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Gain-of-function mutations in complement factor B are associated with atypical hemolytic uremic syndrome.

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ABSTRACT

Hemolytic Uremic Syndrome (HUS) is an important cause of acute renal failure in children. Mutations in one or more genes encoding complement regulatory proteins have been reported in approximately one third of non-diarrheal, atypical HUS patients, suggesting a defect in the protection of cell surfaces against complement activation in susceptible individuals. Here we identified a subgroup of aHUS patients showing persistent activation of the complement alternative pathway and found within this subgroup two families with mutations in the gene encoding factor B (*BF*), a zymogen that carries the catalytic site of the complement alternative pathway convertase (C3bBb). Functional analyses demonstrated that F286L and K323E aHUS-associated *BF* mutations are gain-of-function mutations that result in enhanced formation of the C3bBb convertase or increased resistance to inactivation by complement regulators. These data expand our understanding of the genetic factors conferring predisposition to aHUS, demonstrate the critical role of the alternative complement pathway in the pathogenesis of aHUS and provide support for the use of complement inhibition therapies to prevent or reduce tissue damage caused by dysregulated complement activation.

INTRODUCTION

Hemolytic uremic syndrome (HUS, MIM 235400) is characterized by thrombocytopenia, Coomb's test negative microangiopathic haemolytic anemia and acute renal failure (1). The typical form of HUS follows a diarrhoeal prodrome and is frequently associated with 0157:H7 *E.coli* infections (2). However, five to ten percent of HUS patients lack a particular relationship with infection and have the poorest long-term prognosis (1). This atypical form of disease (aHUS) usually occurs in adults and in very young children and is associated with mutations or polymorphisms in the genes encoding the complement regulators factor H (*CFH*) (3-8), membrane cofactor protein (*MCP*) (9, 10), and factor I (*IF*) (11, 12).

The molecular mechanisms underlying aHUS are only now beginning to be understood. In contrast to patients with classical complement deficiencies, aHUS patients present relatively normal complement levels and activities in plasma (5). Crucially, functional analysis of several *CFH*, *MCP* and *IF* aHUS-associated mutations has demonstrated that they affect primarily the control of complement activation on cellular surfaces, while complement regulation in plasma is not greatly affected (4, 7, 10, 13). These findings have led to the hypothesis that aHUS results from a defective protection of host cells within the context of a "normal" complement function in fluid phase (7, 14). Under this perspective, a situation that triggers complement activation in the microvasculature will not be properly controlled, and the amplification of the initial damage will result in tissue destruction.

Despite these advances in our understanding of the molecular basis of aHUS, no genetic defect has yet been found in two-thirds of aHUS patients, and incomplete penetrance of the disease in individuals carrying factor H, MCP or factor I mutations is relatively frequent, suggesting the existence of additional genetic factors that predispose to aHUS.

To provide further insights into the genetic factors predisposing to aHUS we have examined the hypothesis that genetic variants of complement activators, resulting in excessive activation of the complement system, are also aHUS risk factors. Like mutations in the complement regulators, genetic variations in complement activators have the potential to disrupt the balance between activation and regulation, dysregulating the complement system and predisposing to pathology when the complement system undergoes activation.

Here we describe mutations in the gene encoding the complement activator factor B (*BF*) associated with aHUS. We report their functional characterization and demonstrate that they are gain-of-function mutations. These data offer a complementary view to the previously found loss-of-function mutations in complement regulators and definitively establish the critical role of the complement alternative pathway in the pathogenesis of HUS. In addition, we analyze the issue of incomplete penetrance of disease in *BF* mutation carriers and show that, in a large multiple-affected Spanish HUS pedigree, the concurrence of an enhanced activation of the alternative pathway of complement with an impaired protection of host surfaces likely results in unrestricted activation of the alternative pathway at the cellular surfaces and triggers the development of aHUS.

PATIENTS, MATERIALS AND METHODS

Patients

This study includes a total of 74 aHUS patients selected on the basis of a clinical history of HUS with non-diarrhoea-associated origin. Of special relevance for this study are patients H55P, H55, H85 and H112, all members of a large multiple-affected Spanish pedigree, referred to as family PER, and H21, the propositus in family BRA. H55P is a male who presented aged 48 with HUS. Prolonged treatment with plasmapheresis failed to recover his renal function. He had no recurrences and has been on hemodialysis since then. H55, the daughter of H55P, presented in 1994, aged 23, two years after her first pregnancy, with the diagnosis of HUS. She was treated with plasma infusions, plasmapheresis and haemodialysis and showed improvement of renal function, but after four recurrences, she completely lost renal function in 2005 and is currently on peritoneal dialysis. H85, the nephew of H55P, presented with HUS in 1978, aged 2, following an upper respiratory tract infection. He improved after haemodialysis treatment and multiple blood transfusions and currently shows a moderate renal insufficiency. H112, the daughter of H85, presented aged 4 months with HUS with mild neurological signs in 2006, following an upper respiratory tract infection. She improved with plasma infusions which allowed her to stop haemodialysis. H21 is a boy who presented with HUS at 4 months in 2000 following a *M. morganii* respiratory tract infection. An initial recovery of renal function was followed by several recurrences that ended in complete lost of renal function two years after the first episode. Several neurological crises and persistent and severe hypertension made necessary bilateral nephrectomy at the age of 4.

All protocols included in these studies have been approved by national and/or local institutional review boards, and all subjects gave their informed consent.

Complement analyses

C3, C4, factor H and factor I levels were measured in serum or plasma samples as previously described (5, 15, 16). Factor B plasma levels were determined by a sandwich ELISA method using a polyclonal goat anti-human factor B antibody (NSC) as the capture antibody and a monoclonal mouse anti-human factor B as the detection antibody. To assess complement function and regulation we used the standard hemolytic assays CH50 and AP50 and a factor H-dependent hemolytic assay (13). Expression levels of the membrane regulators MCP and DAF in PBLs were determined by flow cytometry as previously described (17).

