Factor H and Atypical Hemolytic Uremic Syndrome: Mutations in the C-Terminus Cause Structural Changes and Defective Recognition Functions

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Atypical hemolytic uremic syndrome is a disease that is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. Mutations in the complement regulator factor H are associated with the inherited form of the disease, and >60% of the mutations are located within the C terminus of factor H. The C-terminus of factor H, represented by short consensus repeat 19 (SCR19) and SCR20, harbors multiple functions; consequently, this study aimed to examine the functional effects of clinically reported mutations in these SCR. Mutant factor H proteins (W1157R, W1183L, V1197A, R1210C, R1215G, and P1226S) were recombinantly expressed and functionally characterized. All six mutant proteins showed severely reduced heparin, C3b, C3d, and endothelial cell binding. By peptide spot analyses, four linear regions that are involved in heparin, C3b, and C3d binding were localized in SCR19 and SCR20. A three-dimensional homology model of the two domains suggests that these four regions form a common binding site across both domains. In addition, this structural model identifies two types of residues: Type A residues are positioned on the SCR surface and are represented by mutants W1157R, W1183L, R1210C, and R1215G; and type B residues are buried within the SCR structure and affect mutations V1197A and P1226S. Mutations of both types of residue result in the same functional defects, namely the reduced binding of factor H to surface-attached C3b molecules and reduced complement regulatory activity at the cell surfaces. The buried type B mutations seem to affect ligand interaction of factor H more severely than the surface-exposed mutations.

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Hemolytic uremic syndrome (HUS) is a disease that is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure (reviewed in [1]). The typical epidemic- or diarrhea-associated form of HUS, which is nonrecurring and shows almost complete recovery, is most common in infants and young children. A second form, termed atypical, non–diarrhea-associated HUS (aHUS), occurs predominantly in adults. This form is rare, is often recurrent, and generally has a poor outcome. Most patients with aHUS develop end-stage renal failure and require long-term dialysis. Several studies have shown that aHUS is associated with defective complement regulation, which is caused by genetic alterations of complement regulators or by autoantibodies to individual regulators (2). The majority of aHUS mutations (65 cases) have been identified in the multidomain complement regulator factor H (3–8). Five patients showed mutations within the membrane co-factor protein (CD46) (9,10), four patients had mutations in the serine protease factor I (11,12), and for three aHUS patients, autoantibodies for factor H have been reported (13).

Factor H is a central complement regulator that is composed of 20 short consensus repeat (SCR) domains, in which the complement regulatory region is located in the N-terminal SCR1 to SCR4, whereas its cell binding recognition domain is located in its C-terminal part (14). Two separate binding sites for heparin and sialic acid have been identified in SCR7 and SCR19/SCR20, whereas a third site may exist in SCR13 (15–19). The SCR19 and SCR20 form the major recognition region of the protein (20,21). In support of this conclusion, functional analyses of recombinant factor H proteins mutated in SCR19/SCR20 at sites identified in patients with HUS display limited capacities to bind to cell surfaces (21). Moreover, a mAb with an epitope spanning SCR19 and SCR20 inhibits heparin binding by factor H (14,22). More than half of the HUS-associated mutations are clustered in SCR20 and SCR19, and different residues are affected (1–5). So far, defective C3b and endothelial cell binding has been demonstrated for two mutant proteins

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(4,21). It therefore is of interest to analyze whether additional C-terminal mutants result in the same or at least similar defects.

To correlate the reported aHUS mutations with factor H recognition functions, we used peptide spot assays to localize the linear binding regions in SCR19 and SCR20. In addition, recombinant factor H proteins that incorporate six distinct aHUS-associated mutations were expressed and used for functional analyses. Molecular modeling studies of SCR19 and SCR20 were performed to provide structural insights of the mutations. This approach identified in SCR19 and SCR20 a total of four linear, mostly overlapping regions that are responsible for binding to the various ligands. All six mutant proteins showed defective ligand and cell binding. These results demonstrate that defective factor H is related directly to aHUS.

Materials and Methods

Peptide Spot Analysis

Forty peptides that represent SCR19 and SCR20 of factor H (residues 1104 to 1231) with lengths of 13 amino acids and an overlap of 10 amino acids were synthesized and coupled to a cellulose membrane (Jerini Peptide Technologies, Berlin, Germany). The membranes were treated and analyzed as described (23).

