), the key regulator of AP in plasma, consists of 20 short consensus repeat domains (SCRs or sushi domains), each about 60 residues long. SCR domains are compact all- β elliptical structures stabilized by two disulphide bridges. SCR-containing proteins, such as FH, contain multiple SCRs arranged head-to-tail (Barlow *et al*, 1993). FH regulates AP activation by competing with factor B in binding to C3b, by acting as a cofactor for factor I to proteolytically inactivate C3b, and by enhancing dissociation of the C3bBb complex. In addition, FH is practically the only regulator that downregulates AP activation on host structures that lack the membrane-bound regulators, such as basement membranes in kidney glomeruli (Meri *et al*, 1992; Pickering *et al*, 2002). Furthermore, FH helps protect endothelial cells (Manuelian *et al*, 2003) and certain tumour cells (Junnikkala *et al*, 2000) from complement attack. Recently, FH polymorphism was also found to be associated with age-related macular degeneration, the most common cause of blindness in the elderly in the industrialized countries (e.g. Edwards *et al*, 2005).

The surface regulatory activity of FH is based on its ability to discriminate between the nonactivating ‘self’ structures and ‘foreign’ activator structures (Meri and Pangburn, 1990). This depends on interactions of the carboxyl-terminal end of FH (Hellwage *et al*, 2002; Pangburn, 2002). The carboxyl-terminal region (SCRs 19–20, FH19–20) is unique in that it can bind to C3b/C3d (Sharma and Pangburn, 1996; Jokiranta *et al*, 2000), heparin/heparan sulphate-containing surfaces (Blackmore *et al*, 1998), and endothelial cells (Manuelian *et al*, 2003), and even be utilized by pathogenic microbes for complement evasion. For instance, the OspE proteins of *Borrelia burgdorferi* also bind to FH19–20 (Alitalo *et al*, 2004).

Recent studies have shown that mutations in the gene encoding FH lead to atypical haemolytic uremic syndrome (aHUS), a usually rapidly progressing disease leading to end-stage renal failure. The mutations are either truncation of FH or point mutations mainly within FH19–20 (Manuelian *et al*, 2003; Neumann *et al*, 2003; Dragon-Durey *et al*, 2004). Since

aHUS is characterized by endothelial cell damage, microthrombosis, haemolysis, kidney failure, and alternative complement pathway activation (Ruggenti *et al*, 2001), it appears that surface protection of endothelial cells, platelets, erythrocytes, and kidney glomeruli from complement attack is impaired by a dysfunction of the carboxyl-terminus of FH.

We have now solved the structure of the carboxyl-terminus of FH (FH19–20) to facilitate analysis of the molecular basis of aHUS. Our data obtained with recombinant mutant constructs of FH19–20 clearly show that a cluster of residues on FH20, the hotspot for aHUS-associated mutations, mediates binding to C3b and C3d. In contrast, these mutations do not affect heparin binding, indicating that the primary lesion in aHUS is a defect in C3b/C3d binding. This finding has direct implications for our understanding of the pathophysiology of aHUS and of the role of FH in protecting endothelial cells from C attack.

Results

Overall structure of FH19–20

A recombinant construct containing the carboxyl-terminal domains 19–20 of human FH was expressed and purified (Supplementary Figure 1). The crystal structure of FH19–20 (G¹¹⁰⁹ to K¹²³⁰) was solved by single wavelength sulphur anomalous dispersion and refined to an *R*-factor of 20.5% with good stereochemistry (Table I). The electron density quality was good throughout the structure, but four side chains (R¹²⁰², R¹²⁰⁶, R¹²¹⁰, and K¹²³⁰) are disordered. FH19–20 is about 30 × 30 × 70 Å and consists of two SCR domains, each with a well-defined hydrophobic core and two cysteine bridges. The overall structure approximately resembles a leg, with FH19 being the thigh, the interdomain hinge region the

knee, and FH20 the calf, ankle, and foot. This is because the domains are essentially collinear. The angles (Lehtinen *et al*, 2004) between FH19 and FH20 are: tilt 25.4°, with very little twist (7.3°) and skew (−23.9°). FH19–20 has an hour-glass electrostatic surface with positively charged ends and a negatively charged band around the middle, where the two SCRs join (Figure 1). Unexpectedly, FH20 contains a short α -helix (R¹¹⁷¹ to Tyr¹¹⁷⁷), although an α -helix has never before been seen in the common SCR fold, and is unusual in atypical SCR folds. FH20 also contains one well-ordered buried water molecule (Wat17) which hydrogen bonds three residues: C¹¹⁶⁷, K¹¹⁸⁸, and S¹¹⁹¹ (Supplementary Figure 2).

The crystal structure showed that the FH19–20 monomers form a tight D2 tetramer (Figure 2). The A–B and A–C interfaces (the two antiparallel dimers) bury surface areas of 992 and 886 Å² per monomer, respectively; the parallel A–D dimer is not packed as tightly. FH20 R¹¹⁷¹ and E¹¹⁷² at the base of the α -helix of monomer A interact with FH19 from monomer C and the Q¹¹³⁹ residues of monomers A and C interact with each other across the two-fold axis. The size and

Figure 1 Electrostatic potential of FH19–20 on the surface in two views 90° apart. Positive surface is blue and negative red.