Genotyping

Patients and their relatives were screened for mutations and polymorphisms in *CFH, MCP, IF* and *BF* genes. DNA from these individuals was extracted from PBLs or from buccal mucosa cells collected with cheek brushes (MasterAmp buccal swab brush, Epicentre Technologies) using standard procedures. Each exon of the *CFH, MCP* and *IF* genes was amplified from genomic DNA of patients using specific primers derived from the 5' and 3' intronic sequences as previously described (5, 10, 11). All *BF* exons were amplified from genomic DNA in three amplicons of 1800, 2500 and 2000 bp, respectively using three pairs of primers: 5`-CCA AGC CAG GAC ACA CCA TC-3` and 5`-TCT TCC GCT TCT GTT GTT CC-3`, 5`-TCA TGT ACG ACA CCC TCA A-3` and 5`-AGC TCT CGT GTC CCT TTG AA-3`, 5`-ATG AAA GCC AGT CTC TGA-3` and 5`-CCTGTCCAGCAGGAAACC-3`. Several primers were used for sequencing the *BF* amplicons: 5`-GTT TCT CAG TGA CAT GGT CTC C-3`, 5`-TAT AGT GTT ACA AGT GGA CTT AAG G-3`, 5`-CTC AAC TTG CTC ACC CTG C-3`, 5`- CAG ACT ATC AGG TGA GAG CGT C-3`, 5`-ACTAGCCACATGCATTGAGCT-3`, 5′- CTGATTCCTTTAGGTCAGCTAAG-3`

and also for the generation of the amplicons. Automatic sequencing was performed in an ABI 3730 sequencer using a dye terminator cycle sequencing kit (Applied Biosystems, NJ). Genotyping of HUS-associated *CFH* and *MCP* SNPs was performed by resequencing or by allelic discrimination using TaqMan probes (Applied Biosystems; Foster City, CA).

Factor B purification

Factor B from patients and controls was purified in one chromatographic step as follows. Two to five ml of plasma-EDTA were directly applied to a Sepharose column coated with the JC1 mouse monoclonal anti-human factor B antibody and previously equilibrated with Tris-HCl (20 mM pH 7.4) containing NaCl (50 mM). After extensive washes with equilibration, acetate (0.1 M, p H4, containing NaCl 500 mM) and borate (0.1 M, pH 8, containing NaCl 500 mM) buffers, the proteins bound to the column were eluted with 1 ml of glycine 100 mM pH 2.5 followed by equilibration buffer. 20 µl of Tris-HCl 2M pH 8 were added to the 500 µl eluted fractions to equilibrate glycine pH. Those fractions containing factor B were pooled and dialysed against Hepes 10 mM pH 7.4 containing NaCl 50 mM.

PAGE and Immunoblotting

PAGE in the presence of SDS was performed using the buffer system described by Laemmli (1970). Samples were reduced at 100ºC for 5 min in the presence of 700 µM mercaptoethanol (Sigma). Proteins were transferred onto nitrocellulose membranes and factor B revealed by using rabbit anti-factor B polyclonal antibody.

SPR analysis of convertase formation

Interactions of factor B with C3b were analysed on a BIAcore 3000 (BIAcore AB, Uppsala, Sweden) at 25°C. C3b was deposited on the surface of a CM5 chip as described previously (18). In brief, human C3. factor B and factor D (Complement Technology Inc, Tyler, Tx) were flowed to seed the chip surface with small amounts of C3bBb; C3 was then flowed to densely coat the chip with C3b coupled via the thioester to hydroxy groups on the carboxy-methyl dextran surface. Covalent coupling of C3b to the surface therefore occurred in the 'native' conformation. The surface was stabilised by a short injection of 50mM diethylamine, pH 11. Factor B preparations to be tested were purified by affinity chromatography from plasma of HUS patients (H21 and H55P) or a control subject and quantified by ELISA as described above. These factor B proteins (37µg/ml) were then flowed over the C3b surface at 20µl/min in HBS/Mg (10mM Hepes pH7.4, 50mM NaCl, 1mM $MgCl_2$) with or without 2µg/ml factor D. Interactions were monitored and analysed using BIAcore BIAevaluation software, data from a blank reference cell were subtracted to account for any changes in refractive index of solutions flowing across the chip surface.

Plate assay for AP convertase decay

Assays were carried out essentially as described previously (18, 19). ELISA plates (MediSorp, Nunc, Roskilde, Denmark) were coated for 1 hour at 37°C with 3µg/ml C3b. C3b was prepared as described previously (18); coating of the plate with either C3b monomer or dimer (potential to form C5 convertase) did not alter assay results, hence all assays were performed with C3b monomer. After coating, plates were washed with VBS/Ni^{2+} (2.5mM sodium barbitone pH 7.4, 71mM NaCl, 1mM Ni^{2+}, 0.1% Tween 20) and blocked with VBS/Ni^{2+}/1% BSA. Plates were washed in VBS/Ni^{2+} and

convertase was formed by incubating 0.5µg/ml factor B and 50ng/ml factor D in wells for 30 minutes at 37°C. Plates were washed and wells were then incubated with either buffer or known concentrations of a decay-accelerator, soluble recombinant DAF (sDAF; gift of Dr Susan M Lea, Biochemistry, Oxford University, UK) or soluble recombinant CR1 (sCR1, gift of T Cell Science, Needham, MA) for 20 minutes at 25°C. Plates were washed and remaining factor B was detected using polyclonal sheep anti-factor B (The Binding Site, Birmingham, UK) followed by HRPO-conjugated anti-sheep Ig. Colour was developed using OPD (Dako, Ely, UK). Background values were obtained by omitting factor B/factor D from some wells. Absorbance was measured at 490nm and % convertase remaining was calculated as follows: % convertase remaining =100x(A490inh – A490Bgnd)/(A490buffer-A490bgnd). In some instances factor D was omitted from the incubations in order to assess formation of proenzyme and incubation with CD55 or sCR1 was omitted.

RESULTS AND DISCUSSION

Complement profiles in aHUS patients identifies subgroup with evidence of alternative pathway activation

Our registry of aHUS patients includes 74 individuals who have been extensively studied with regard to complement profiles and genomic analyses of the complement regulatory genes *CFH*, *MCP*, *IF* and *DAF*. In concordance with other aHUS cohorts we found mutations in *CFH*, *MCP* or *IF* in approximately 30% of the aHUS patients (Figure 1a). No mutations were identified in the *DAF* gene.