Generation of Mutant Factor H Fragments

Single nucleotide exchanges in SCR20, representing the W1157R, W1183L, V1197A, and P1226S substitutions, were introduced by the QuikChange site-directed mutagenesis technique (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The following primers were used to generate mutant Factor H sequences in the expression vector pBSV-FH8–20 (24) (modified nucleotides in the sequence are in boldface):

ForW1157R 5′-C A T G T A G A A A T G G A C A A **C** G G T C A G A A C C A C C A A A A T G C-3′

RevW1157R 5′-G C A T T T T G G T G G T T C T G A C C **G** T T G T C C A T T T C T A C A T G-3′

ForW1183L 5′-C A T A G C A T T A A G G T **T** G A C A G C C A A A C A G A A G C T T T A T T C G-3′

RevW1183L 5′-C T G T T T G G C T G T C **A** A C C T T A A T G C T A T G T T A T A A T T T T C C-3′

ForV1197A 5′-C G A G A A C A G G T G A A T C A G **C** T G A A T T T G T G T G T A A A C G G-3′

RevV1197A 5′-C C G T T T A C A C A C A A A T T C A **G** C T G A T T C A C C T G T T C T C G-3′

ForP1226S 5′-C A T G T T G G G A T G G G A A A C T G G A G T A T **T** C A A C T T G T G C A A A A A G-3′

RevP1226S 5′-C T T T T T G C A C A A G T T G **A** A T A C T C C A G T T T C C C A T C C C A A C A T G-3′

The sequence of the various mutant vectors was verified by sequence analysis. The generation of the wild-type factor H fragment SCR8 to SCR20 and mutants R1210C (FH8-20/R1210C) and R1215G (FH8-20/R1215G) was described previously (21).

Insect Cell Culture

Spodoptera frugiperda Sf9 cells were cultivated as described (24). Cells were infected with recombinant virus, and purification of the recombinant proteins was performed according to standard procedures (24)

Heparin Chromatography of Mutant Proteins

The binding of the recombinant mutant and wild-type factor H proteins to a heparin matrix was analyzed using an ÄKTAPrime system (GE Health Care, Uppsala, Sweden). Supernatant that was prepared from infected insect cells was diluted in 0.5 × PBS, and 5 to 10 ml of this solution was applied to a heparin matrix (HiTrap; GE Health Care) at a flow rate of 1 ml/min. After loading, the matrix was washed with 100 ml of 20 mM NaCl in 0.5 × PBS. Subsequently, bound proteins were eluted using a linear salt gradient that ranged from 100 to 500 mM NaCl. Fractions of 500 μl were collected, separated by SDS-PAGE, and used for immunoblotting. The elution profiles of the various proteins were compared using the Prime View software (GE Health Care). For each protein, this separation was repeated three times, and in all cases, identical elution profiles were obtained.

Binding to C3b and C3d by Surface Plasmon Resonance

The binding to C3b and C3d of mutant recombinant and wild-type factor H SCR8-SCR20 was assayed using a Biacore 3000 instrument (21). Briefly, C3b or C3d was immobilized *via* standard amine coupling to the flow cells of a sensor chip (carboxylated dextran chip CM5; Biacore, Uppsala, Sweden). The surface of the flow cells was activated, and C3b or C3d was diluted in coupling buffer (10 mM acetate buffer [pH 5.0]) was injected until an appropriate level of coupling was reached (approximately 4000 resonance units). Each analyte was injected separately into a flow cell coupled with C3b or C3d or into a blank control using a flow rate of 5 μl/min at 25°C. Each binding experiment was performed three times.

ELISA to Determine C3b and C3d Binding

MaxiSorp plastic plates (Nunc, Wiesbaden, Germany) were coated with 10 μg/ml C3b or C3d (Calbiochem-Novabiochem Corp., San Diego, CA) in PBS. After blocking with 3% BSA in PBS, the various factor H fragments were added. Binding of the factor H proteins was detected by serial incubation with goat anti-human factor H (Calbiochem-Novabiochem Corp.) diluted 1:1000 in PBS that contained 0.05% Tween-20 and horseradish peroxidase–conjugated rabbit anti-goat Ig 1:1000 (Dako, Hamburg, Germany). The reaction was developed using 100 μg/ml 3′3′5′5′-tetramethylbenzidine (Roth, Karlsruhe, Germany) in 0.1 M Na-acetate buffer (pH 5.5) as chromogene, 2 μl/ml 30% H_2O_2 was used as substrate, and the color reaction was stopped with 2 M H_2SO_4. The absorbance was measured at 450 nm on a Spectra Max 190 photometer (Molecular Devices, Eugene, OR).

Cultivation of Endothelial Cells and Binding Assay

Human umbilical vein endothelial cells (American Type Culture Collection, Rockville, MD) were grown as described (21). For binding experiments, cells were grown to confluence either in cell culture flasks or on Lab-Tek chamber slides (Nalgene-Nunc International, Wiesbaden, Germany), washed, and cultivated in serum-free DMEM for 20 to 30 h.

SDS-PAGE and Western Blot Analysis

Purified recombinant proteins were separated by SDS-PAGE under nonreducing conditions as described (25).

