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Mutations in components of complement influence the outcome of Factor I-associated atypical hemolytic uremic syndrome

Frank Bienaime[1,2,3], Marie-Agnes Dragon-Durey[1,2,3,4], Catherine H. Regnier[1,2,3,8], Sara C. Nilsson[5], Wing H. Kwan[1,2,3], Jacques Blouin[4], Mathieu Jablonski[1,2,3], Nicolas Renault[6], Marie-Anne Rameix-Welti[4], Chantal Loirat[7], Catherine Sautés-Fridman[1,2,3], Bruno O. Villoutreix[6], Anna M. Blom[5] and Veronique Fremeaux-Bacchi[1,2,3,4]

[1]Cordeliers Research Center, INSERM UMRS 872, Paris, France; [2]Université Pierre et Marie Curie (Paris-6), Paris, France; [3]Université René Descartes, Paris, France; [4]Assistance Publique-Hopitaux de Paris, Hopital Europeen Georges-Pompidou, Service d'Immunologie, Paris, France; [5]The Department of Laboratory Medicine, University Hospital Malmö, Lund University, Malmö, Sweden; [6]INSERM U648, University Paris V, Paris, France; [7]Assistance Publique-Hopitaux de Paris, Hopital Robert Debre, Paris, France

Genetic studies have shown that mutations of complement inhibitors such as membrane cofactor protein, Factors H, I, or B and C3 predispose patients to atypical hemolytic uremic syndrome (aHUS). Factor I is a circulating serine protease that inhibits complement by degrading C3b and up to now only a few mutations in the *CFI* gene have been characterized. In a large cohort of 202 patients with aHUS, we identified 23 patients carrying exonic mutations in *CFI*. Their overall clinical outcome was unfavorable, as half died or developed end-stage renal disease after their first syndrome episode. Eight patients with CFI mutations carried at least one additional known genetic risk factor for aHUS, such as a mutation in *MCP*, *CFH*, *C3* or *CFB*; a compound heterozygous second mutation in *CFI*; or mutations in both the *MCP* and *CFH* genes. Five patients exhibited homozygous deletion of the Factor H-related protein 1 (*CFHR-1*) gene. Ten patients with aHUS had one mutation in their *CFI* gene (Factor I-aHUS), resulting in a quantitative or functional Factor I deficiency. Patients with a complete deletion of the *CFHR-1* gene had a significantly higher risk of a bad prognosis compared with those with one Factor I mutation as their unique vulnerability feature. Our results emphasize the necessity of genetic screening for all susceptibility factors in patients with aHUS.

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Correspondence: *Veronique Fremeaux-Bacchi, INSERM UMRS 872, Service d'Immunologie Biologique, Hôpital Européen Georges Pompidou, 20-40 rue Leblanc, PARIS, Cedex, 75908, France.*
E-mail: veronique.fremeaux-bacchi@egp.aphp.fr

[8]Current address: Novartis Institutes for Biomedical Research, Disease Area Oncology, Basel, Switzerland

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Several studies have emphasized the role of the alternative complement pathway in the pathogenesis of atypical hemolytic uremic syndrome (aHUS), an uncommon form of thrombotic microangiopathy.[1] Mutations have been identified in genes encoding fluid-phase complement inhibitors Factor H (FH), the serine protease (SP) Factor I (FI), as well as the surface-bound inhibitor membrane cofactor protein (MCP, CD46) and more recently thrombomodulin and, in Factor B (FB) and in C3, which are included in the alternative pathway C3 convertase.[2–5] In addition, a few common complement regulator gene polymorphisms, such as p.Arg240His in the C4b-binding protein (C4BP) gene (*C4BPA*), and deletion of two closely related genes, such as complement FH-related 1 and 3 (*CFHR-1*, *CFHR-3*), or variants in *CFH* and *MCP* genes have been reported to increase the risk for aHUS.[6–8] Recently collected data from registries allowed a generalized analysis of the clinical characteristics of aHUS patients according to the identified risk factors.[9,10] Indeed, mutations reported in *CFH* and *MCP* genes account for 20–30 and 10% of aHUS cases, respectively. Patients with *MCP* mutations have a good renal prognosis and are characterized by aHUS that reoccurs frequently in the native kidney, but not after transplantation, compared with those with defects in *CFH*. Several studies reported low frequency ($<5\%$) of *CFI* mutations in aHUS, with the exception of the French cohort of patients with a early onset of aHUS (12% in a cohort of 46 aHUS with pediatric onset).[9–12] FI is a two-chain SP predominantly synthesized by the liver, in which the 38-kDa light chain carries the catalytic domain (SP or SP domain) with the catalytic triad His362, Asp411, and Ser507,[13] although the function of the heavy chain is unclear. The crucial step of the alternative pathway activation is the formation of the C3 convertase, an enzymatic complex composed of C3b and Factor Bb (C3bBb), which proteolytically activates C3. In the presence

Table 1 | Summary of mutations and their functional consequences

	FI domains					References	
	FIMAC	CD5	LDLr	Linker	Serine protease	Original report	Functional studies
Functional deficiency[a]				R299W		Caprioli *et al.*[9]	Kavanagh *et al.*[17]
				I322T		Geelen *et al.*[18]	Kavanagh *et al.*[17]
					D501N	Caprioli *et al.*[9]	Kavanagh *et al.*[17]
					D506V (P22)	Fremeaux-Bacchi *et al.*[19]	Kavanagh *et al.*[17]
					I415V (P8)	Sellier-Leclerc *et al.*[10]	New
Quantitative deficiency					1446–1450del TTCAC	Caprioli *et al.*[9]	Kavanagh *et al.*[17]
					W528X	Fremeaux-Bacchi *et al.*[19]	Fremeaux-Bacchi *et al.*[19]
			c.922 delC			Kavanagh *et al.*[12]	Kavanagh *et al.*[12]
					c.1624 ins AT	Esparza-Gordillo *et al.*[11]	Esparza-Gordillo *et al.*[11]
		W127X				Kavanagh *et al.*[12]	Kavanagh *et al.*[17]
					R456X (P10)	Fremeaux-Bacchi *et al.*[19]	New
					I398L (P5;P6)	Sellier-Leclerc *et al.*[10]	New
	C25F (P1)	**N133S (P3)**	**c.784delA (p.G243fsX46) (P4, P15)**		**A413T (P7)**	New	New
	P32A (P2; P11)				**W438L (P9)**	New	New
No functional defect identified		M120I				Kavanagh *et al.*[17]	Kavanagh *et al.*[17]
			G243D (P16; P17)			Nilsson *et al.*[16]	Nilsson *et al.*[16]; Kavanagh *et al.*[17]
Undetermined		A222G				Caprioli *et al.*[9]	
					Y369S	Chan *et al.*[20]	
		H165R (P14)				Sellier-Leclerc *et al.*[10]; Boyer *et al.*[21]	
		Gly101R (P12)			**D385N (P19)**	New	
		His100R (P13)	**I288S (P18)**		**G406D(P12)**	New	
					Y441S (P21)	New	
					P535S (P23)	New	