Most patients in our HUS cohort present normal C3 and C4 levels (Figure 1b) and haemolytic complement activities (not shown). This is also in agreement with previous reports describing that, in general, HUS patients are not hypocomplementemic (5). We noticed, however, that some of the patients without mutations in the complement regulatory genes including patients H21, H49, H55P, H65 and H71, present evidence of alternative pathway activation. They show very low levels of C3 and normal or elevated levels of C4 (Figure 1b) and analysis of their plasma by western blot showed the presence of the activation product Ba (Figure 1c). Interestingly, H55P, belongs to a large multiple-affected aHUS Spanish kindred in which disease segregated with the MHC region in chromosome 6q24, a region that includes the complement factor B gene (*BF*) (20).

Identification of *BF* mutations in patients with alternative pathway activation.

The above observations pointed to *BF* as a new candidate gene predisposing to HUS and prompted us to search for mutations in *BF* in the group of patients showing evidence of alternative pathway activation. Two patients in this subgroup (H21;

pedigree BRA and H55P; pedigree PER) were found to carry missense mutations in heterozygosis in the *BF* gene. Genomic sequencing of *BF* in H55P revealed a heterozygous mutation (c.987C>G; F286L) in exon 6, encoding the von Willebrand type A domain of factor B. The F286L missense mutation segregates with the low levels of C3 that are present in different members of family PER and is inherited by all HUS-affected members of this pedigree (Table 1, Figure 2a). Genomic sequencing of *BF* in H21 revealed another heterozygous mutation (c.1096A>G; K323E) in exon 7, also encoding a residue within the von Willebrand type A domain of factor B. K323E is a *de novo* mutation that is not present in H21 parents (Figure 2b). Parental sample integrity in family BRA was confirmed by genotyping several markers in the RCA gene cluster in chromosome 1q32. Neither F286L nor K323E mutations were present in a control population including more than 100 normal individuals.

Structure-function analyses identify *BF* gain-of-function mutations in aHUS.

Factor B is a zymogen that carries the catalytic site of the complement alternative pathway convertase C3bBb. Upon interaction with C3b, factor B is cleaved by factor D into two fragments, Ba and Bb. Ba is released while Bb remains bound to C3b forming the C3bBb alternative pathway convertase, an active serine protease enzyme that cleaves additional C3 into C3b. The Bb fragment of factor B consists essentially of two protein domains: a von Willebrand factor type A domain and a serine protease (SP) domain (21). From structural studies, the factor B type A domain is a globular structure composed of several parallel β sheets surrounded by seven αhelices (22, 23). Site directed mutagenesis of factor B residues at the C3b-Bb interface in the factor B type A domain have identified a number of residues that are critical for the interaction between C3b and factor B and that influence the normal dissociation of C3b from Bb, whether it

is spontaneous or promoted by the complement regulatory proteins CFH, DAF or complement receptor 1 (CR1). In particular, the mutation D279G, in close proximity to the Mg2+ binding site within the factor B type A domain, caused resistance to decay acceleration mediated by all three regulators and also increased C3b binding affinity and C3bBb stability (24). Both of the *BF* mutations F286L and K323E identified here in the aHUS patients alter residues at the C3b-Bb interface in the type A domain of factor B in close proximity to the D279 (Figure 3). This finding led us to suspect that they have the potential for altering the stability of the C3bBb convertase and the activity of the alternative pathway. The mutation K323E changes a fully exposed residue with a positive charge to one with a negative charge. The mutation F286L influences Y319, another fully exposed residue, modifying the edge-to-face stacking interaction between F286 and Y319. Substituting F286 with leucine likely decreases mobility restrictions to Y319 at the C3b-Bb interface (Figure 3).

To functionally characterize the effects of the K323E and F286L *BF* mutations we purified the factor B from the plasma of appropriate carriers and from normal controls and tested the purified factor B proteins for their capacity to form the C3 convertase and influence its decay, either spontaneous or promoted by complement regulatory proteins. Each protein was purified to homogeneity (Figure 4a). We have previously monitored formation and decay of the C3 convertase in real-time using surface plasmon resonance (SPR) (18). To assess the effects of mutations, factor B from controls or patients was flowed across a surface coated with C3b via its thioester group, with or without the factor B cleaving enzyme factor D (2µg/ml). Formation of C3bB (no factor D) was similar for control factor B and for K323E factor B isolated from patient H21; in marked contrast, F286L factor B isolated from patient H55P demonstrated a much more rapid and efficient formation of the C3bB complex (Figure 4b). In the presence of factor

D, the expected enhanced formation of the C3bBb complex was seen for control factor B and K323E factor B, while the kinetics of complex formation for F286L factor B were not further enhanced from that obtained in the absence of factor D (Figure 4c).

The above data show a clear functional consequence of the F286L mutation with enhanced formation of C3bB and no further enhancement of complex formation in the presence of factor D. However, the K323E factor B resembled normal factor B in the assay. To corroborate these findings a different assay system was used to investigate formation of the alternative C3-convertase by control and mutant factor B. C3b was immobilised on ELISA plates via the thioester and wells incubated with equal amounts of control and mutant factor B. Formation of C3bB was much enhanced with F286L factor B with two to three-fold more C3bB formed compared to control and K233E factor B, supporting the findings from SPR analysis (Fig 5a). When the C3bBb enzyme was formed in the presence of factor D the difference was much less apparent, with increased levels of complex formed by control factor B and K323E factor B (Fig 5b), supporting the SPR data. To further explore the effects of the K323E and F286L *BF* mutations and to rule out the possibility that the presence of normal factor B in the patient samples, a consequence of heterozygosity, have masked subtle alterations caused by the K323E mutation, we then chose to analyse decay acceleration by DAF and sCR1. C3bBb enzymes formed on the plates were incubated with different amounts of DAF or sCR1 and the loss of Bb from the complex measured (Fig 5c, 5d). C3bBb formed from F286L factor B was less sensitive to decay by either sCR1 or CD55, demonstrating that the C3bBb complex made with this mutant is not only more efficiently generated but is also more resistant to decay by complement regulators. C3bBb complex formed by K323E factor B was sensitive to decay by sCR1 but reproducibly demonstrated a reduced sensitivity to decay by DAF, suggesting that C3bBb complex made with this

mutant will resist natural decay. It is important to emphasize that the functional differences described were observed despite the purified proteins from the patients comprise a mixture of normal and mutant factor B. Taken together, the data demonstrate that each of the mutant factor B proteins will produce increased alternative pathway activation. Formation of the C3bB complex by mutant F286L factor B is much enhanced, and decay acceleration by DAF and CR1 much reduced, producing more active enzyme *in vivo*. The functional consequences of the K323E mutation are more subtle and may be restricted to reduced susceptibility to decay, likely sufficient for increased enzyme activity *in vivo*. The mutations likely introduce conformational changes in the factor B type A domain that decrease the sensitivity of the C3bBb complex to decay by DAF and perhaps other complement regulators. It is not yet clear whether reduced decay is the result of an enhanced binding of Bb to C3b or decreased affinity for DAF. Nevertheless, these data demonstrate, for the first time, the existence of gain-of-function mutations in the *BF* gene and their association with HUS, illustrating the important contribution of the alternative pathway C3 convertase to the pathogenesis of HUS.