Immunofluorescence Staining

Endothelial cells that were grown on eight-well chamber slides for 24 h in FCS-free medium were washed and fixed with 4% paraformaldehyde for 30 min. Cells were treated with 1% BSA/PBS for 30 min to prevent unspecific binding and incubated for 1 h with purified recombinant protein diluted in 0.5 × PBS using identical amounts of recombinant wild-type factor H SCR8-SCR20 or mutant protein (W1157R, W1183L, V1197A, R1210C, and R1215G). Cells were incubated overnight with polyclonal anti–factor H antiserum (Calbiochem-Novabiochem) diluted 1:200 in blocking buffer. Excess antibody was removed

by washing with PBS, and the cells were incubated further at room temperature with an Alexa fluor-488–conjugated donkey anti-goat antibody (Molecular Probes, Eugene, OR). After 2 h, cells were washed with PBS and mounted in fluorescence-preserving medium. The nuclei were stained with DAPI (10 μg/ml; Sigma Aldrich, Taufkirchen, Germany) and the cell wall with Alexa 633–labeled wheat germ agglutinin (Molecular Probes). Fluorescence staining was visualized with appropriate filter settings using a confocal laser scanning microscope (Zeiss-LSM-510 META; Carl Zeiss, Jena, Germany) equipped with the META detector.

Homology Modeling

The homology modeling of the factor H SCR domains using the MODELLER program and the PROCHECK verification program is described elsewhere (26). The models for SCR19 and SCR20 were based on the crystal structures of SCR3 and SCR1, respectively, in vaccinia virus coat control protein (VCP) (27) because these showed the highest sequence identity (40 and 18%, respectively, with SCR19 and SCR20) and the minimum number of insertions and deletions. The SCR19 and SCR20 models are available from the factor H aHUS web database (http://www.FH-HUS.org) (26). Whereas the secondary structure is expected to be conserved and modeled accurately, individual sidechains may not be positioned correctly in all cases. SCR19 and SCR20 are joined by a short three-residue linker, but this linker was not modeled because of the great variability in conformation seen between adjacent SCR domains (28).

Results

Peptide Spot Analysis

Peptide spot analyses were performed to identify within the C-terminus of factor H the linear binding regions and amino acids that are relevant for interactions with the various ligands. Peptides that represent the sequence of SCR19 and SCR20 were spotted and probed with heparin, C3b, and C3d. These analyses identified four linear regions that interact with heparin (Figure 1A, top). Region I is 10 residues long (amino acids 1145 to 1154), is located in SCR19, and binds relatively weakly. Three major binding sites were located in SCR20 (region II, amino acids 1178 to 1187; region III, amino acids 1202 to 1215; and region IV, residues 1229 to 1231; Figure 1B). Incubation of a membrane with an unrelated control protein showed no positive spots (data not shown). In addition, separate membranes were used to map the interaction sites for C3b and C3d (Figure 1A, middle and bottom). This approach identified the same or very similar regions for the three ligands and revealed a substantial overlap of the binding sites, together with evidence of ligand specificity. C3b binding is mediated by regions II, III, and IV, whereas C3d binding is meditated by regions I, II, and III. It also seems that for heparin binding, a longer stretch of region II is used as for C3b and C3d binding. Hence, region IV binds heparin and C3b but not C3d. Thus, SCR19 and SCR20 of factor H use similar and overlapping residues for binding to the three ligands.

Generation and Expression of Mutant Factor H Proteins

Six recombinant mutant factor H proteins were expressed to assay and compare the role of six single amino acid substitutions on factor H ligand interaction. For exclusion of the contribution of the additional heparin and C3b binding sites, mutant proteins were expressed using an SCR8 to SCR20 backbone. The recombinant mutant factor H proteins have single residue exchanges in SCR19 (W1157R) or in SCR20 (W1183L, V1197A, R1210C, R1215G, or P1226S). The substitutions are positioned within or adjacent to the four binding regions identified from the spot analyses (Figure 1B). All purified mutant and wild-type factor H proteins showed comparable mobilities (Figure 2A).