Abbreviations: FI, Factor I; FIMAC, Factor I membrane attack complex; LDLr, low-density lipoprotein receptor.
Mutations reported in this series are represented in bold.
[a]Defect in C3b cofactor activity.

of FH, C4BP, or MCP as cofactors, FI inactivates C3b by further proteolytic cleavage yielding iC3b.[14] The *CFI* gene is located on chromosome 4 and comprises 13 exons.[15] Until now, 17 heterozygous mutations in *CFI* have been reported in patients with aHUS, but their functional consequences were characterized in approximately half of the cases. A summary of *CFI* mutations and their functional consequences is presented in Table 1.[9–12,16–21] In some patients, the mutant FI protein is present in normal amounts, and the presumed functional deficiency has not yet been defined.[16,17,20]

We screened 202 patients suffering from aHUS for *CFI* mutations, and identified 18 different mutations in exonic sequences in 23 patients. This study establishes the high frequency of *CFI* mutations associated with a second genetic abnormality or with rare polymorphisms in complement proteins, which may contribute to the severity of aHUS in these subjects. Furthermore, using *in vitro* expression system and molecular modeling, we identified the nature of functional defects of eight new mutations in the *CFI* gene.

RESULTS

Genetic screening, complement component assessment

Among the 202 aHUS patients included in the current study, 23 (12 women and 11 men) had *CFI* gene mutations resulting in a frequency of 11.3% (Figure 1, Tables 2 and 3). None of the detected mutations were found in 100 healthy individuals. All mutations were heterozygous except one. Two patients presented two mutations in the *CFI* gene (one compound heterozygous and one homozygous). We identified 17 missense mutations, one nucleotide deletion leading to a premature stop codon and one nonsense mutation. Nine mutations were located within the SP domain. Of the 23 patients, 8 presented low FI concentration at the time of investigation.

Among the group of patients with *CFI* mutations, eight patients carried a single gene mutation in *MCP* ($n=2$), *CFH* ($n=1$), *CFI* ($n=1$), *C3* ($n=2$), or *CFB* ($n=1$), or with a mutation in both *MCP* and *CFH* genes ($n=1$). Five patients exhibited homozygous deletion of the *CFHR-1* gene, and one patient had the polymorphism

(p.Arg240His) in *C4BPA*. Of the 13 patients, 4 presented low C3 levels at the time of investigation. Overall, 10 patients were negative for mutations/deletions in *CFH MCP*, *C3*, *CFB*, *CFHR-1*, and *C4BPA* genes and formed the group of aHUS-FI patients. In three of these patients, C3 and FB plasma concentrations were decreased, which is consistent with alternative pathway activation. C4 and FH were in the normal range in all patients. Except for one (P17), none of the patients with a CFHR-1 deletion presented with anti-FH antibodies.

Clinical outcome of patients with *CFI* mutations

Of 23 patients with *CFI* mutations, 3 presented a familial form. The age at onset varied from neonatal to early adulthood (Tables 1 and 2). The first episode of aHUS occurred at <2 months of age in four patients. All patients but one exhibited acute renal failure (defined as an acute increase in the serum creatinine level from the baseline level). None of the patients in the aHUS-FI group, except one, presented a severe initial outcome defined by end-stage renal disease (ESRD) or death 1 year after the onset. Patient 6, who presented severe facial malformed syndrome, died a few days after the onset. Three patients from this group were on chronic dialysis 18 months, 2 years, or 12 years, respectively, after the first flare. No relapse occurred during the follow-up for five patients. In the group of patients with additional genetic defects in complement genes, 10 out of 13 presented a severe outcome. Patient 16 died from *Staphylococcus aureus* septicemia.[10] Five patients had ESRD as soon as the first episode of the disease, and three had a rapid evolution to ESRD during the first year after the onset. Kaplan–Meier curves suggested longer overall kidney function among patients with exclusively *CFI* mutations than among those with *CFI* mutations associated with a deletion of the *CFHR-1* gene ($P = 0.006$ by log-rank test) (Figure 2).

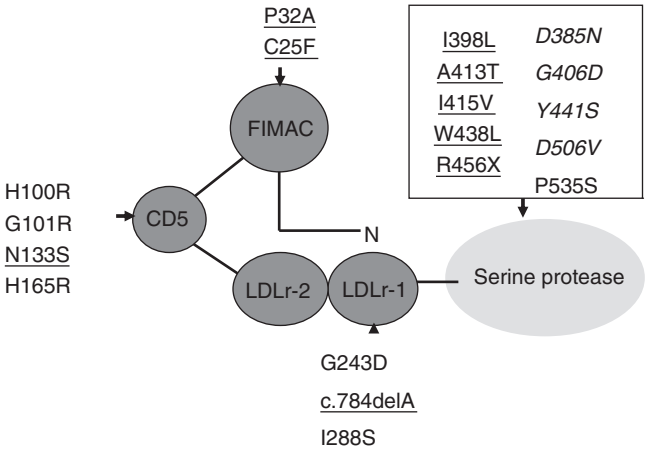

Figure 1 | Schematic localization of *CFI* mutations identified in the French cohort. *In vitro* expression studies in mammalian cells have been conducted for the underlined mutations. FIMAC, Factor I membrane attack complex.

Analysis of the consequences of mutations on recombinant CFI expression and processing

The impact of *CFI* mutations found in aHUS-CFI patients (that is, those lacking additional genetic abnormalities) on FI production and function was assessed using an *in vitro* expression model. We first cloned the cDNA coding for human wild-type (WT) *CFI* into a eukaryotic expression vector and transiently expressed the protein in human embryonic kidney (HEK)293 cells. Both pro-FI and mature FI were detected in the culture supernatant at day 2 (data not shown). Mutations were introduced by site-directed mutagenesis, and their consequences on FI production were evaluated 2 days after transfection. FI mutant D506V previously identified to be associated with a functional

Table 2 | Patients with *CFI* mutations

		CFI sequence change			Summary of clinical history and biological findings						
		Numbering									
Patients	Genetic status	Nucleotide from ATG	Nucleotide from Met1	Nucleotide from mature protein	C3 (mg/l)	FB (mg/l)	FI (mg/l)	S/F	Age of onset	ESRD or death 1 year after the onset	Late outcome
P1	Het	c.128G>T	p.Cys43Phe	C25F	704	107	40	S	30	No	ESRD (12 y)
P2	Het	c.148C>G	p.Pro50Ala	P32A	777	129	40	S	0.04	No	No relapse (6 y)
P3	Het	c.452A>G	p.Asn151Ser	N133S	583	80	23	S	39	No	No relapse (4 y)
P4	Het	c.784delA	c.784delA (p.G243fsX46)		891	104	28	S	32	No	ESRD (18 m)
P5	Het	c.1246A>C	p.Ile 416Leu	I398L	1030	ND	43	S	0	No	No relapse (6 y)
P6	Het	c.1246A>C	p.Ile 416Leu	I398L	410	67	ND	S	0.08	Death	
P7	Het	c.1291G>A	p.Ala431Thr	A413T	857	139	44	S	18	No	Relapses (2, 3, and 8 m) and ESRD (2 y)
P8	Het	c.1297A>G	p.Ile433Val	I415V	1000	190	112	S	0.16	No	No relapse (10 y)
P9	Het	c.1367G>T	p.Trp456Leu	W438L	738	128	20	S	22	No	No relapse (5 y)
P10	Het	c.1420C>T	p.Arg474Stop	R456X	601	55	24	S	31	No	Relapses (2, 4 m) and ESRD (18 m)