Polymorphisms in the *BF* gene have recently been associated with age-related macular degeneration (AMD) (25), a disease that has previously been shown to strongly associate with polymorphisms in the *CFH* gene and where the macular pathology is driven by complement activation (26-29). Two *BF* polymorphisms were identified that were protective for AMD, one of which had previously been shown to reduce the haemolytic activity of factor B and the other, in the signal peptide, was suggested to modulate secretion of factor B. The observed protection in each case was ascribed to a reduced activity of the complement alternative pathway. This report provides timely

support for our suggestion that genetic changes that alter the function of factor B may influence pathology driven by alternative pathway activation.

Disease penetrance in carriers of *BF* mutations is modulated by other complement-associated risk alleles.

Within the HUS pedigree reported here, we have identified a total of 11 individuals carrying the F286L *BF* mutations, but only 7 of them have developed HUS thus far and the majority of the healthy carriers are old enough to assume that they are not at risk (Figure 2 and Table 2). Incomplete penetrance of the disease has also been reported for the aHUS-associated mutations in the *CFH*, *MCP* and *IF* genes (7, 9-11). Previously we, and others, have reported that two relatively frequent *CFH* and *MCP* SNPs haplotype blocks are strongly associated with aHUS (17, 30). We have proposed that concurrence of different susceptibility alleles greatly influences predisposition to aHUS and provides an explanation for the incomplete penetrance of aHUS in carriers of mutations in the complement regulator genes (17, 31). To investigate whether penetrance of the disease in *BF* mutations carriers is influenced by other aHUS genetic risk factors, we genotyped the *CFH* and *MCP* aHUS-associated SNP haplotype blocks and found that among carriers of the F286L mutation, all the HUS patients presented the *MCPggaac* risk allele, whereas among the healthy carriers (I-2, II-5, III-12 and IV-1) only the youngest, and probably still at risk, individual IV-1 harboured the HUS-associated *MCPggaac* risk allele. Of note, the single affected individual with the K323E mutation also presented the *MCPggaac* risk allele. These data strongly suggest that the HUS phenotype associated with the *BF* gain-of-function mutations is modulated by common complement regulator gene variants, like the *MCPggaac* allele, associated with increased risk of developing HUS (17).

Complement dysregulation is the unifying event in aHUS patients.

The efficiency of the complement system as an innate defence mechanism against microbial infections depends on a fine control that avoids the wasteful consumption of its components and restricts its activation to the surface of microorganisms, thus preventing non-specific damage to host tissues. Genetic and functional analyses of the complement regulatory proteins factor H, MCP and factor I have shown that this critical control of complement activation may be impaired in aHUS patients (4, 7, 10). The identification of gain-of-function mutations in *BF* associated with aHUS reinforces this view and illustrates that dysregulation of the complement system may also result from an abnormally increased activity of the alternative complement pathway activators. These findings identify other complement activators in the same pathway as *BF*, like *C3* and *CFP* (encoding properdin), as potential candidate genes contributing to HUS susceptibility.

In conclusion, these data demonstrate the critical role of the alternative complement pathway in the pathogenesis of aHUS and expand our understanding of the genetic factors conferring predisposition to aHUS. Furthermore, they support the hypothesis that susceptibility to atypical HUS involves the concurrence of multiple factors (genetic and environmental) contributing to the dysregulation of the alternative complement pathway. This hypothesis provides an explanation for the incomplete penetrance of the disease in carriers of aHUS-associated mutations and may also be relevant in explaining the severe or fatal outcome of a small percentage of individuals with the more common diarrhoea-associated typical HUS. The findings provide further support for the use of complement inhibition therapies in aHUS and perhaps also in severe forms of typical HUS to prevent or reduce tissue damage caused by dysregulated complement activation.

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FOOTNOTES FOR THE FIGURES

Figure 1.

Activation of the alternative pathway in a subgroup of aHUS patients without mutations in the *CFH*, *MCP* and *IF* genes.

a) Distribution of *CFH*, *MCP* and *IF* mutations in the aHUS Spanish cohort.

b) C3 and C4 plasma levels in aHUS patients. C3 levels (0 to 200 mg/dL) are depicted in the x-axis with a vertical line indicating the lower C3 limit (77 mg/dL) for the normal range of variation of the C3 levels in the Spanish population. C4 levels (0 to 60 mg/dL) are depicted in the y-axis with a horizontal line indicating the lower C4 limit (14 mg/dL) for the normal range of variation of the C4 levels in the Spanish population. The area shadowed in grey includes patients who present low or very low C3, but normal C4 levels indicating activation through the alternative pathway. Patients with detected mutations in *CFH, MCP* and *IF* are shown with filled circles and those without mutations with empty circles. C3 and C4 values are mean values in at least three bleedings at different times.

c) Western blot of factor B with a goat polyclonal antibody of EDTA plasmas from H65, H21 and H55P HUS patients and EDTA-plasmas from 4 controls (N1 to N4). The presence of the activation Ba fragment of factor B is indicated.

Figure 2.

Mutations in the *BF* gene in two aHUS pedigrees.

Pedigrees of families PER (a) and BRA (b) are described. Individuals are identified by numbers within each generation (in Roman numbers). Affected individuals are indicated with solid symbols. Deceased individuals are crossed. Carriers of *BF* mutations are

indicated by an asterisk. For each *BF* mutation, the chromatogram corresponding to the DNA sequence surrounding the mutated nucleotide in *BF* is shown for the appropriate HUS patient and for a control sample. The corresponding amino acid sequences for the wild type and the mutated alleles are shown. The amino acid numbering is referred to the translation start site (Met +1) and the nucleotide nomenclature is referred to the transcription start site described in (32).