Table I Statistics on data collection, phasing, and refinement of the FH19–20 structure

Data collection statistics		
Crystal (space group)	Native (I4 ₁ 22)	Samarium-SAD/SAS (I4 ₁ 22)
Unit cell parameters (Å)	<i>a</i> = <i>b</i> = 91.41, <i>c</i> = 110.88	<i>a</i> = <i>b</i> = 91.25, <i>c</i> = 110.32
Wavelength (Å)	0.931	1.542
Resolution (Å) ^a	15.0–1.75 (1.80–1.75)	30.0–2.40 (2.49–2.40)
Completeness (%) ^a	99.8 (99.9)	99.4 (100)
Redundancy ^a	6.9 (5.1)	53.0 (25.6)
<i>R</i> _{sym} ^{a,b}	0.059 (0.441)	0.117 (0.425)
<i>I</i> / σ ^a	17.6 (3.7)	43.7 (11.8)
Overall figure of merit for phasing		
Overall (no. of observations)/after density modification ^c		0.28 (9413)/0.71
Refinement and geometry		
Total unique reflections/test set (% size of the reference set)	23 961/1239 (5.2%)	
<i>R</i> _{work} / <i>R</i> _{free} (%) ^a	20.5/22.4 (27.6/29.7)	
Bond r.m.s.d. from ideality (Å)	0.012	
Angle r.m.s.d. from ideality (deg)	1.245	
Ramachandran plot		
Residues in most favoured regions (%)	91.4	
Residues in additionally allowed regions (%)	8.6	
Current model		
Protein atoms/water oxygen	1995/174	
Mean <i>B</i> -value main/side chain and water oxygen (Å ²)	17.2/26.1	
Residues built ^d	G ¹¹⁰⁹ to K ¹²³⁰	

^aValues for the highest resolution shell are given in parentheses.

^b $R_{\text{sym}} = \sum_{hkl} |I(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} I(hkl)$, where $\langle I(hkl) \rangle$ is the mean of the symmetry equivalent reflections of $I(hkl)$.

^cShows the results before and after density modification by the program RESOLVE.

^dAmino-acid numbering based on FH with signal peptide (accession number P08603); first residue of FH19 is K¹¹⁰⁵.

Figure 2 Tetrameric assembly of FH19–20, in stereo view. Each of the monomers is in a different colour. The B and C monomer amino-termini are on top (blue and purple), while the A and D amino-termini are at the bottom (red and green). A schematic view from the top is shown.

hydrophobicity of the interface is not entirely consistent with the tetramer being a crystallization artefact.

Localization of aHUS-associated mutations on FH19–20

So far 22 different aHUS-associated non-frame shifting mutations have been reported to be located within FH19–20, and plasma levels of FH have been reported for 15 of these (Sanchez-Corral *et al*, 2002; Neumann *et al*, 2003; Dragon-Durey *et al*, 2004). Apart from the stop mutation (R1172Stop), which leads to loss of practically the entire FH20 domain, only V1197A and F1199S are associated with low FH levels in patient sera (Table II). This indicates that the aHUS-associated carboxyl-terminal FH mutations usually influence structure and function, not protein expression. The crystal structure allowed us to group the aHUS-associated mutations into three categories (Table II): (i) those that disrupt the tertiary structure; (ii) those that affect the exposed positively charged residues putatively involved in polyanion binding; and (iii) those that affect a cluster of exposed residues on FH20, involved in C3b and C3d binding (see below). In addition to these categories, two amino acids within FH19–20 have been reported to be mutated in aHUS (residues D1119 and L1189). These mutations are remote from the above-mentioned clusters and are located at the interface between monomers in the crystallographic tetramer (D1119G in FH19 and L1189R/F in FH20).

Four FH19 and five FH20 mutations are likely to disrupt the structure of the FH19–20 monomer since they alter the hydrophobic core of the domains (V1134G, Y1142D, W1157R, and C1163W in FH19 and S1191L/W, V1197A, F1199S, and P1226S in FH20) (Figure 3A). The mutation C1163W also destroys the cystine bridge, presumably leading to unfolded FH19–20. In addition, the surface-exposed G¹¹⁹⁴ is in α_L geometry in a β -turn between two strands. The aHUS-associated mutation G1194D disrupts this geometry and is likely to lead to a dramatic change in the overall structure of the whole loop.

Binding of FH19–20 to negatively charged polyanions, such as heparin, could be reduced if the surface-exposed positive charge of FH19–20 was decreased. Five such point

mutations have been reported in aHUS: Y1142D, G1194D, R1210C, and R1215G/Q. Two of these increase negative charge within an already negatively charged region (Y1142D, G1194D) and cause misfolding (above), rather than impaired heparin binding (Figure 3A). Two of the mutations, R1210C and R1215G, have been shown to cause impaired binding to C3b/C3d (Manuelian *et al*, 2003) or surface-bound C3b (Sanchez-Corral *et al*, 2002) and only slightly impaired binding to heparin (Manuelian *et al*, 2003).