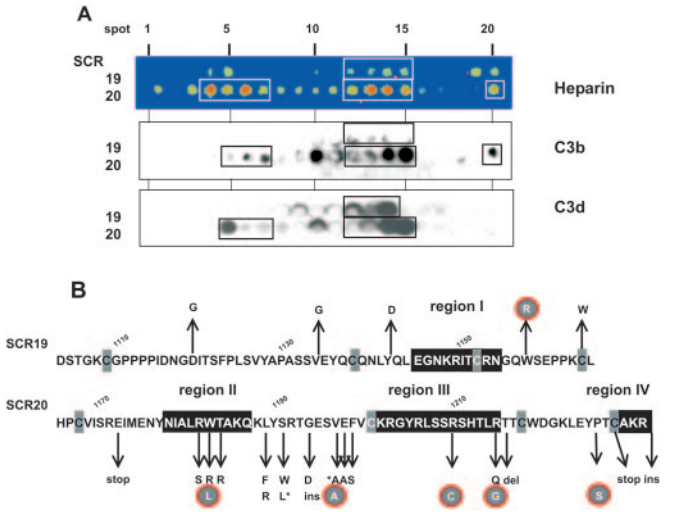

Figure 1. Localization of linear binding motifs in the C-terminal recognition region of factor H. (A) Peptides with a length of 13 amino acids and an overlap of 10 amino acids that covered the complete short consensus repeat 19 (SCR19) and SCR20 domains of factor H were synthesized; spotted onto the membrane; and analyzed for the binding of heparin, C3b, and C3d. The four identified binding regions are boxed. (B) Linear protein sequences of SCR19 and SCR20 of factor H. The four binding regions identified by peptide spot analyses are boxed, and the atypical hemolytic uremic syndrome (aHUS)-associated mutations with their corresponding amino acid exchanges are shown. The circled amino acids were those studied in this work. Structural cysteins are marked by gray shading. The double mutations at S1181 and V1187 are marked by *.

Binding of Mutant Factor H Proteins to Heparin

Five mutant and the wild-type factor H SCR8-SCR20 proteins were assayed using heparin affinity chromatography. Bound proteins were eluted with a linear salt gradient, and the various elute fractions were collected and analyzed by SDS-PAGE and Western blotting. All mutant proteins bound to the heparin matrix but were eluted before the wild-type protein (Figure 2B). Wild-type factor H SCR8-SCR20 was maximally eluted in fraction 34 at approximately 340 mM NaCl. The W1157R mutant showed maximal elution in fraction 32 (320 mM NaCl). The two mutant proteins with W1183L and V1197A showed very similar characteristics and were eluted in fractions 29 and 28 (290 mM NaCl), whereas mutant R1210C was eluted in fraction 30 (305 mM NaCl) and mutant P1226S was eluted in fraction 27 (285 mM NaCl). These experiments show that single amino acid exchanges within SCR19 and SCR20 significantly weakened heparin binding. The mutation in SCR19 (W1157R) caused a

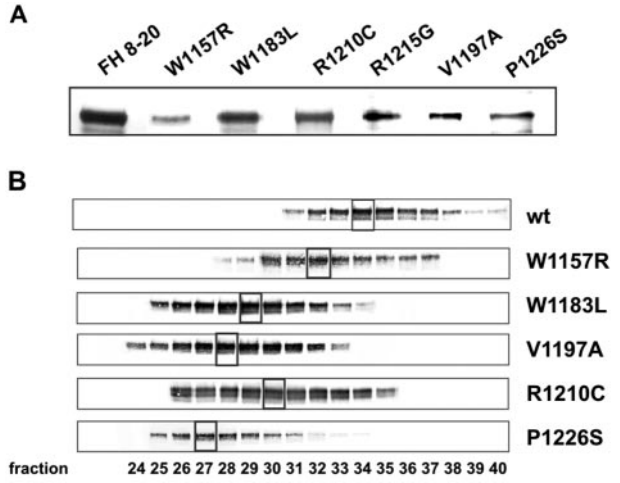

Figure 2. Expression of recombinant mutant factor H proteins and heparin affinity chromatography. (A) Recombinant factor H proteins with the indicated aHUS-associated mutations were generated on a factor H 8-20 framework and expressed in insect cells. The mutant proteins were purified by nickel affinity chromatography, separated by SDS-PAGE, and identified by silver staining. (B) Heparin affinity chromatography of mutant factor H proteins. The proteins were loaded onto a heparin column, the matrix was washed thoroughly, and bound proteins were eluted using a linear NaCl gradient. The various fractions were analyzed by SDS-PAGE and Western blotting. As the elutions were performed under identical conditions, the elution profiles of the mutants can be compared directly.

minor reduction in heparin affinity, whereas the other five in SCR20 had a larger effect.

Binding of Mutant Factor H Proteins to C3b and C3d

The C-terminus of factor H is central for interaction with complement component C3. The C3 interaction was analyzed by surface plasmon resonance. C3b or C3d was coupled to the sensor chip, and the five mutant and the wild-type factor H SCR8-SCR20 proteins were injected. The wild-type fragment showed prominent binding to C3b and C3d, as judged by the strong association and dissociation profile (Figure 3, A and B). The five mutant proteins showed severely reduced binding to immobilized C3b and C3d (Figure 3, A and B). A low but residual binding affinity was observed for the mutant proteins W1183L and R1210C, whereas no interactions with C3b or C3d was observed for the three mutant proteins W1157R, V1197A, and P1226S.

In addition, an ELISA test was used to assay C3 binding. C3b or C3d was immobilized to the surface of a microtiter plate, and the binding of six mutant factor H proteins was tested. The binding activities of the mutated proteins W1183L, V1197A, R1210C, and R1215G was reduced to approximately 50% of the wild-type protein. Binding of the mutant W1157R was strongly reduced, whereas that for the P1226S mutant was completely abolished (Figure 3C).