Abbreviations: aHUS, atypical hemolytic uremic syndrome; CFI, complement Factor I; ESRD, end-stage renal disease; F, familial aHUS; FB, Factor B; FI, Factor I; m, month; ND, not determined; S, sporadic aHUS; y, year.
Individual clinical histories have been previously reported for four patients (P2, P5, P8, and P10).[10,19]
Normal values: C3 (660–1275 mg/l); FI (42–78 mg/l); FB (42–78 mg/l). ESRD was defined as the need for chronic renal replacement.

Table 3 | Patients with *CFI* mutations combined with a second genetic abnormality

	CFI sequence change					Clinical history						
	Genetic status	Nucleotide from ATG	Amino acid	According to mature protein	Associated genetic change	C3 (mg/l)	FB (mg/l)	CFI (mg/l)	S/F	Age of onset	ESRD or death 1 year after the onset	Late outcome
P11	He	c.148C>G	p.Pro50Ala	P32A	MCP (p.Arg103Trp;R69W)	915	176	67	F	2	No	Two relapses (5, 7 y) and lost from follow-up
P12	He	c.355G>A	p.Gly119Arg	G101R	CFI (p.Gly424Asp; G406D)	893	129	50	S	42	No	No relapse (10 y)
P13	He	c.353A>G	p.His118Arg	H100R	MCP (IVS 2+2)	1140	166	71	S	32	Yes (ESRD)	
P14	He	c.348A>G	p.His183Arg	H165R	CFH (p.Asn767LysfsX7)	290	150	71	S	1.4	Yes (ESRD)	
P15	He	**c.784delA**	**p.G243fsX46**		Del CFHR1(HO)	545	72	24	S	31	Yes (ESRD)	
P16	He	c.782G>A	p.Gly261Asp	G243D	C3 (p.Lys155Gln)	1150	206	76	F	0.5	Death	
P17[a]	He	c.782G>A	p.Gly261Asp	G243D	Del CFHR1(HO)	694	118	82	S	28	Yes (ESRD)	
P18	He	c.917T>G	p.Ile 306Ser	I288S	Del CFHR1(HO)							
					Del CFHR3 (HO)	892	128	77	S	32	Yes (ESRD)	
P19	He	c.1207G>A	p.Asp403Asn	D385N	Del CFHR1(HO)							
					Del CFHR3 (HO)	839	99	72	S	43	Yes (ESRD)	
P20	Ho	**c.1246A>C**	**p.Ile 416Leu**	**I398L**	Del CFHR1(HO)							
					Del CFHR3 (HO)	523	36	17	S	46	Yes (ESRD)	
P21	He	c.1376A>C	p.Tyr459Ser	Y441S	CFB (p.Val455Ile)	1050		46	S		Yes (ESRD)	
P22	He	c.1571A>T	p.Asp524Val	D506V	C3 (p.Pro1114L)	624	76	65	S	1.5	No	No relapse (8 y)
P23	He	c.1657C>T	p.Pro553Ser	P535S	CFH (p.Arg1210Cys) MCP (p.Tyr29X)	1260	151	76	F	30	Yes (ESRD)	

Abbreviations: CFI, complement Factor I; ESRD, end-stage renal disease; FB, Factor B; FI, Factor I; m, month; y, year.
Individual clinical histories have been previously reported for five patients (P11, P14, P16, P17, and P22).[10,16,19,21,25]
[a]The screening for anti-Factor H for P17 was positive at the diagnosis and during follow-up. Screening for anti-FH antibodies was negative for patients 15, 18, 19, and 20.
Mutations reported in this series are represented in bold.
Normal values: C3 (660–1275 mg/l); FI (42–78 mg/l); FB (42–78 mg/l).

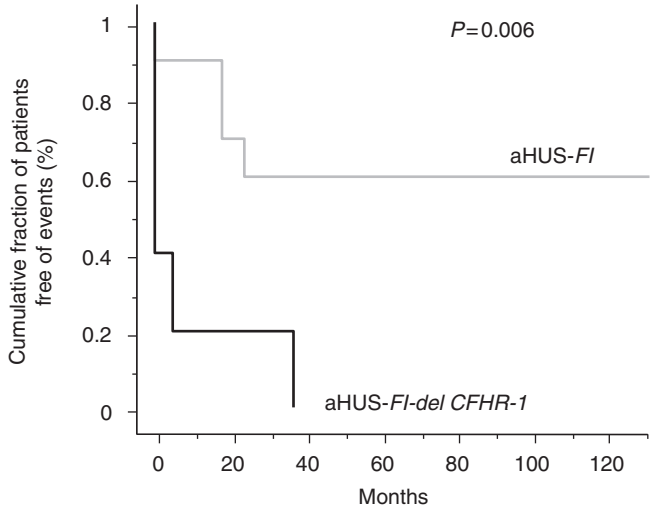

Figure 2 | Kaplan–Meier curves for kidney survival in patients with aHUS and *CFI* mutations. Cumulative fraction of patients free of events (or overall renal survival), defined as the combination of the occurrence of initiation of dialysis or death, whichever occurred in aHUS-*CFI* patients without additional genetic defect (aHUS-*FI*) or with a complete deletion in the *CFHR-1* gene (aHUS-*FI-del CFHR-1*). aHUS, atypical hemolytic uremic syndrome; FI, Factor I.

deficiency was used as a control.[17] As shown in Figure 3, C25F, N133S, I398L, A413T, and W438L mutations led to a dramatic decrease in the FI level detected in cell culture supernatants by enzyme-linked immunosorbent assay (ELISA) and western blotting. The P32A mutation resulted in a 60% decrease in the FI level in the supernatant. I398L, A413T, W438L, and P32A mutations reduced the amount of intracellular FI. As expected, the two mutations predicted to generate premature stop codon resulted in the absence of detectable recombinant FI in both the supernatant and the cell lysate, despite transfection efficiency equivalent to WT. Only the W438L mutation led to the slightly reduced electrophoretic mobility of intracellular recombinant FI in contrast to all the other missense mutations that migrated as the WT. To further investigate the mechanisms leading to a decrease in FI secretion, we studied the subcellular localization of FI in HEK293 cells transfected with WT, I398L, or A413T FI constructs. In cells expressing the WT protein, colocalization of the FI protein with wheat germ agglutinin was observed, resulting in a dot pattern consistent with a trans-Golgi localization. In cells expressing either I398L or A413T mutant FI, there was no colocalization with wheat germ agglutinin, but instead with protein disulfide isomerase, a marker for the endoplasmic reticulum (Figure 4). Furthermore, a fraction of intracellular WT FI was resistant to endoglycosidase H digestion, whereas I398L and A413T FI were not (data not shown).