The rest of the aHUS-associated mutations form a cluster on FH20 (Figure 3A). Within approximately 10 Å of R¹¹⁸² and E¹¹⁹⁸ are nine residues, where 12 different mutations have been identified in association with aHUS in a total of 17 patients: R1182S, W1183L/R, T1184R, L1189F/R, V1197A, E1198A, F1199S, R1215G/Q, and P1226S (Table II). Of these disease-associated mutations, four *add* a positive charge or *remove* a negative charge and therefore are unlikely to cause impaired binding to heparin. All of those analysed (W1183L, V1197A, and R1215G) have shown impaired binding of FH to C3d or to surface-bound C3b (Sanchez-Corral *et al*, 2002; Manuelian *et al*, 2003).

Sequence and structural comparisons of C3b/C3d-binding SCR domains

The distribution and characteristics of the known aHUS-associated mutations in FH19–20 suggested that the functional FH defect in aHUS is in C3b/C3d binding instead of in heparin binding. To test this, the sequence of FH20 was compared to the carboxyl-terminal domain of FH-related protein 4 (FHR-4) and murine FH, which also bind C3b and C3d (Hellwage *et al*, 1999; Cheng *et al*, 2006). FHR-4, which does not bind heparin, should contain only a C3b/C3d-binding region. There are eight surface-exposed residues in domain 20 of human FH that are identical or similar in murine FH and FHR-4 (Figure 3B). R¹¹⁸², K¹¹⁸⁸, Y¹¹⁹⁰, E¹¹⁹⁵, and E¹¹⁹⁸ form a cluster in the FH20 structure. It is unlikely that they are involved in heparin binding since they all are found in FHR-4 that does not bind heparin. Homology-based modelling of domains 4–5 of FHR-4 using FH19–20 as a template showed that the positively charged patch on the

Table II Summary of observed mutations within domains 19–20 of FH in patients with aHUS

Mutation	Location	Main effect of the mutation	FH plasma level	Reference
FH19				
D1119G ^a	Exposed	Destabilization of quaternary structure?	Normal	Richards <i>et al</i> (2001)
V1134G	Buried	Destabilization of tertiary structure	High	Neumann <i>et al</i> (2003)
Y1142D	Buried	Destabilization of tertiary structure	High	Neumann <i>et al</i> (2003)
W1157R	Buried	Destabilization of tertiary structure	High	Neumann <i>et al</i> (2003)
C1163W	Buried	Destabilization of tertiary structure	NR ^b	Caprioli <i>et al</i> (2003)
FH20				
E1172Stop		Loss of majority of domain 20	Low	Caprioli <i>et al</i> (2003)
R1182S^c	Exposed	Defect in C3b/C3d binding	NR	Unpublished ^d
W1183R	Exposed	Defect in C3b/C3d binding	High/normal	Remuzzi <i>et al</i> (2002)
W1183L	Exposed	Defect in C3b/C3d binding	High/normal	Dragon-Durey <i>et al</i> (2004)
T1184R	Exposed	Defect in C3b/C3d binding	Normal	Richards <i>et al</i> (2001)
L1189F	Exposed	Destabilization of quaternary structure?	NR	Rodriguez de Cordoba <i>et al</i> (2004)
L1189R	Exposed	Destabilization of quaternary structure?	High	Perez-Caballero <i>et al</i> (2001)
S1191L	Buried	Destabilization of tertiary structure	NR	Richards <i>et al</i> (2001)
S1191W	Buried	Destabilization of tertiary structure	NR	Rodriguez de Cordoba <i>et al</i> (2004)
G1194D	Exposed	Destabilization of tertiary structure	NR	Caprioli <i>et al</i> (2003)
V1197A	Buried	Destabilization of tertiary structure	Low	Caprioli <i>et al</i> (2001)
E1198A	Exposed	Defect in C3b/C3d binding	NR	Caprioli <i>et al</i> (2003)
F1199S	Buried	Destabilization of tertiary structure	Low	Dragon-Durey <i>et al</i> (2004)
R1210C	Exposed	Destabilization of tertiary structure	High/normal	For example, Caprioli <i>et al</i> (2001)
R1215G	Exposed	Defect in C3b and heparin binding	Normal	Richards <i>et al</i> (2001)
R1215Q	Exposed	Defect in C3b and heparin binding	Normal	Caprioli <i>et al</i> (2001)
P1226S	Buried	Destabilization of tertiary structure	High	Neumann <i>et al</i> (2003)

^aThe amino-acid numbering is based on the FH sequence with the signal peptide (18 residues).^bNR, not reported.^cMutations that form a cluster at the suggested C3d-binding site are indicated in bold.^dThe mutation has been published only in an electronic database (<http://www.fh-hus.com/>).

carboxyl-terminal domain occurs only in FH19–20, not in FHR-4 (Supplementary Figure 3). These results provide further support for our model.

Analysis of site-directed mutants of FH19–20

We therefore made five FH19–20 mutants, each with one amino-acid mutation within the identified cluster of aHUS-associated mutations: R1182A, W1183L, K1186A, K1188A, and E1198A. We chose to mutate residues located in the cluster on the surface, which should not have dramatic effects on the overall conformation of the domain, as they primarily lead to side-chain deletion. All the mutants were expressed in *Pichia pastoris*, purified and subjected to heparin-, C3b- and C3d-binding studies. The mutants W1183L and E1198A have been found in aHUS patients, and R1182A is like the aHUS R1182S mutation (<http://www.fh-hus.com/>).