Figure 3.

Structural implications for the HUS-associated *BF* mutations.

Diagram of the von Willebrand type A domain of factor B. The positions of the residues Phe286 and Tyr323 that are mutated in the HUS patients are indicated. The position of the Mg2+ ion and that of the Asp279 and Tyr319 are also indicated. Notice the edge-to-face stacking of F286 and Tyr319 residues. Numbering of residues is referred to the initial methionine and therefore includes the sequence of the N-terminal signal peptide. Residue Asp279 was described previously as Asp254 by (19).

Figure 4.

SPR analysis of factor B purified from controls and carriers of HUS-associated BF mutations.

a) Coomassie and western blot illustrating the purification of factor B from patient and control EDTA plasmas and the absence of activation factor B fragments in all preparations.

b) Formation of C3bB complexes. Control factor B ($fB_{WT,}$ dotted line) and factor B purified from H21 (fB_{K323E} grey line) formed similar levels of the proenzyme whereas factor B purified from H55 (fB_{F286L} solid line) formed C3bB complexes much more rapidly and to a much higher level.

c) Formation of C3bBb complexes. fB_{WT} (dotted line) and fB_{K323E} (grey line) formed similar levels of active enzyme and those were increased compared to the levels of proenzyme (4b). fB_{F286L} (solid line) formed high and similar levels of complexes with C3b in the absence or presence of factor D.

Figure 5.

Convertase formation and decay monitored by plate assay.

a) Microtitre plates were coated with C3b. Wells were subsequently incubated with differing amounts of factor B purified from control (WT) or patients H55 (F286L) and H21 (K323E). Bound factor B was detected as described in Methods and expressed relative to C3bB formation by factor B F286L as 100%.

b) C3b-coated wells were incubated with factor B (0.5µg/ml) and factor D (50ng/ml), plates were incubated in buffer for 20 minutes and bound Bb was detected as described in Methods. Results are expressed relative to C3bBb formation by factor B F286L as 100%

c) and d) Decay by sDAF or sCR1. Wells were incubated as in (b) and different amounts of sDAF (c) or sCR1 (d) were added. C3bBb complexes were then subjected to 20 minutes decay by soluble regulator. The experiments shown are representative of three different experiments. Each point was measured in quadruplicate and error bars represent standard errors of these replicates.

TABLE 1. Complement profiles in members of families PER and BRA.

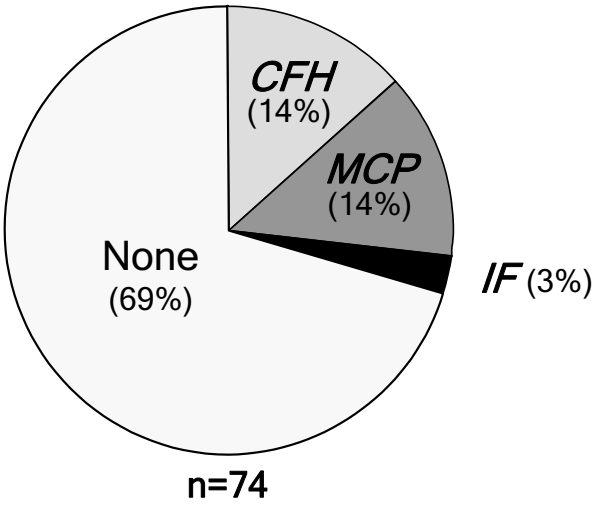
		Status	C3[1,2] (77-210mg/dL)	C4[2] (14-47mg/dL)	Factor H[3] (12-56mg/dL)	Factor B[3] (7.5-28mg/dL)	Factor I[3] (75-115 %)	MCP[4] (91-109%)
PER	I-2	Healthy	68	24	38	15	n.a.	n.a.
	II-5	Healthy	75	25	37	16	n.a.	n.a.
	II-7 (H55P)	Affected	46	29	36	17	118	100
	II-10	Healthy	122	28	35	22	n.a.	121
	II-11	Healthy	123	28	38	26	n.a.	n.a.
	III-7 (H55)	Affected	91	36	32	16	104	110
	III-12	Healthy	67	16	42	13	n.a.	n.a.
	III-13 (H 85)	Affected	60	19	23	12	98	118
	III-14	Healthy	117	21	33	12	n.a.	98
	IV-1	Healthy	134	17	27	15	n.a.	n.a.
	IV-2 (H 112)	Affected	65	25	24	22	95	66
BRA	I-1	Healthy	102	28	38	11	84	n.a.
	I-2	Healthy	89	28	28	12	85	n.a.
	II-1 (H21)	Affected	23	32	26	21	103	84

1) Normal range of variation in controls for each variable is shown between brackets.
2) C3 and C4 were determined by nephelometry.
3) Factor H, Factor B and Factor I plasma levels were determined by ELISA. Factor I levels are referred to a reference serum; each value corresponds to the mean of three independent determinations.
4) Levels of MCP in PBLs (MFI, mean fluorescence intensity) were determined by flow cytometry and referred to a series of control samples drawn and analysed the same day. The average MFI value for the control samples was set to 100%. Range of variation for all control samples (n=17) was within 10% of the average value (91 to 109%).
5) Pedigree codes as in Figure 2. *BF* mutation carriers are labelled with black triangles. HUS patients are identified by their code in parenthesis.
6) n.a.: not available.