Mutations I415V and D506V had no effect on either intracellular or extracellular FI levels. Therefore, we investigated the functional activity of both mutants produced *in vitro*. No differences were detected for their ability to cleave C3b and C4b in solution as compared with WT (Figure 5). We also tested the ability of WT D506V and I415V FI to inactivate membrane-bound C3b in the presence of FH. We found that WT FI inactivated C3b in a time-dependent manner. On the contrary, the supernatant of cells transfected with the I415V mutant was not effective in inactivating membrane-bound C3b, and the slight decrease in stabilized C3b sites was comparable with the decrease observed using the supernatant of mock-transfected cells. The D506V mutant FI showed a slightly reduced activity (Figure 5c).

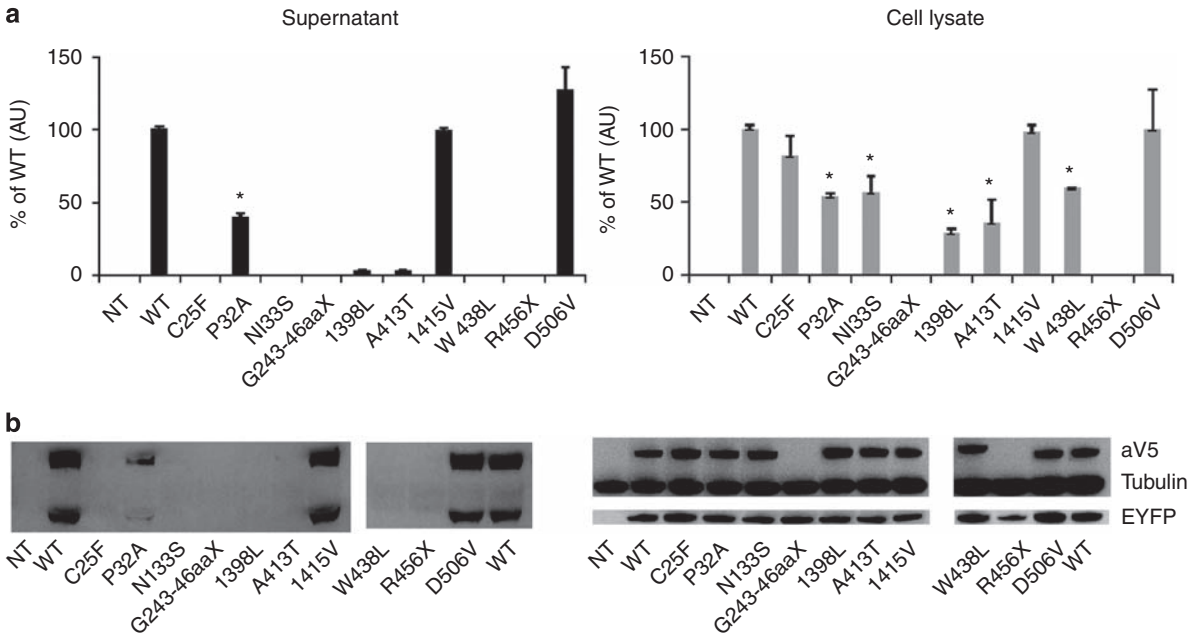

Figure 3 | Consequences of mutations on FI production. The FI synthesis was assessed 48 h after transfection by ELISA (**a**) and western blotting with anti-V5 antibody (**b**) in cell supernatants and cell lysates. Untransfected (NT) cells were used as negative control. The membrane used for western blotting was reprobed with anti-tubulin and anti-EYFP antibodies, which served as loading and transfection controls, respectively. *Significantly different from WT FI level according to Student's *t*-test ($P<0.05$). The results are representative of three independent experiments, with bars corresponding to s.d. ELISA, enzyme-linked immunosorbent assay; FI, Factor I.

Structural analysis of point mutations

To evaluate the potential consequences of point mutations on the protein structure, we produced high-resolution homology models of FI domains (Figure 6) and mapped the corresponding mutations in appropriate domains. Predicted consequences of the mutations are summarized in Table 4.

DISCUSSION

This report is focused on aHUS patients carrying mutation in the *CFI* gene. Less than 20 cases of patients with aHUS with a mutation in the *CFI* gene have been reported previously (reviewed in Chan *et al.*[20] and Kavanagh *et al.*[22]; Table 1), and the aHUS-phenotype associated with *CFI* gene mutations appears to be variable in contrast to the cases of FH and MCP.[10] In this study, we report results of CFI genetic screening in a large cohort of non-Stx–HUS patients.

Mutations in *CFI* account for 11.3% of aHUS cases in the cohort of 202 patients, which is slightly higher than the frequency reported by the other groups.[9,12] All *CFI* mutations but one are heterozygous and located throughout the entire gene. Of 10 mutations, 9 that were investigated in depth in our study led to reduced protein levels *in vivo* and/or *in vitro*. *In vivo* plasma FI was occasionally detected at normal levels in patients (or in unaffected relatives bearing the mutation), although mutations resulted in quantitative defects *in vitro*. As previously postulated by Kavanagh *et al.*,[17] our current results emphasize that the FI level within normal range does not exclude a mutation impairing FI secretion or production.[17] We report the first case of homozygous mutation with a subtotal FI deficiency in an aHUS patient. The corresponding recombinant FI I398L mutant was very weakly secreted *in vitro* into the supernatant of transfected cells, explaining the low concentration of FI in the patient's plasma. According to previous reports, patients with complete FI deficiency have increased susceptibility to recurrent infection.[14] The exceptional association of aHUS in this patient could be due to the presence of only partial consumptive loss of C3, in contrast to patients with complete FI deficiency having undetectable C3 levels.[23] Using a membrane-bound C3b inactivation assay, we identified I415V as a new mutation associated with functional deficiency. It had similar behavior as the D506V substitution.[17] Hemolytic test demonstrated a functional defect for these two mutant proteins; however the A415V mutation carried the more severe defect for these two mutant proteins. In a different experimental setting of fluid-phase cofactor assay, mutants were capable of performing the catalytic act. In these experiments, the FI/FH ratio was ~1/1 (FI was not the limiting factor for the reaction) in an isotonic buffer (rather than in low ionic strength, characteristic for the hemolytic test and for the previously published cofactor activity data for D506V). Under these favorable experimental conditions, mutant proteins cleaved C3b and reached the efficiency of the WT. Thus, in corresponding patients, a partial protection of the endothelium may be achieved because of reduced, but existing, enzymatic activity of mutant proteins. Taken together, as reported for aHUS-MCP, ~30% of mutations in the *CFI* gene result in a functional deficiency.[24]