All the mutants bound to the heparin-affinity chromatography column. The monomer forms eluted either at the same salt concentration as *wt* (R1182A, W1183L, K1186A, elution started at 300 ± 16 mM NaCl), slightly earlier (K1188A, at 210 ± 20 mM NaCl), or clearly later (E1198A, at 500 ± 18 mM NaCl) (Figure 4). The oligomeric form of the W1183L mutant clearly eluted later than the monomer.

Binding of the FH19–20 mutants to C3b/C3d was analysed by a radioligand assay, where the mutants were in the solid phase and radiolabelled C3b in the fluid phase, and by an SPR assay. In the radioligand assay, the binding of all five mutants was decreased compared to *wt* FH19–20. The most clearly reduced binding was observed with R1182A and K1188A ($P < 0.001$), followed by W1183L and E1198A ($P < 0.01$) (Figure 5A). The decrease in the binding of K1186A was barely statistically significant.

In the SPR assay, we measured binding of the constructs to solid phase-coupled C3b. In this setup, fluid phase FH19–20 bound to C3b with a K_d of 5.4 ± 0.7 μ M (s.d.) (Figure 5B). The constructs K1188A, R1182A, and W1183L had K_d s of 10.9 ± 5.8 , 8.9 ± 0.6 , and 11.7 ± 3.5 μ M, respectively, indicating that they bound more weakly than *wt* (Figure 5B). The binding of E1198A was similar to *wt* (K_d of 4.2 ± 1.0 μ M), and K1186A could not be tested with this technique. Similar results were obtained with solid-phase C3d (Supplementary Figure 4). The small difference in the results of the radio-ligand and SPR assays, as well as the anomalous binding curve of the W1183L mutant to C3d, might be due to FH oligomerization in solution. The anomalous result with E1198A may be due to diminished electrostatic repulsion by the negatively charged carboxymethylated dextran surface.

Docking of FH19–20 with C3d

As the C3b/C3d-binding site on FH20 was now identified, we docked FH19–20 to the structure of C3d (PDB entry 1C3D) (Nagar *et al*, 1998) using Hex 4.0 (Ritchie, 2003). Various docking models were possible, but we rejected those where the FH19–20-binding region on C3d overlapped with its binding site for complement receptor 2 (CD21) (Szakonyi *et al*, 2001), since FH and CD21 cannot inhibit each other's binding to C3d *in vitro* (Drs W Proding and T Sakari Jokiranta, unpublished data). The best model of the C3d–FH19–20 interaction has good electrostatic and spatial complementarity to the putative C3b/C3d-binding site on FH20 (Figure 6). Approximately 800 Å² is buried in the interacting surface. In the model, C3d binds to FH19–20 with a part of its concave negatively charged surface (Nagar *et al*, 1998); in particular, $\alpha 2$ and the carboxyl-termini of $\alpha 1$ and $\alpha 12$ interact with FH19–20. D¹⁰²⁹ (numbering of C3 with its signal

Figure 3 Location of aHUS mutations in the FH19–20 structure. **(A)** A stereo view of FH19–20 with locations of residues mutated in aHUS patients. The mutated residues are coloured according to the putative consequence: brown, disrupting normal folding; green, interference with binding to C3b/C3d; and blue, putative interference with oligomerization. Disulphide bridges are orange. **(B)** Sequence alignment of the carboxyl-terminal domains of human and murine FH and human FHR-4. The conserved cysteines are shown in black. The residues of FH mutated in aHUS patients are indicated with arrows. Surface-exposed residues of human FH20 and identical or highly homologous residues in murine FH20 or human FHR-4 are shown in gray. Surface-exposed residues shared by these three proteins, and therefore potentially involved in interactions with C3b/C3d, are indicated below the alignment. The site-directed mutants we made in FH19–20 are shown at the bottom.

sequence) and E¹⁰³⁰ of C3d appear to interact with R¹¹⁸² of FH, E¹⁰³² with R¹²¹⁵, R¹⁰⁴² with E¹¹⁹⁸, and E¹⁰⁴⁷ with R¹²¹⁰, respectively (Figure 6B). FH K¹¹⁸⁸ may bind C3d residue E¹⁰³², H¹²¹² and K¹¹⁸⁶ are also close to C3d and FH W¹¹⁸³ may form a hydrogen bond to C3d K¹²⁸⁴.

Docking of FH19–20 with heparin

Positively charged residues form heparin-binding sites in all known heparin-binding protein structures. In FH19–20, R¹¹⁸², K¹¹⁸⁶, R¹²⁰³, R¹²⁰⁶, R¹²¹⁰, R¹²¹⁵, and K¹²³⁰ form a positively charged belt around the distal end of FH20 (Figure 6C). However, site-directed mutagenesis of R¹¹⁸² and K¹¹⁸⁶ did not affect heparin binding of FH19–20 (Figure 4), suggesting that these residues are not important in heparin binding. Conversely, previous mutation data (Manuelian *et al.*, 2003; Jokiranta *et al.*, 2005) suggest that R¹²⁰³, R¹²⁰⁶, R¹²¹⁰, and R¹²¹⁵ all may participate in heparin binding. This is consistent with the recent crystal structure of Vaccinia Complement control Protein (VCP) complexed with a heparin decamer (Ganesh *et al.*, 2004). Superimposing only the heparin-binding region (32 residues) of VCP with the corresponding part of FH20 yielded an r.m.s.d. of 0.97 Å. In this superposition model, FH20 R¹²⁰³, R¹²⁰⁶, and K¹²³⁰ would be within 4 Å of the sugar phosphates in the VCP–heparin complex, and R¹¹⁸² from the ankle region within 6 Å (Figure 6C). A full-length heparin, not just the decamer, could interact with several other positively charged residues in both the ankle and heel regions of FH20.