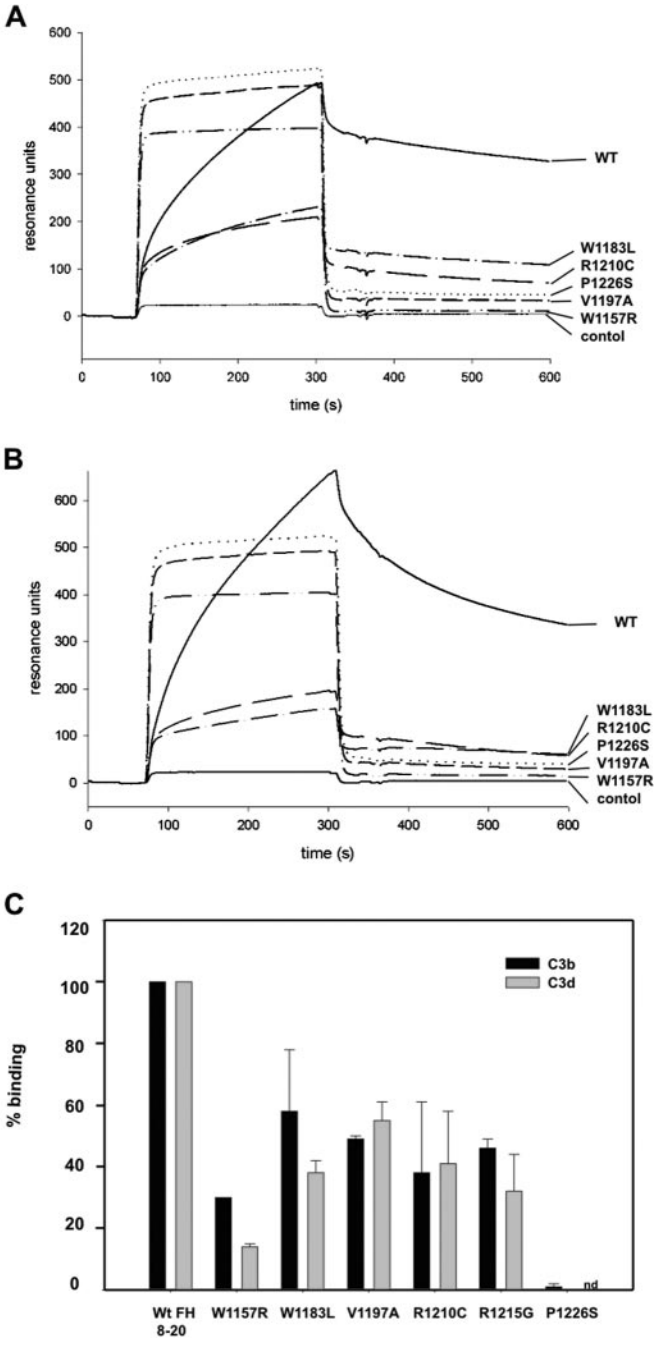

Figure 3. Binding of recombinant mutant factor H proteins to C3b and C3d. (A) Binding of mutant factor H (W1157R, W1183L, V1197A, R1210C, R1215G, and P1226S [dashed lines]) and wild-type factor H SCR8-SCR20 (solid line) to immobilized C3b was analyzed by surface plasmon resonance. Upon injection of the protein into the flow cell, both the association and the dissociation profiles were recorded. (B) The corresponding binding of recombinant mutant and wild-type factor H proteins to C3d was analyzed. (C) Binding of the mutant and wild-type factor H proteins to C3b and C3d using an ELISA approach. nd, not determined.

Binding to Endothelial Cells

The binding of five aHUS-associated mutant proteins to human umbilical vein endothelial cells was assayed by confocal

microscopy (Figure 4). Cells that were cultivated in serum-free medium were incubated with each protein and stained with factor H antiserum. The binding of factor H is indicated by a specific green fluorescence signal. Again, all five mutant factor H proteins showed weak cell binding activity, in contrast to the wild-type factor H SCR8-SCR20 fragment, which showed prominent binding (Figure 4).

Localization of Peptides and Mutations in a Molecular Model

Molecular models for the SCR19 and SCR20 domains were generated by homology modeling with two crystal structure models showing the highest sequence identity with these domains (27). Even though the linker between SCR19 and SCR20 is short at three residues, the high variability in known inter-SCR orientations (29) meant that it was not possible to predict the relative arrangement of SCR19 and SCR20. Nonetheless, the mapping of the active regions from the peptide spot analysis showed that all three regions in SCR20 were located in proximity to each other at the C-terminal end of SCR20, which then is presumed to interact with all three of the heparin, C3b, and C3d ligands (Figure 5). The active region in SCR19 is located at the loop between β-strands β4 and β5 and along β-strand β5. If SCR19 and SCR20 are orientated to form a V-shaped structure, then it is possible that the four regions may form a single binding surface across SCR19 and SCR20.