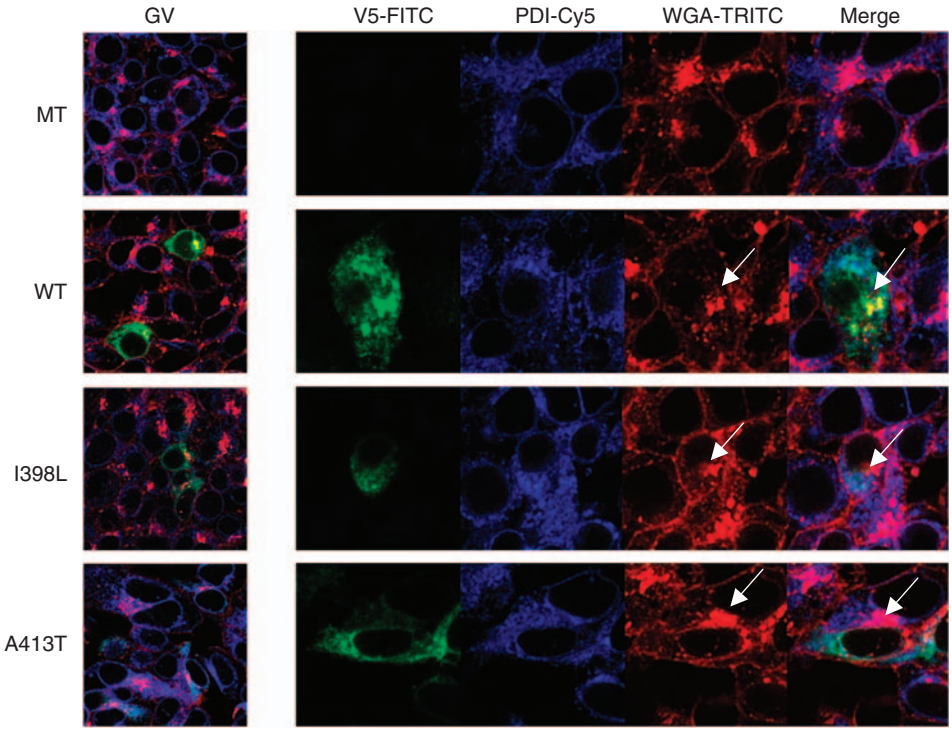

Figure 4 | Subcellular localization of WT, I398L, and A413T FI. Subcellular localization of FI was studied using confocal microscopy 48 h after transient transfection. Anti-V5 antibody was used to detect FI. WGA stained trans-Golgi (dot-like juxtanuclear aspect; arrow) and endosomes (granular aspect), whereas anti-PDI stained endoplasmic reticulum. WT FI colocalized with the marker of the trans-Golgi in a dot-like aspect, which is consistent with a predominant trans-Golgi localization. I398L and A413T mutant FI proteins did not colocalize with the trans-Golgi marker but with the endoplasmic reticulum marker. GV, global view using the same amplification in each channel; V5-FITC, V5 tag marked by fluorescein isothiocyanate; PDI-Cy5, PDI revealed by Cyanin5; WGA-TRITC, WGA revealed by tetramethyl-rhodamine iso-thiocyanate; MT, mock-transfected cells; WT, HEK293 cells transfected with the WT FI construct; I398L and A413T HEK293 cells transfected with the corresponding mutated FI constructs. FI, Factor I; HEK293, human embryonic kidney293; PDI, protein disulfide isomerase; WT, wild type; WGA, wheat germ agglutinin.

In general, patients with aHUS have only one discovered mutation.[9] Surprisingly, in the group of patients with *CFI* gene mutations, 30% of patients (7 of 23) had an additional mutation in genes known to represent the susceptibility factor for aHUS. We identified four patients who presented with heterozygous quantitative or functional MCP or FH deficiency. In particular, both R69W and R1210C FH mutations in MCP and CFH genes, respectively, were deficient in their ability to control the alternative pathway on a cell surface.[25,26] Patients with simultaneous *CFI* and *MCP* mutations were also reported in other studies.[9,11] Three patients had previously unreported mutations in recently identified aHUS susceptibility genes *C3* and *CFB*.[2,3,27] Further experiments are currently underway to assess their functional role.

In the current study, 5 out of 23 (21%) patients with the *CFI* mutation have no *CFHR-1* gene, which appears to be higher than the number reported in Caucasian control subjects (between 2 and 7%).[28,29] FH-related (CFHR) proteins share common features with FH, but their exact functions are unclear, although a general role in the regulation of complement activation is emerging.[29,30] Several studies support the hypothesis that polymorphisms or haplotypes in *MCP* and *CFH* genes greatly influence predisposition to aHUS and provide an explanation for the incomplete penetrance of aHUS in carriers of mutations in complement proteins.[7,31,32] Recently, Zipfel *et al.*[8] showed in two independent cohorts of aHUS patients that the deletion of *CFHR-1* and *CFHR-3* increases the risk of aHUS. However, the disease risk associated with this deletion has been found to be restricted to the group of patients with anti-FH antibodies and interestingly also to the group of patients with *CFI* mutations.[33]

In our series of 23 patients, clinical outcome was unfavorable with 50% of patients dying or progressing to ESRD immediately or within 2 years after the first episode of the disease. However, seven patients presented with complete remission of the disease without aHUS relapses (30%). Following these observations, we tried to identify factors, which may influence the severity of the disease. Despite the small size of the cohort, our study provides evidence for the influence of the complement genetic background. Indeed, the common polymorphism of the *CFHR-1* gene (or a gene in linkage disequilibrium with the *CFHR-1* gene) had a dramatic impact on disease severity. Owing to the small number of patients with *CFI* + *CFH*, *CFI* + *MCP*, *CFI* + *CFB*