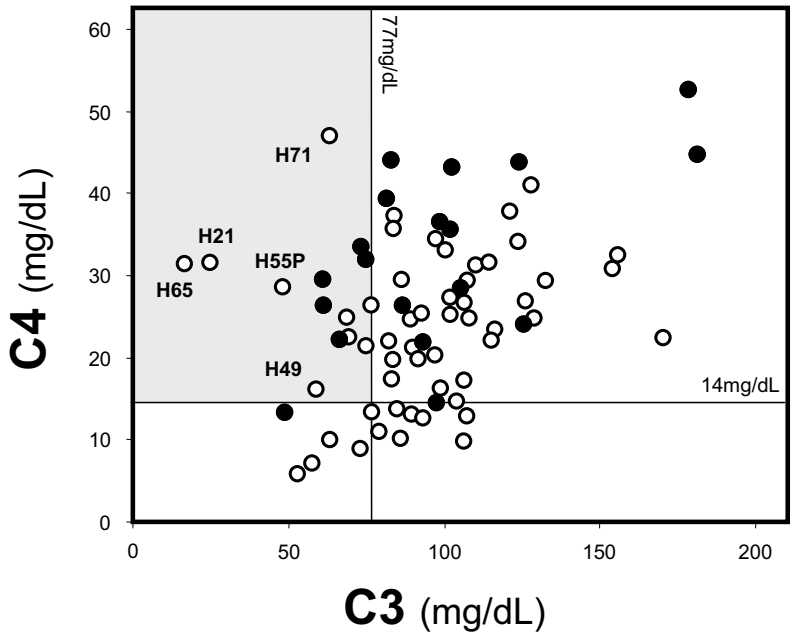
TABLE 2. aHUS-associated *CFH* and *MCP* SNPs haplotype blocks and disease status in carriers of *BF* mutations.

		CFH -257C>T	*CFH c.994C>A*	*CFH C.2089A>G*	*CFH C.2881G>T*	*MCP -547A>G*	*MCP -261A>G*	*MCP IVS9-78G>A*	*MCP IVS12+638G>A*	*MCP c.2232T>C*	**Disease status (age at onset; current age)**
PER (F286L)	**I-2**	T T	C C	A A	G G	A A	A A	G G	G G	T T	Healthy (88)
	II-5	T C	C A	A A	G G	A A	A A	G G	G G	T T	Healthy (60)
	II-7 (HUS55P)	T C	C A	A A	G G	A G	A G	G A	G A	T C	Affected (48y; 59y)
	II-9 (HUS85P)	T C	C A	A A	G G	A G	A G	G A	G A	T C	Affected (35y; deceased)
	III-7 (HUS55)	C T	A C	A G	G T	G G	G G	A A	A A	C C	Affected (23y; 34y)
	III-12	T T	C C	A G	G T	A A	A A	G G	G G	T T	Healthy (20)
	III-13 (HUS85)	C C	C A	A A	G G	A G	A G	G A	G A	C C	Affected (2y; 29y)
	IV-1	T C	C C	G A	T G	A G	A G	G A	G A	T C	Healthy (15y)
	IV-2 (HUS112)	T C	C A	G A	T G	G G	A G	G A	G A	T C	Affected (4mo; 9mo)
BRA (K323E)	**II-1 (HUS21)**	T C	C A	G A	T G	A G	A G	G A	G A	C T	Affected (4mo; 6y)

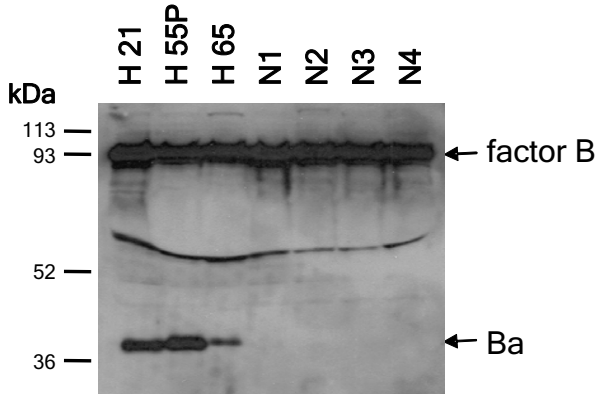
a)


CFH
(14%)
MCP
(14%)
None
(69%)
IF (3%)
n=74

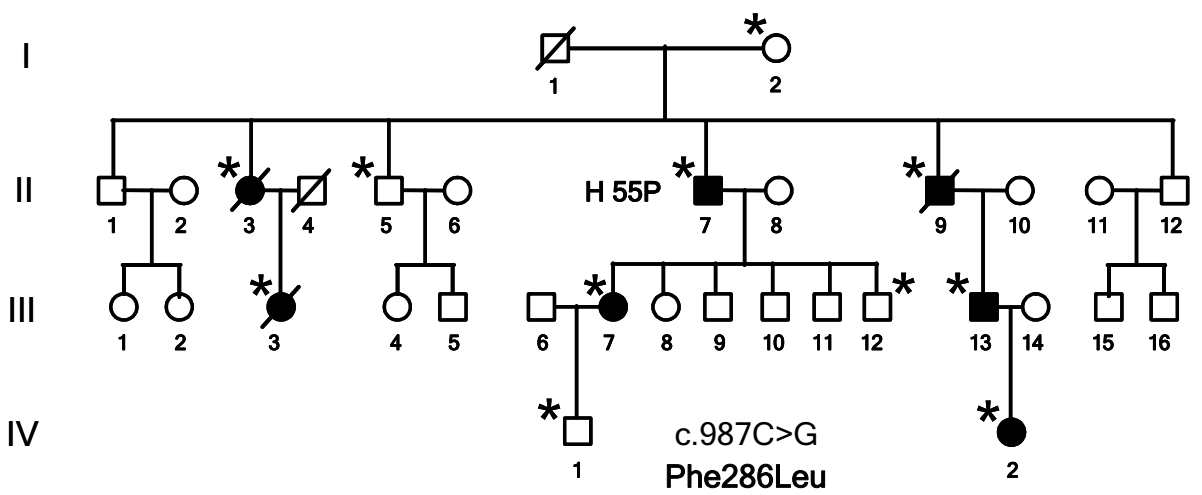
b)

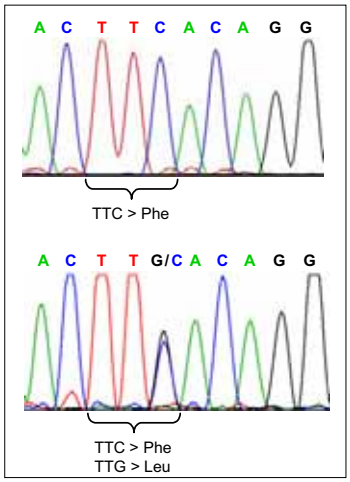

77mg/dL
14mg/dL
H71
H21
H55P
H65
H49
C4 (mg/dL)
C3 (mg/dL)
0
10
20
30
40
50
60
0
50
100
150
200

c)