Discussion

The carboxyl-terminus of complement factor H makes key interactions with C3b and helps to determine whether AP activation should stop or not. This is demonstrated by the fact that mutations in this region of FH lead to the atypical, that is, familial form of haemolytic uremic syndrome. To understand these important molecular events and the pathogenesis of aHUS, we solved the structure of FH19–20. Structural and sequence analysis coupled with mutagenesis and binding assays have shown the ankle region to be critical in FH binding to C3b and C3d. This region is also the location of a cluster of disease-associated aHUS mutations.

The structure of FH19-20 is unusual

Our tertiary structure of FH19–20 shows two unusual features. First, FH19–20 contains a short α -helix, seen before only in the unusual SCR-like domain 5 of β 2-GPI (Schwarzenbacher *et al.*, 1999). The ‘hypervariable loop’ of FH19–20 is longer (33 residues between the first and second cysteine) than in previously solved SCR structures. Such long hypervariable loops occur in the carboxyl-terminal SCR domain of FHRs and domain 6 of the α -chain of C4b-binding protein; these, too, may contain an α -helix. Secondly, FH19–20 has a structural water molecule that forms three hydrogen bonds in the hydrophobic core of FH20 (Supplementary Figure 2). Such water molecules are not uncommon, although they have not been reported in SCR domains before.

Figure 4 Heparin affinity chromatography of FH19-20 and mutants. (A) Coomassie blue-stained SDS-PAGE gel of FH19-20 and purified mutants. (B) Western blot of column fractions for *wt* and the mutants. Each construct was injected onto the heparin column in PBS and eluted using a linear NaCl gradient. The fractions were run on SDS-PAGE and blotted with anti-FH antibody. Fractions collected for Western blotting are indicated with vertical lines; average and minimum/maximum NaCl concentration is shown at the top and the mobility of size markers is indicated on the left.

Interestingly, two of the aHUS-associated mutations, S1191L and S1191W, involve S¹¹⁹¹, which binds the water molecule. We believe that the water molecule may be involved in stabilizing the structure of FH20 to make it C3b/C3d-binding competent.

The heel region binds heparin

We recently showed that the R1203E/R1206E/R1210S mutant completely lacks heparin binding (Jokiranta *et al*, 2005). These three Arg are in the 'heel' of FH20, some 8–13 Å from each other. In another study, two aHUS mutations, R1210C and R1215G, impaired binding of FH8-20 to C3b/C3d and slightly impaired heparin binding (Manuelian *et al*, 2003). These residues are at least 14 Å apart from each other and so cannot interact with adjacent heparin saccharides. In

Figure 5 Binding of FH19-20 and the mutant constructs to C3b and C3d. (A) Binding of ¹²⁵I-labelled C3b to microtiter plate-coupled *wt* or mutant FH19-20 constructs. The results are shown in comparison to *wt* FH19-20. Standard deviations are indicated with bars and statistical significance of the difference of each mutant in comparison to *wt* FH19-20 are indicated by asterisks (**P* < 0.01 and ***P* < 0.001). (B) SPR analysis (Biacore[®]) of binding of the fluid-phase FH19-20 constructs to solid-phase C3b. The interaction between the fluid-phase FH19-20 constructs and solid-phase C3b is shown as a function of the fluid-phase construct concentration. Each construct was injected separately to a blank control flow cell and a C3b-coupled flow cell using a flow rate of 30 μl/min and several protein concentrations (3–200 μg/ml).

addition, the primary heparin-binding site of VCP is in the heel region of the most carboxyl-terminal domain (Ganesh *et al*, 2004); six basic residues are close enough to bind to a heparin decamer. Although some of the positively charged residues in VCP do not occur in FH20, the overall tertiary arrangement is relatively similar. The FH residues thus putatively involved in heparin binding include R¹²⁰³, R¹²⁰⁶, and K¹²³⁰ (Figure 6C).

The region around the short α-helix in FH20 is flanked by R¹¹⁷¹ and R¹²⁰² and contains two solvent-exposed negatively charged residues (E¹¹⁷² and E¹¹⁷⁵). Heparin might interact with R¹¹⁷¹ and R¹²⁰². However, this is not consistent with our current model, and R¹¹⁷¹ is unlikely to be critical since murine FH, which also binds heparin, lacks a positively charged residue at this position.