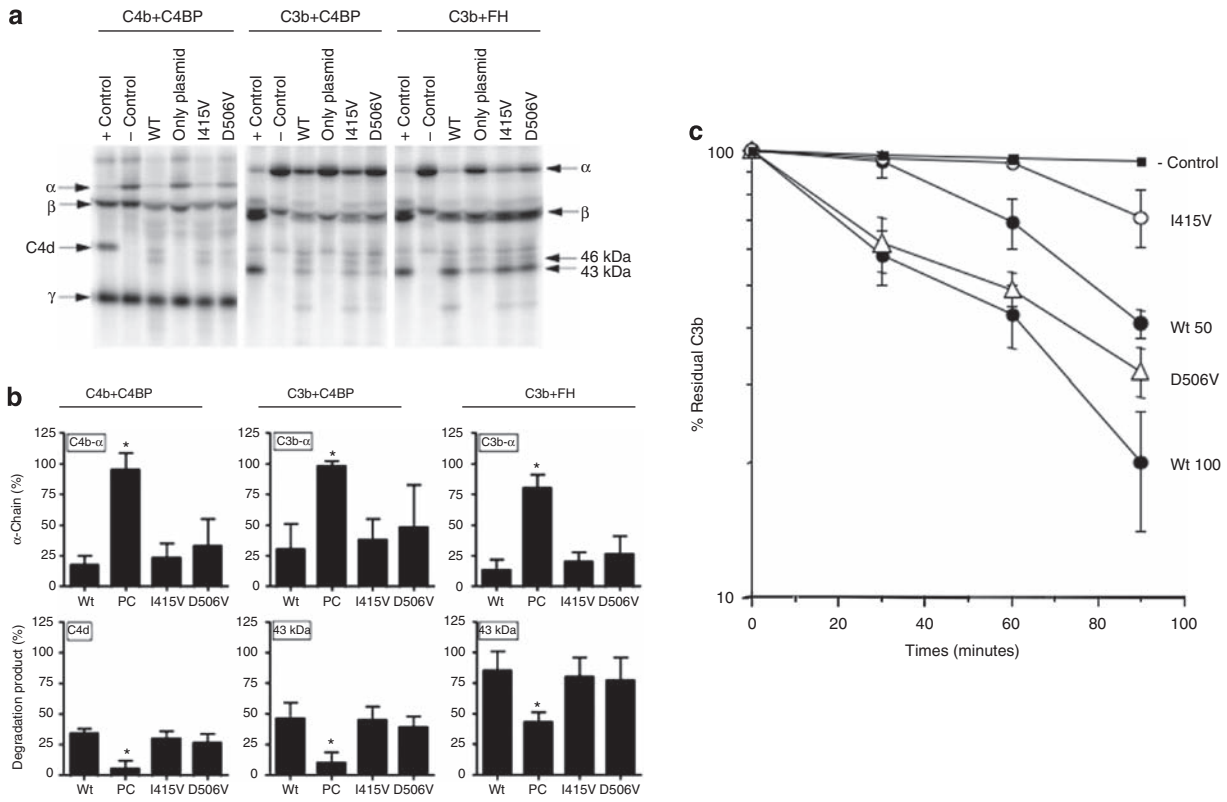

Figure 5 | Inactivation of C3b and C4b by WT, I415V and D506V FI. (**a**) (Representative raw data) and (**b**) (densitometric quantification): fluid-phase C4b and C3b degradation assay. Partially purified WT, I415V, D506V, and mock-transfected HEK293 cells (only plasmid, PC) were assayed for their capacity to degrade C4b and C3b in fluid phase in the presence of C4BP or FH as cofactors. As a negative control (−control), FI was omitted in the incubation mixture and highly purified WT FI (20 μg/ml) was used as positive control (+ control). All samples containing FI contain C3b degradation products of 43 and 46 kDa, and C4b degradation product C4d. The experiment was repeated four times. One-way ANOVA tests were performed to test significant differences between WT and plasmid only, I415V or D506V, $*P > 0.05$. (**c**) The kinetics of C3b inactivation by 120 ng of FI WT (WT100) and 60 ng of FI WT (WT50) was assessed and compared to activities of FI I415V and FI D506V. No significant activity was observed in the absence of FI (concentrated supernatant of mock-transfected HEK293; −control) and with FI I415V supernatant during 90 min of the kinetic experiment. In the presence of FI D506V, the residual C3b decreased with time but the effect was less pronounced than that for the WT. The results are representative of three independent experiments with bars corresponding to s.d. ANOVA, analysis of variance; FH, Factor H; FI, Factor I; HEK293, human embryonic kidney293; WT, wild type.

or *CFI + C3* mutations, clinical findings were analyzed descriptively without statistical comparisons. It is known that patients with defective MCP have a better prognosis than do those with dysfunctional FH.[9,10] The two patients with CFI and CFH mutations developed ESRD after the first episode of the disease. In association with the *CFI* mutation, the influence of *MCP* or C3 mutations on the prognosis might be variable as half of the patients presented a severe outcome. Analysis of the clinical history of the patients suggests that aHUS-associated *CFI* mutations as sole risk factors have a good prognosis if there are no severe sequelae after the first episode. Except recurrences within the first year after the onset, patients did not present any new episodes during the long follow-up (on average 6 years). We demonstrated that all mutations identified in patients without other genetic abnormalities have consequences on production/secretion or function of the protein. Our results illustrate that reduction in FI activity secondary to a quantitative or a functional defect leads to a 'mild' thrombotic process.

There are some limitations to our study that should be pointed out. The functional study was restricted to 11 out of 19 mutations identified in our population. Only all mutations found in the aHUS-FI group were included in the *in vitro* analysis. Therefore, we can conclude that mutations in *CFI* as a single genetic abnormality are related to the pathophysiology of aHUS. The functional consequences of the nine missense mutations found in association with another genetic defect in the complement gene remain speculative. As all genetic changes in *CFI* may not be associated with a functional defect, and also because of the small number of patients with *CFH*, *MCP*, *FB*, or *C3* mutations in the cohort, the impact of an additive CFI mutation on the course of the disease could not be assessed by our study. For the same reason, the more severe renal phenotype seen in CFHR-1 patients might be related only to