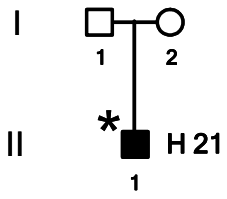

H 21
H 55P
H 65
N1
N2
N3
N4
kDa
113
93
52
36
factor B
Ba

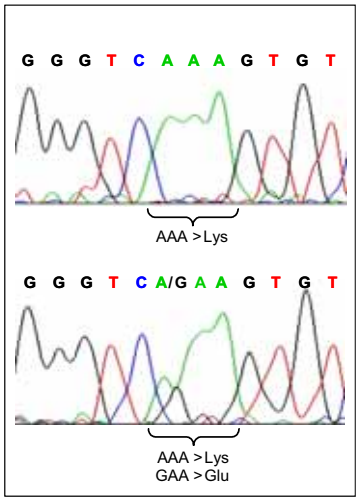
Family PER

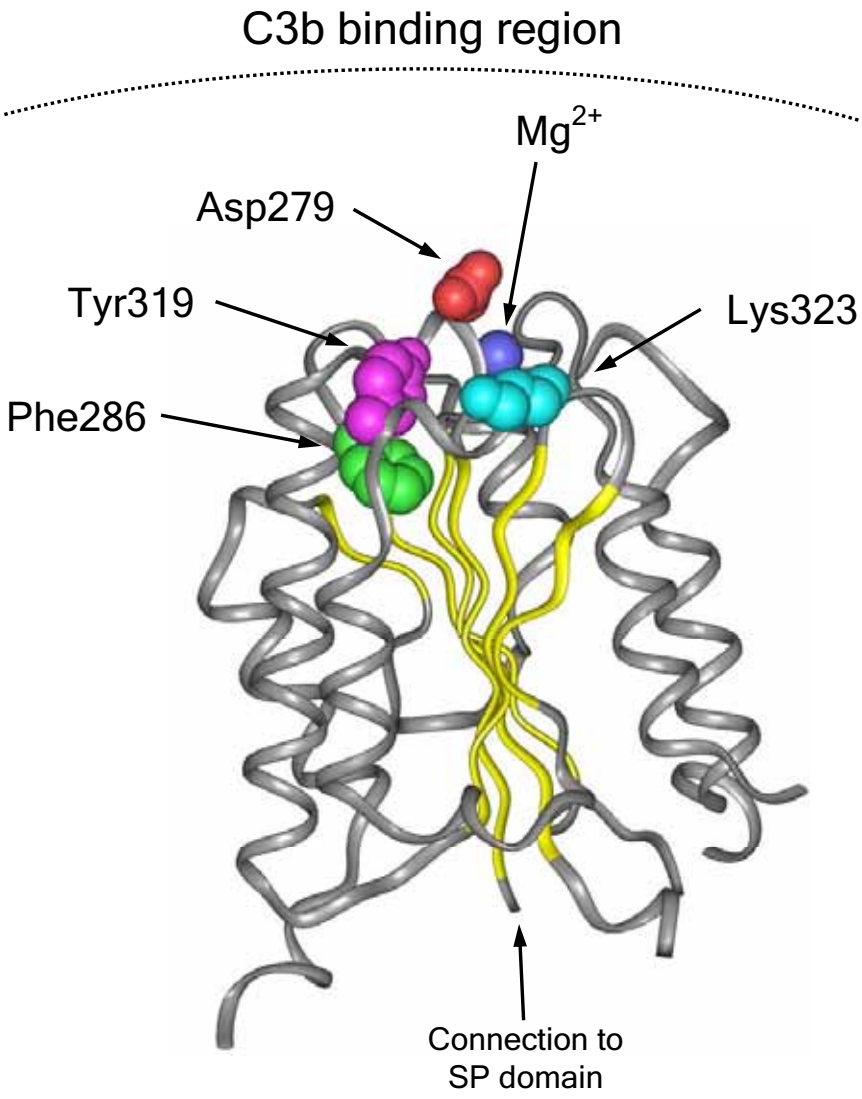




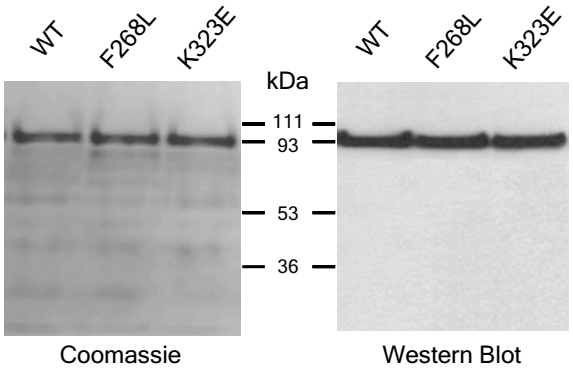
Family BRA



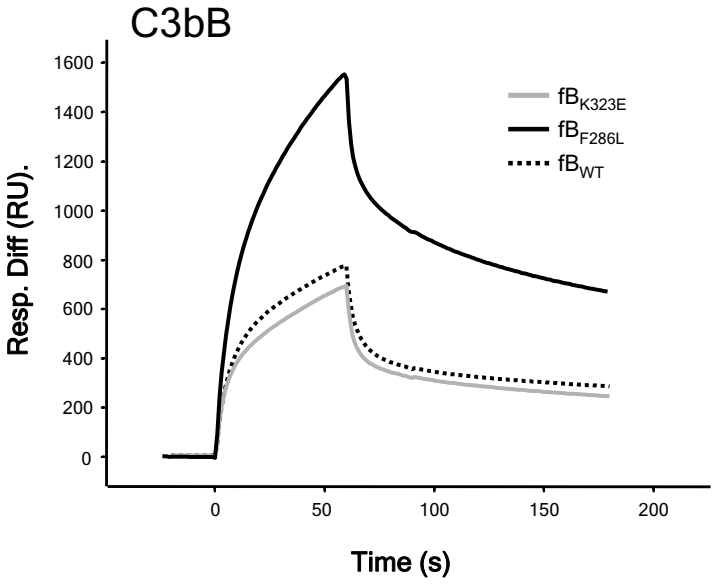



C3b binding region
Mg2+
Asp279
Tyr319
Lys323
Phe286
Connection to
SP domain

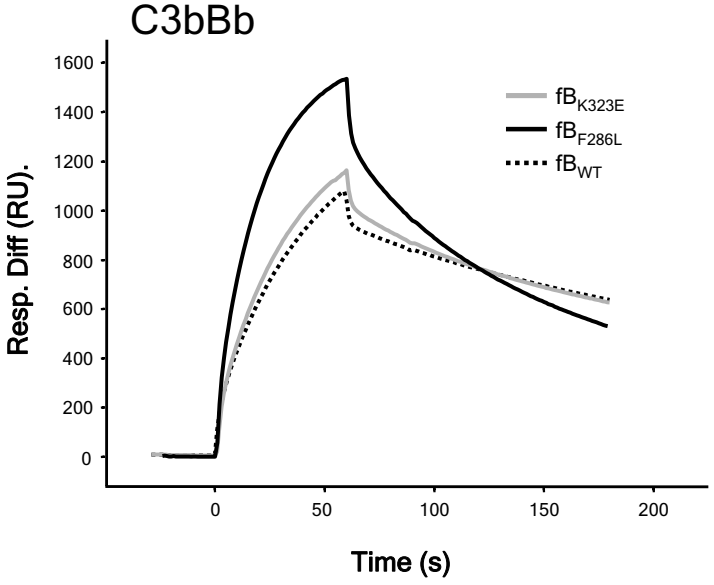
a)


WT
F268L
K323E
kDa
111
93
53
36
WT
F268L
K323E
Coomassie
Western Blot

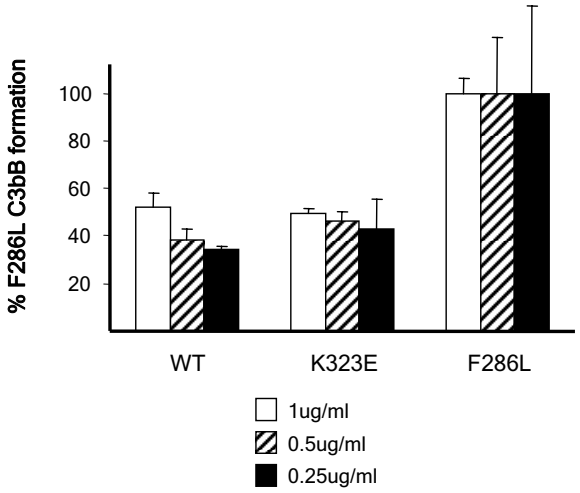
b)


C3bB
Resp. Diff (RU).
Time (s)
fB$_{K323E}$
fB$_{F286L}$