Defects in C3b/C3d binding cause aHUS

We now focus only on the surface-exposed residues mutated in aHUS. The FH19-20 structure (Figures 2 and 3A), sequence analysis (Figure 3B), and docking model (Figure 6) all suggest that the hot-spot for aHUS-associated mutations within the ankle region of FH20 is the primary binding site for C3b/C3d, not heparin. We verified this by making point mutations to five surface-exposed residues within this area (Figures 4 and 5). The result is novel, but supported by earlier mutagenesis results. In addition, in the recently published

Figure 6 Docking model of FH19–20 binding to C3d and heparin. (A) The mutations in FH19–20 that have been characterized in aHUS patients are colored as in Figure 3A. On C3d, the FH-interface area is marked with red, while the surface region which interacts with CD21 (Szakonyi *et al*, 2001) is shown in magenta. (B) The molecules have been dragged apart and rotated away from each other to better visualize the hypothetical interacting regions. (C) Location of a heparin decamer after superimposition of vaccinia complement control protein–heparin complex with FH20. The positively charged residues previously proposed to interact with heparin are in cyan.

crystal structure of C3 (Janssen *et al*, 2005) (PDB 2A73), the suggested FH19–20-binding site on C3b/C3d is on the surface of intact C3 but its accessibility is partially obscured by the adjacent CUB domain. Limited accessibility in intact C3 is expected since FH binds to the cleavage fragment C3b, which probably adopts a radically altered overall conformation (Janssen *et al*, 2005).

In all, 12 of the reported aHUS-associated mutations affect the surface-exposed area that we now show to be involved in C3b/C3d binding (Table II). For instance, the aHUS-associated mutations that lead to impaired C3b/C3d binding, W1183L, V1197A (Sanchez-Corral *et al*, 2002), and R1215G (Manuelian *et al*, 2003), are located in this ankle region. Of the residues mutated in aHUS (W1183R/L, R1182S, T1184R, L1189R, V1197A, E1198A, and R1215G/Q, Table II), all except T¹¹⁸⁴ are in our modelled FH20–C3d interface and make potentially favorable interactions.

Some aHUS-associated mutations appear to have more than one effect, suggesting that the heparin- and C3b/C3d-binding sites are close to each other. This is consistent with the structure (Figures 2 and 3) and our recent data (Hellwage *et al*, 2002; Jokiranta *et al*, 2005). For instance, the E1198A mutation resulted in increased heparin affinity but decreased C3b binding (Figures 4 and 5). In addition, the region between the heparin- and C3b/C3d-binding sites contains K¹¹⁸⁸ and H¹²¹² that could be part of an extended heparin-binding site. The slightly decreased heparin binding of the K1188A mutant (Figure 4) fits well with this hypothesis. This multipurpose surface may explain why heparin binding can compete with C3d binding, but not *vice versa* (Jokiranta *et al*, 2005); the ankle in FH20 that binds C3d is also part of the heparin-binding surface (see above), but C3d does not interact with the heel.

Our data thus show that *none* of the mutations causes a defect in heparin binding only, while several affect selectively only C3b/C3d binding. The most probable cause for the pathogenetic processes of aHUS is thus defective C3b/C3d binding, although, in some cases, a defect in heparin binding may also be important. The homology-based molecular models of FH19–20 (Hellwage *et al*, 2002; Perkins and Goodship, 2002) are inaccurate; for instance, they—unsurprisingly—do not contain the unusual α -helix. As a result, the cluster of C3b/C3d-binding residues we identify are not as close

together, and the electrostatic surface is incorrect. It is thus hardly surprising that these models have led to incorrect interpretations of aHUS mutations.

Oligomerization of FH19–20

The FH19–20 crystal structure is clearly tetrameric, with two interfaces that look typical for protein–protein interfaces in oligomers. However, analytical ultracentrifugation of FH has been interpreted as not showing oligomers (Aslam and Perkins, 2001). It is therefore unclear if oligomerization occurs *in vivo* or not, and whether it has any physiological or pathophysiological significance. However, an unexpected role for oligomerization is suggested by two observations. (i) One aHUS-associated mutation, W1183L, shows enhanced oligomer formation and the oligomers bind tighter to the heparin column than the monomer (Figure 4B). (ii) The pathophysiological consequences of three of the aHUS-associated mutations (D1119G, L1189F, and L1189R) cannot be rationally explained by a disruption of the fold or interference of the remote heparin- or relatively remote C3b/C3d-binding sites. These three particular residues mediate oligomerization in the crystal structure. Therefore, we hypothesize that oligomerization might occur in certain local microenvironments even though it is not visible in solution by ultracentrifugation analyses. Binding of FH to surface-associated C3b, and therefore one of the major functions of FH, might be improved by oligomerization of FH. This is supported by our structural data since the oligomerization interfaces do not overlap with the observed C3b/C3d-binding site. Furthermore, the avidity of FH oligomers to the self-surfaces might be higher than that of the monomer, thereby enhancing the complement regulatory function of FH on those surfaces. This is supported by our recent results showing that binding of FH to endothelial cells is mediated by the heparin-binding site (Jokiranta *et al*, 2005). This may explain why only a fraction of FH molecules in solution are capable of high-affinity interaction with surfaces containing C3b deposits (Conrad *et al*, 1978).