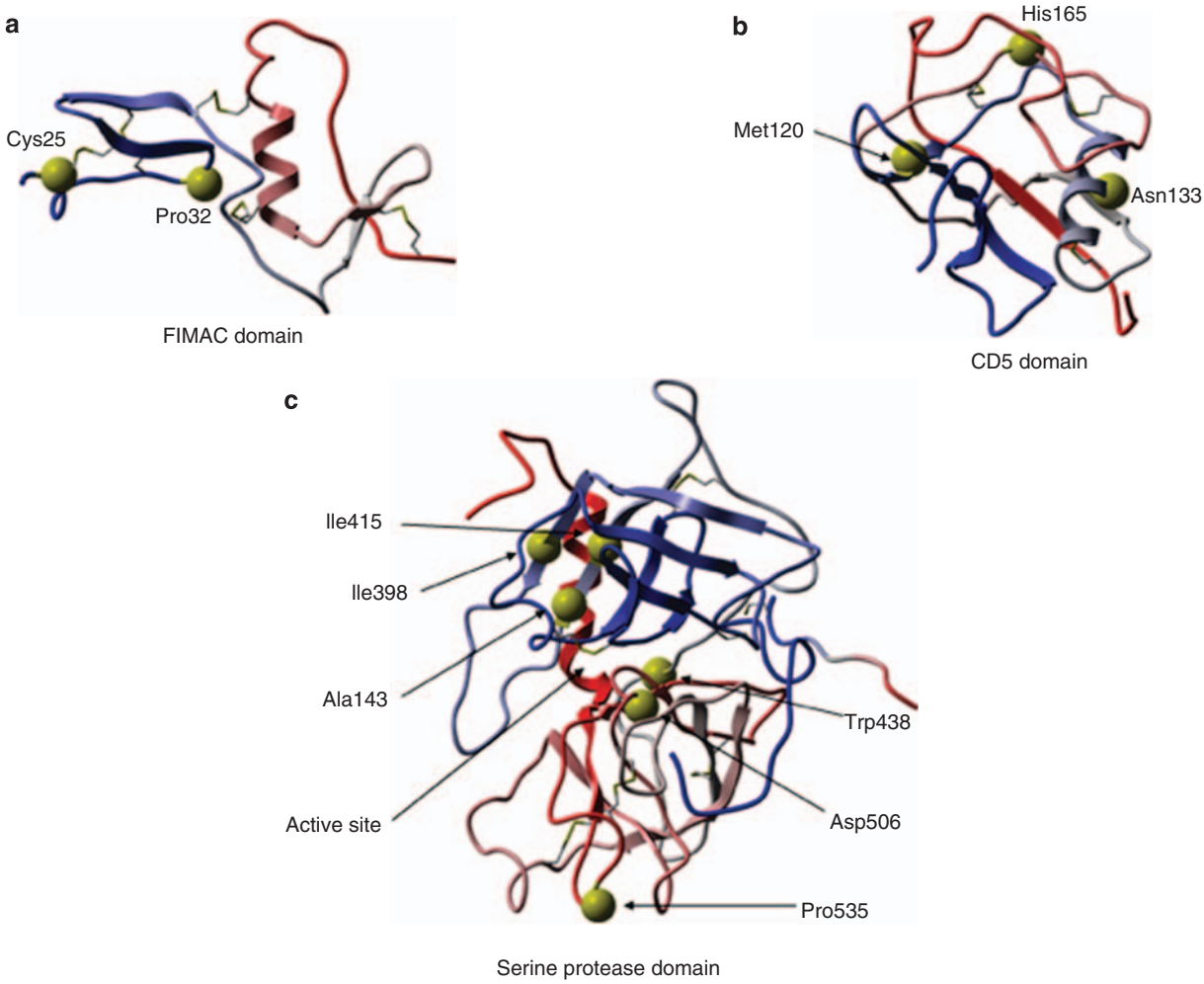

Figure 6 | CFI homology models. Mutations identified in patients were mapped onto the 3D structures (ribbon rendering generated with ICM Molsoft (La Jolla, CA, USA), colored from blue, N terminus to red, C terminus) and are shown as CPK spheres (yellow). (**a**) FIMAC domain; (**b**) CD5 domain; (**c**) serine protease domain. CFI, complement factor I; CPK, Corey-Pauling-Koltun; FIMAC, Factor I membrane attack complex; 3D, three dimensional.

Table 4 | Structural analysis of the identified point mutations and summary of functional significance

Missense mutation	Domain	Location in the 3D models	Possible structural/functional impacts	Summary
C25F	FIMAC	Intra-domain disulfide bond	Stability/folding problems	Quantitative deficiency
P32A	FIMAC	Turn	Solvent exposed Stability/folding problems	Quantitative deficiency
N133S	CD5	α-Helix	Solvent exposed Stability/folding problems through quality-control mechanisms and/or inter-domain interactions	Quantitative deficiency
I398L	SP	β-Strand near α-helix and C terminus	Buried, formation of a small buried cavity most likely destabilizing the structure by 2–4 kcal/mol Stability/folding problems	Quantitative deficiency
A413T	SP	β-Strand	Buried, creation of steric clashes that may not be well tolerated in a buried and tightly packed environment[43,44]	Quantitative deficiency
I415V	SP	β-Strand	Buried, creation of a small cavity, can potentially modify the orientation of the catalytic Asp 411	Functional deficiency
W438L	SP	Loop	Solvent exposed Stability/folding problems through quality-control mechanisms and/or inter-domain interactions	Quantitative deficiency

Abbreviation: 3D, three dimensional; FIMAC, Factor I membrane attack complex.

an undefined functional consequence of the *CFI* mutation found in these patients. However, it is unlikely, as the five patients with CFHR1 deficiency have all different *CFI* mutations. Moreover, two of these mutations (I398L and G243fsX46) lead to quantitative FI deficiency. Finally, although the high prevalence of *CFHR-1* deletion in the

aHUS-CFI population may reflect an impact of this genetic variant on the penetrance of the disease, the severe course in these patients may have also influenced their recruitment in our cohort and may explain the threefold higher prevalence of CFHR-1 deficiency in *CFI* mutant aHUS patients.

Our findings establish that a deficiency in the *CFI* gene itself has a key role in the development of the disease and seems to have a specific phenotype. Our data suggest that the complement genetic background might explain the variability of the expression of the disease in aHUS patients with the *CFI* mutation, and emphasize the necessity to perform screening for *CFHR-1* deletion and for a second genetic abnormality in patients with mutations in the *CFI* gene.

MATERIALS AND METHODS

Clinical and biological screening

We studied 202 patients with aHUS (21 familial and 181 sporadic forms) referred from several Departments of Nephrology across France. We report 23 patients from this series, who were screened for mutations in *CFH*, *CFI*, *MCP*, *CFB*, and *C3* genes and for polymorphisms (p.Arg240His in the *C4BPA* gene and deletion of the *CFHR-1* gene).

Clinical data and results of standard biochemical assays were collected from each referring unit. Informed consent was obtained from all patients. The clinical history of 10 out the 23 patients with *CFI* mutations has been reported previously.[10,16,19,21]

Complement assays and genetic screening

Plasma concentrations of FH and FI were measured by ELISA, whereas concentrations of C4, C3, and FB were determined by nephelometry (Dade Behring, La Defense, Paris, France).[19,34] The membrane expression of MCP was analyzed on granulocytes from patients using phycoerythrin-conjugated antibodies (Serotec, Oxford, UK).[35] All exons were sequenced as described previously.[19,34,35] The primer sequences for *CFH*, *MCP*, *CFI*, *CFB*, *C3*, and *C4BPA* genes screening are available from the authors on request. We used the multiplex ligation-dependent probe amplification reaction for screening *CFHR-1* and *CFHR-3* genes using specific *CFHR-1* and *CFHR-3* probes (MRC-Holland, The Netherlands). In this study, we used numbering starting from the first amino acid of the FI mature protein lacking signal sequence as previously used for the majority of *CFI* mutations.[12,17–19,23]

CFI cDNA cloning and mutagenesis

Human liver RNA was retrotranscribed using the AMV Kit (Roche, Mannheim, Germany). *CFI* cDNA was amplified by PCR (35 cycles: 95 °C 1 min, 55 °C 1 min, 72 °C 1 min) using proofreading turboPFU DNA polymerase (Stratagene, La Jolla, CA, USA) and the following primers: 5′-GAACACCTCCAACATGAAGC-3′ and 5′GACATTGTACTGAGAAATAAAAGGCC-3′. cDNA was cloned in pcDNA3.1/V5-His-TOPO (Invitrogen, Paisley, Great Britain, UK) according to the manufacturer's recommendations. Point mutations were introduced by site-directed mutagenesis using the QuikChange II XL kit (Stratagene). The whole cDNA sequence of all mutants was verified by sequencing.