The SCR19 and SCR20 models were also used to interpret position of the individual mutant residues. On the basis of the accessibility of the various sidechains, the mutations can be subdivided. Type A corresponds to surface-exposed residues (W1157R, W1183L, R1210C, and R1215G), whereas type B cor-

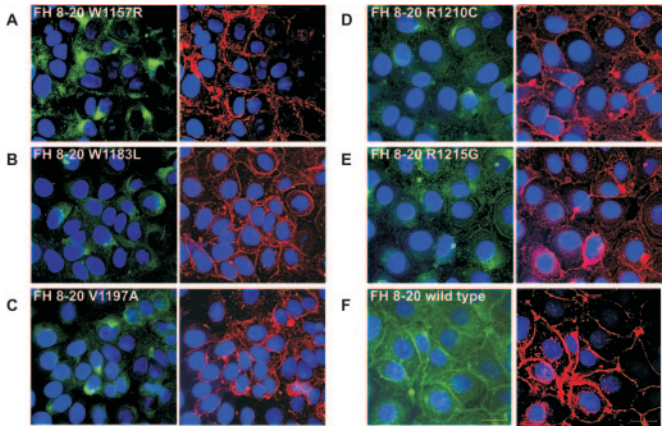

Figure 4. Binding of recombinant mutant and wild-type factor H proteins to human umbilical vein endothelial cells. Cells that were cultivated in serum-free medium were incubated with supernatant that contained either the indicated recombinant proteins W1157R (A), W1183L (B), V1197A (C), R1210C (D), and R1215G (E) or wild-type factor H SCR8-SCR20 (F). Cells were used directly for staining with factor H antiserum in combination with Alexa 488–labeled secondary antiserum (left). Cell surface structure was visualized with Alexa 633–labeled wheat germ agglutinin (right). Staining of the DNA with DAPI identifies the position of the nuclei. The bars in wild-type factor H show the length of 20 μm.