Pathophysiology of aHUS

The influence of aHUS-associated mutations on the binding of FH to C3b/C3d has previously been suggested (Hellwage

Figure 7 Schematic model of how mutations in FH might affect complement regulation on endothelial cell surfaces. Under normal circumstances FH has sufficient avidity for surface-associated C3b to be able to promote its inactivation into iC3b in concert with membrane-bound complement regulator CD46 (left). Reduced avidity of FH to cell-bound C3b by a mutation in FH20 can result in a failure to efficiently inactivate surface-bound C3b molecules thereby leading to complement-mediated cell damage (right).

et al, 2002; Sanchez-Corral *et al*, 2002; Manuelian *et al*, 2003), but no pathogenetic explanation or a structural basis has been proposed. HUS is characterized by thrombotic microangiopathy that is believed to follow erythrocyte and platelet damage and endothelial cell damage in multiple vascular beds, especially in kidneys. Normally, a low amount of C3b is deposited on all cells in contact with plasma. Concerted actions of the membrane-bound complement regulator CD46 (membrane cofactor protein) and plasma FH, together with the proteolytic factor I, rapidly lead to the inactivation of deposited C3b to iC3b on endothelial cells preventing propagation of the C3b-amplification loop (Figure 7). Mutations in CD46 and factor I have also been reported in aHUS (Richards *et al*, 2003; Kavanagh *et al*, 2005). All these mutations could lead to a failure in the control of C3 AP amplification and subsequent cell lysis. The level of FH is relatively high in plasma (116–600 µg/ml) (Esparza-Gordillo *et al*, 2004) and provides some redundancy for controlling complement activation. The aHUS-associated point mutations in FH are almost always heterozygous. Despite having approximately half the normal plasma level of FH, individuals with a mutation within the FH19–20 region may develop aHUS. Consequently, more than half the normal amount of functional FH appears to be needed to prevent aHUS. The role of FH as a gatekeeper of the alternative

pathway activation operates within a relatively narrow avidity range using three binding sites between FH and C3b (Jokiranta *et al*, 2000). The difference in avidity when FH binds to C3b on a nonactivator surface versus on an activator surface is only 3–10-fold (Pangburn and Müller-Eberhard, 1978; Pangburn, 1989). Therefore, the relatively small drop in affinity of one of the three binding sites in the studied aHUS-associated mutants can have a clear and pathophysiologically significant effect by impairing even slightly the control of AP activation on the nonactivator surfaces.

FH binds endothelial cells in the absence of C3b or C3d (Manuelian *et al*, 2003), but the presence of C3b or its fragments iC3b and C3d significantly increases the avidity. When an FH molecule is bound via its carboxyl-terminus simultaneously to glycosaminoglycans and C3b, iC3b, or C3d on the endothelial cell surface, it presumably can act efficiently to control C3 convertases via its amino-terminal effector site. We thus suggest that, in aHUS, impaired C3b/C3d binding by FH19–20 is the primary cause of the decreased avidity of FH to the C3b-challenged cells. This results in decreased action of FH on the cells and subsequent damage to the endothelial cells, erythrocytes, and platelets (Figure 7). Alterations in heparin/glycosaminoglycan binding may play a secondary role.

Materials and methods

Expression and crystallization of recombinant FH19–20

For recombinant expression of FH19–20, the corresponding recombinant DNA was created by PCR using Uni-ZAP[®] XR human liver cDNA library (Stratagene, La Jolla, CA, USA) as a template (cloning primers are given in the Supplementary data). The DNA was cloned into the pPICZαB expression vector and the sequence confirmed by automatic sequencing. The recombinant construct was expressed in *P. pastoris* cells as described in the manufacturer's protocols. The culture supernatant was diluted in 1/2 PBS, filtered (0.22 μm filter), and subjected to heparin affinity chromatography (HiTrap Heparin, Amersham Bioscience). The bound protein was eluted with a salt gradient (75 mM–1.0 M). The pooled fractions were gel filtrated (Sephacryl S-100 High Resolution gel, 300 ml, 80 cm, Amersham Pharmacia Biosciences) and repurified with a secondary heparin affinity column. The protein purity was confirmed by SDS-PAGE (>99%). Before crystallization, the protein was concentrated to 10 mg/ml and dialysed into 20 mM Tris, 50 mM NaCl, pH 7.0. The protein was crystallized in sitting drops by mixing 1 μl of protein solution at 10 mg/ml with 1 μl of reservoir solution of 2.2 M (NH₄)₂SO₄, 0.1 M Tris-HCl, pH 8.5 at 4°C. In this study, we have used amino-acid numbering based on the FH sequence with signal peptide (accession number P08603) for consistency with earlier literature.

Construction of FH1920 mutants

Five mutations were introduced to the FH19–20 sequence using the QuikChange[®] Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) (see Supplementary data for primers). Constructs were expressed and purified similar as for *wt* FH19–20 above.

X-ray crystallography

The crystals belong to the tetragonal space group I4₁22 (Table I), with one molecule per asymmetric unit and a solvent content of ~68%. The native crystals diffracted to 1.75 Å resolution on a synchrotron source and 2.4 Å on our in-house diffractometer (Table I). Since FH19–20 contains one Met and eight Cys, we decided to phase the structure using single wavelength anomalous dispersion from sulphur atoms (SAS) at 1.54 Å. The SAS data were collected on an R-Axis IV image plate using Cu Kα radiation. Native data were collected at beam line ID14-1 using a MarCCD detector at the European Synchrotron Radiation Facility (ESRF), Grenoble. The data were processed using the programs DENZO/SCALEPACK (Otwinowski and Minor, 1997) and XDS (Kabsch, 1993).