Expression analysis of WT and mutant FI proteins

Human embryonic kidney293 cells were seeded at 1×10^6 cells in Dulbecco's medium supplemented with 5% fetal calf serum. The next day, transient transfections were carried out with 2 μg of expression vectors encoding the WT or various mutants of FI together with 0.03 μg of green fluorescent protein expression vector (transfection control) using the FUGENE6 transfection reagent (Roche). Forty-eight hours after the transfection, supernatants were collected and cells were lysed in 50 μl of lysis buffer (1% Triton X100, 10 mM Tris-HCl). Transfection efficiency was assessed by measuring green fluorescent protein expression in cell lysates by flow cytometry and western blotting, using anti-tubulin and anti-EYFP antibodies. Independent experiments were repeated thrice. Stable transfectants of FI WT, I415V, and D506V HEK293 cells were obtained by selection with 0.6 mg/ml geneticin (Invitrogen). The quantity of FI in the supernatants was determined by ELISA using sheep anti-FI (Abcam, Paris, France).

Immunofluorescence

Human embryonic kidney293 cells were transfected as described above and grown for 48 h on microscope cover slips in the culture medium. After fixation in phosphate-buffered saline–4% paraformaldehyde for 15 min, cells were permeabilized for 5 min in phosphate-buffered saline with 0.1% Triton X-100 and washed twice for 5 min in phosphate-buffered saline containing 1% fetal calf serum. Cells were incubated for 45 min at room temperature with mouse anti-protein disulfide isomerase monoclonal antibody (Abcam) in phosphate-buffered saline–1% fetal calf serum. After washes, cells were first incubated with Cyanin 5-conjugated anti-mouse antibody (Jackson Immunology, West Grove, PA, USA) and tetramethyl−rhodamine iso-thiocyanate-conjugated wheat germ agglutinin (wheat germ agglutinin, Sigma, St Louis, MO, USA) for 45 min at room temperature, and then with mouse fluorescein isothiocyanate-conjugated anti-V5 monoclonal antibody (Invitrogen) for 45 min at room temperature. Slides were mounted with Vectashield (AbCys, Paris, France) and analyzed by laser-scanning confocal microscopy (Zeiss LSM 510, Zeiss, Le Pecq, France).

Recombinant CFI purification and fluid-phase C3b and C4b inactivation assay

Media from cells stably transfected with FI WT, mutants, or mock-transfected cells were collected, centrifuged, and filtered before loading on a HiTrap chelating HP 5 ml column (GE Healthcare, Uppsala, Sweden). The column was washed with 20 mM sodium phosphate buffer containing 500 mM NaCl, pH 7.4, and the protein was eluted with 20 mM sodium phosphate, 500 mM NaCl, and 500 mM imidazole pH 7.4. Fractions containing FI were pooled and dialyzed against Tris-buffered saline pH 8.0 overnight. FI mutants were only partially purified and their concentrations were measured by ELISA.[19]

Factor I (8 μg/ml) was mixed with 50 μg/ml C4b or 150 μg/ml C3b, 100 μg/ml C4BP or 5 μg/ml FH, and trace amounts of ^{125}I-labeled C4b or C3b in Tris-buffered saline. As a negative control (−control), FI was omitted in the incubation mixture and WT FI (20 μg/ml) was used as positive control (+ control). The samples were incubated for 3 h at 37 °C, and the reaction was terminated by addition of SDS-PAGE sample buffer with reducing agent (dithiothreitol). The samples were then incubated at 95 °C for 3 min and applied on a 10–15% gradient SDS-PAGE. Separated proteins were visualized and quantified using a PhosphorImager (Molecular Dynamics, Uppsala, Sweden). The intensity of the α-chain and the C4d fragment of C4b and the α-chain and the 43-kDa fragment of C3b were analyzed using ImageGauge (FujiFilm, Tokyo, Japan). The experiments were conducted four times.

Membrane-bound C3b inactivation assay

Modified hemolytic assay was used to assess human plasma FI and cell supernatants for their capacity to degrade C3b to iC3b in the presence of CFI-depleted plasma. Sheep erythrocytes (E) sensitized with antibody (Sigma) and bearing C3b (EAC43b) were prepared as described previously.[36] Approximately 10^7 EAC43b were incubated in gelatin veronal buffer-EDTA (75 mM NaCl) containing concentrated supernatants with various FI mutants and FI-deficient plasma providing FH as the cofactor. We used plasma samples obtained from a patient who carried two heterozygous mutations in the *CFI* gene leading to a complete FI deficiency. All mutants were used at the same concentrations as assessed by FI ELISA. At time intervals, 100 µl samples were removed, centrifuged, and 100 µl of DGVB++ were added to the pellet of cells. Under these experimental conditions, EC3b cells were converted to EC3bi in the presence of functional FI. To each sample, 100 µl of DGVB++ containing FB (0.15 µg) and Factor D (0.04 µg) were added for 30 min at 30 °C to form the C3bBb convertase. Lytic sites were developed by adding 300 µl of rat serum dilute 1:20 in gelatin veronal buffer-EDTA. Activity determinations obtained by titration were expressed as the average number of hemolytic sites per cell (Z). The capacity to form the C3 convertase at each time point was directly related to the input of residual erythrocyte-bound C3b. The percentage of residual C3b was calculated on the basis of complete lysis at the beginning of the kinetic experiment.

Construction of the three-dimensional, homology-based model of CFI

Three-dimensional models of human FI domains were built on a Silicon Graphics workstation (Silicon Graphics, Fremont, CA, USA) using the molecular modeling package Insight II (MSI) and the modules Homology, Biopolymer, Discover, and CHARMm.[37] In addition, the Loopy program was used to predict insertion loops.[38] Multiple sequence alignments and building of the initial structural models were performed using Homology. Different experimental structures were used and downloaded from the Protein Data Bank; the follistatin domain of the human osteonectin crystal (1bmo.pdb)[39] was used for constructing the Factor I membrane attack complex (FIMAC) domain (S24-T89), the human Mac2-binding scavenger receptor crystal (1by2.pdb)[40] for the CD5 domain (A90-A201), the human urokinase-type plasminogen activator crystal (1lmw.pdb),[41] for the SP domain (C309-I316 and I322-G556), and the human coagulation Factor VII (1jbu.pdb)[42] for the C-terminus region (R557-V565) of the SP domain. The sequence identity between FI domains and the templates was 35–40% with few insertions/deletions, allowing for accurate homology modeling experiments. The three-dimensional models were refined with Discover and CHARMm.[43,44]

DISCLOSURE

All the authors declared no competing interests.

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