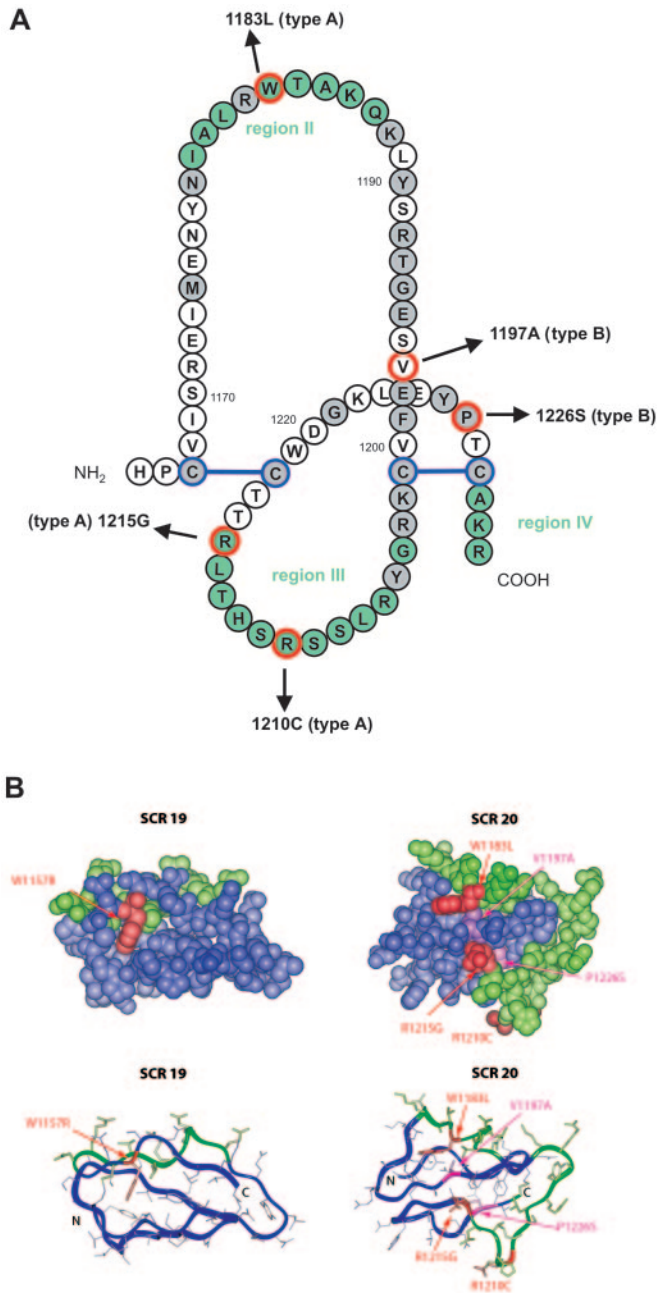

Figure 5. Position of the aHUS-associated mutations in SCR19 and SCR20 of factor H. (A) Sequence of SCR20 of factor H. The disulfide bridges that are formed with CysI-CysIII and CysII-CysIV connectivities are indicated. The residues within regions II, III, and IV involved in heparin binding are shown in green, and the aHUS-associated mutations of this study are highlighted in red. Amino acids that are conserved in all factor H proteins are shown in gray. (B) Homology models of SCR19 and SCR20 to indicate the location of regions I to IV and the six aHUS mutations. The type A W1183L, R1210C, and R1215G mutants (red) are positioned within the heparin binding site at the C-terminal tip of SCR20 and may be proximate to the type A mutant (red) in SCR19 (W1157R). The type B V1197A and P1226S mutants (light red) are buried within the SCR20 structure. The top panel shows a space-filling view of SCR19 and SCR20, whereas the bottom panel shows the same models in a ribbon view.

responds to residues whose side chains are buried within the SCR domain (V1197A and P1226S). It should be noted that SCR20 is atypical of SCR domains in that the conserved Trp residue is adjacent to the third Cys residue of SCR20 and not spaced by four residues (26), and this may affect the predicted sidechain accessibility of R1210C and R1215G. Although the accessibilities are tentative pending an experimental determination of the SCR19 and SCR20 structures, the alignment of V1197A with β-strand 3 in SCR20 and that of P1226S close to the buried C-terminal Cys residue add confidence to the buried accessibility assignments of these two sidechains. Both of the type B mutations show the largest effects when assayed by heparin affinity chromatography (Figure 2B), and likewise on the binding of C3b or C3d when followed by surface plasmon resonance or ELISA analyses (Figure 3).

Discussion

The largest group of aHUS-associated mutations known to date occur in the gene coding for the human immune regulator factor H, and >60% of these reported mutations are clustered within the C-terminal recognition region in SCR19 and SCR20 (1,2,26). Our study shows that at least six of these mutations are related directly with the pathogenesis of aHUS. Six mutant factor H proteins that were reported for patients with aHUS were expressed on a SCR8-SCR20 backbone, and all mutant proteins showed defective ligand binding and endothelial cell interaction (Table 1). By peptide spot analyses, we identified four linear binding regions within SCR19 and SCR20 that mediate binding to heparin, C3b, and C3d. Molecular modeling satisfactorily accounted for the identification of four peptide regions and the six aHUS mutations in terms of a possible common surface in SCR19 and SCR20 that interacts with heparin, C3b, and C3d. The similar functional consequences of the six aHUS-associated mutants of factor H suggest a common mechanism for the pathophysiology of this disease: Upon an inflammatory insult, the defective cell recognition of factor H reduces its regulatory activities at the surface of endothelial cells, which results in cell damage and exposure of the subendothelial matrix.

By peptide spot analysis, we identified four linear binding regions in the recognition domain of factor H that mediate ligand interaction, and the same or very similar four regions mediate heparin, C3b, and C3d binding. Region I is located in SCR19 and is 10 residues long (residues 1145 to 1154). SCR20 includes three binding regions, of which region II is 10 residues long (residues 1178 to 1187), region III is 14 residues long (residues 1202 to 1215), and region IV at the C-terminus is three residues long (residues 1229 to 1231; Figure 1B). The peptide spot analysis showed that C3b interaction is located mainly in SCR20 and to a great extent overlaps with the heparin binding region (Figure 1A). This result agrees with previous reports that showed competition of C3b binding of a factor H SCR15 to SCR20 fragment with heparin (29). The identification of these four regions accounts for discrepancies in three previous studies. Theoretical homology modeling based on 12 aHUS mutations had predicted that four basic residues (R1182, K1186, K1188, and R1192) at the interface between SCR19 and SCR20 may interact with heparin (30). Our work shows that the first two of these mutated residues are located in region II, and the other two follow immediately (Figure 1B). This is the first experimental evidence to support this prediction. A separate theoretical homology model suggested that K1108 in SCR19 and K1202, R1206, R1210, K1222, K1230, and R1231 in SCR20 interact with heparin (29). K1108 is far from regions I to IV, and its predicted role is not supported by the peptide assay, whereas the role of the remaining six residues located within region III (K1202, R1206, and R1210) or region IV (K1230 and R1231) or between them (K1222) is experimentally supported. A recombinant factor H SCR15-SCR20 fragment with five mutations based on three residues in region III (R1203E, R1206E, and R1210S) and two residues in region IV (K1230S and R1231A) did not bind to heparin (29). Hence, the peptide spot analyses are in agreement with a second set of heparin binding residues in SCR20. In a third study, the crystal structure of the complex between decameric heparin and SCR4 of VCP was reported (31). An overlay of the heparin binding residues of SCR4 in VCP with a model of SCR20 in human factor H shows that the seven VCP heparin binding residues correspond to five