The initial phase information was derived using sulphur anomalous scattering at 1.54 Å using SHELX-97 (Schneider and Sheldrick, 2002). The initial polyaniline-peptide fragments model was built using programs RESOLVE (Terwilliger and Berendzen, 1999) and O (Jones *et al*, 1991). The SAS phase information of FH19–20 was then used to generate an initial model using the 1.75 Å native data set. This model was then refined using ARP/wARP (Perrakis *et al*, 1999), REFMAC5 (Murshudov *et al*, 1997), and rebuilt using O. The quality of the final model was analysed with PROCHECK (Laskowski *et al*, 1993) and figures were prepared with PyMOL (DeLano, 2002).

Homology-based molecular modelling of the carboxyl-terminus of FHR-4

Molecular modelling of the two most carboxyl-terminal domains of FHR-4 was performed using InsightII 2000 (Accelrys Inc., San Diego, CA) and the refined FH19–20 structure as a template. Energy minimizations were performed using a 15 Å cutoff and both steepest descent and conjugate gradient energy minimization algorithms until the maximum derivative was <0.001 kcal/mol as described previously (Jokiranta *et al*, 1995).

Heparin affinity analysis

Purified mutant FH19–20 constructs were dialysed in PBS and injected to heparin columns (HiTrap Heparin, Amersham Bioscience). Constructs were eluted with an NaCl gradient (75 mM–2 M in PBS) using an HPLC (Merck Hitachi LaChrom). NaCl concentrations of the collected fractions were calculated on the basis of conductivity measurements using a conductivity meter CDM210 (Radiometer Analytical SAS, Villeurbanne, France). Samples of fractions were run in 12.5% SDS-PAGE and transferred

to nitrocellulose using standard techniques. Proteins were detected using anti FH19–20 mAb VIG8 (Prodinger *et al*, 1998) and enhanced chemiluminescence detection system.

Radioligand assay

Wells of Nunc-Immuno[™] BreakApart[™] plates were coated with FH19–20 or the mutants (10 μg/ml) in 30 mM phosphate buffer (28 mM NaCl, 2 mM phosphate, pH 7.3) for 17 h at 22°C. After washing the wells twice with 1/2 PBS (70 mM NaCl, 5 mM phosphate, pH 7.3), they were blocked for 60 min at 37°C with 0.05 % Tween 20 in 1/2 PBS. After washing the wells twice with 1/2 PBS, a total of 50 000 c.p.m. of ¹²⁵I-labelled C3b (labelled using the Iodogen method, specific activity 8–10 × 10⁶ c.p.m./μg) was added to the wells in 80 μl of 30 mM phosphate buffer and incubated for 3.5 h at 37°C. After washing, the radioactivity was measured using a γ-counter. The assay was performed in quadruplicate and standard deviations and *P*-values were calculated with Microsoft Excel.

Surface plasmon resonance assays

Real-time SPR assays were performed on a Biacore[®] 2000 and the data analysed using BiaEvaluation 3.0 software (Biacore AB, Uppsala, Sweden). For coupling to carboxylated dextran chips (Sensor Chip CM5, Biacore AB), C3d and C3b were dialysed against 20 mM acetate buffer (pH 4.5) and 10–50 μl of a 1–50 μg/ml solution used for coupling as described (Jokiranta *et al*, 2000). The coupling efficiency was 1000–1500 resonance units (RU) and therefore the flow cells contained approximately 2 ng/mm² of C3d or C3b. For the binding assays, all the reagents were dialysed against PBS. Protein concentrations were measured using the BCA Protein Assay (Pierce Chemical Company, Rockford, IL). Binding curves of the FH19–20–C3b interaction were obtained by nonlinear curve fits (GraphPad Prism 3.0) of the values obtained 15 s before the end of the injections against ligand concentration in the fluid phase.

Docking of FH19–20 with C3d and heparin

In docking analyses, the target protein was the refined model of FH19–20 excluding the waters and including the missing side chains in their most favoured rotamer conformations. For automated protein-to-protein docking between FH19–20 and C3d, we used the crystal structure of C3d (pdb-code 1C3D) (Nagar *et al*, 1998) as a probe and Hex 4.0 (Palma *et al*, 2000). Several docking models were possible for the FH19–20–C3d interaction but we rejected those where the FH19–20-binding region on C3d overlapped with its binding site for complement receptor 2 (CD21) (Szakonyi *et al*, 2001), since FH and CD21 cannot inhibit each other's binding to C3d *in vitro* (Prodinger and Jokiranta, unpublished). Heparin was docked to FH19–20 by superimposing the structure of VCP complexed with heparin (pdb-code 1RID) (Ganesh *et al*, 2004) on FH19–20, and using the heparin coordinates without modification.

PDB accession code and atomic coordinates of FH19–20

The coordinates and structure factors for the native FH19–20 are available in the Protein Data Bank (<http://www.rcsb.org/pdb/>) under the accession code 2G71.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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