fB$_{WT}$

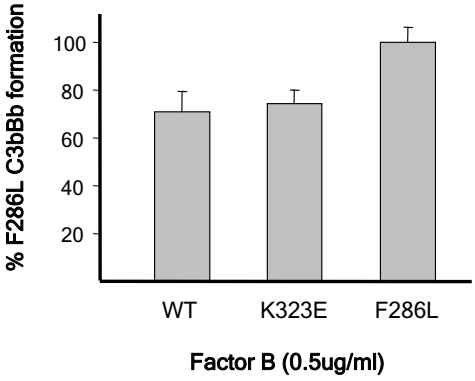
c)


C3bBb
Resp. Diff (RU).
Time (s)
fB$_{K323E}$
fB$_{F286L}$
fB$_{WT}$

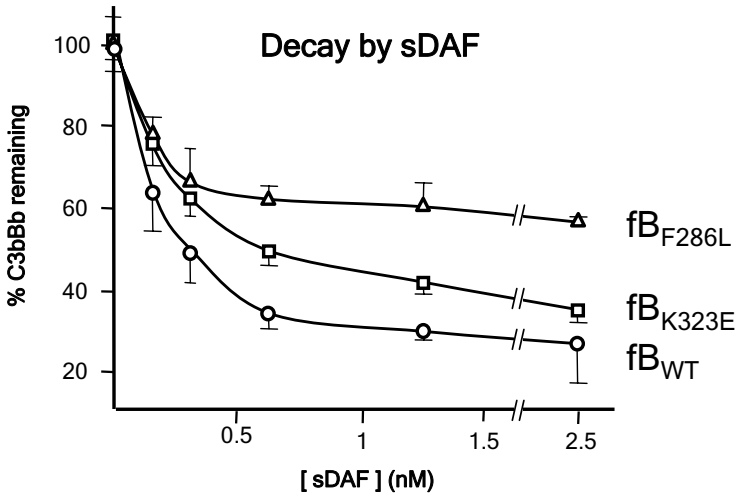
a)


% F286L C3bB formation
100
80
60
40
20
WT
K323E
F286L
1ug/ml
0.5ug/ml
0.25ug/ml

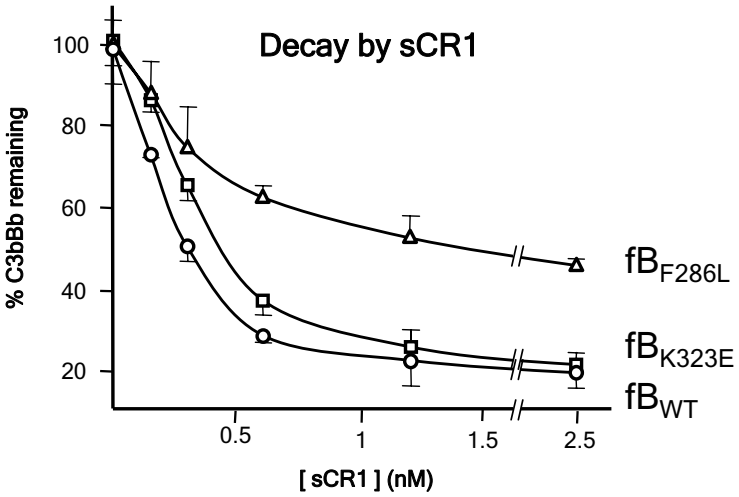
b)


% F286L C3bBb formation
100
80
60
40
20
WT
K323E
F286L
Factor B (0.5ug/ml)

c)


Decay by sDAF
% C3bBb remaining
100
80
60
40
20
0.5
1
1.5
2.5
[sDAF] (nM)
fB$_{F286L}$
fB$_{K323E}$
fB$_{WT}$

d)


Decay by sCR1
% C3bBb remaining
100
80
60
40
20
0.5
1
1.5
2.5
[sCR1] (nM)
fB$_{F286L}$
fB$_{K323E}$
fB$_{WT}$