Table 1. Summary of binding characteristics of the six mutant factor H proteins to three ligands (heparin, C3b, and C3d) and to endothelial cells

Factor H Mutant	Ligand			Endothelial cells	Type
	Heparin	C3b	C3d		
FH 8-20/W1157R	++	(+)	(+)	++	A
FH 8-20/W1183L	+	+	+	+	A
FH 8-20/W1197A	(+)	+	+	(+)	B
FH 8-20/W1210C	+	+	+	+	A
FH 8-20/W1215G	+	+	+	+	A
FH 8-20/W1226S	(+)	(+)	(+)	nd	B
FH 8-20 wt	+++	+++	+++	+++	

Residue W1157R is located in SCR19, and the remaining residues are located in SCR20. nd, not determined. Type A refers to residues that based on a structural model are surface exposed and accessible for ligand interaction. Type B residues are buried and are likely to be essential to maintain the overall architecture and folding of the short consensus repeat domain.

factor H residues in region III (K1202, R1203, R1206, R1210, and R1215) and two Factor H residues in region IV (K1230 and R1231). The peptide spot analyses are consistent with the comparison made with the heparin-VCP crystal structure.

The involvement of the aHUS-associated mutations with the functional properties of factor H was supported by the six mutants of factor H. Clinically, all six mutant proteins are expressed and secreted (2). The mutants showed perturbation of heparin binding (weakest to strongest: W1157R, R1210C, W1183L, V1197A, and P1226S; Figure 2B) and a strong reduction in C3b binding (W1183L, V1197A, R1215G, R1210C, W1157R, and P1226S; weakest to strongest; Figure 3C) and C3d (V1197A, R1210C, W1183L, R1215G, W1157R, and P1226S; again weakest to strongest; Figure 3C). It is interesting that even though factor H molecules with mutations W1183L, V1197A, and R1210C isolated from patient sera had C3b binding sites in the amino terminal ends of factor H, these mutant proteins also showed severe reduction in binding to immobilized C3b (32). These data demonstrate that the C-terminal SCR domains of factor H form a major binding site for C3b. Whereas heparin and C3b binding is concentrated in SCR20, the binding of C3d is mapped to SCR19 and SCR20 (Figure 2) and is overlapping with the heparin binding sites. Although no binding of factor H fragment SCR15 to SCR19 to immobilized C3d was demonstrated (29), SCR19 is supposed to be involved in C3d binding. This is underlined by data showing that mutation W1157R in SCR19 has a significant effect on C3b and C3d binding, but this effect is much reduced for heparin binding (Figures 2B and 4A). Both density and distribution of surface-deposited C3b affect the affinity of factor H to a cell surface. Thus, it seems that the combination of defective heparin and C3b binding contributes to a defective local complement control at the cell surface.

The homology modeling of SCR19 and SCR20 provided further insight into recognition function of the four identified binding regions and the six mutated residues. All four linear binding regions that were identified by peptide spot analyses are surface exposed and accessible to ligands. The SCR20 structure shows that regions II, III, and IV are spatially brought together at the C-terminal tip of SCR20 (Figure 5B). This is consistent with the proposed analogous binding of heparin to the C-terminal tip of VCP in the heparin-VCP crystal structure (31). In SCR19, region I can be brought into proximity with regions II, III, and IV to form a single contiguous surface. This indicates a role of both SCR19 and SCR20 in binding to its three ligands and is consistent with an arrangement of the SCR19 and SCR20 in the four best-fit models for intact factor H (33). The defective binding of the six mutant factor H proteins is most easily explained by different accessibility of individual residues within the SCR domain. Type A residues are accessible, and type B residues are buried (<20% solvent accessibility). In terms of mutations, it is of interest that the two type B mutations V1197A and P1226S showed the largest effect on heparin binding; in addition, P1226S had the largest effect on C3b and C3d binding. The change from a bulky aliphatic sidechain to a small one (V1197A) or that from a hydrophobic amino acid to a small hydrophilic amino acid (P1226S) has a disproportionate effect on factor H activities compared with the type A mutations. It is of interest to see whether functionally active SCR domains are generally sensitive to alterations in their buried residues that can distort the folding of the SCR domain.

So far, 36 of the reported aHUS-associated factor H gene mutations are located in SCR19 and SCR20. By this study, mutations and amino acid substitutions in the two SCR domains disturb the overall recognition function of this region. In consequence mutant factor H cannot bind properly to the endothelial cells and thus cannot inhibit complement activation. This is particularly true during inflammation, when the alternative complement pathway is highly induced and the endothelium needs maximal protection. In this scenario, presumably thrombi that cannot be cleared properly are formed in the kidney, causing kidney damage over the time.

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