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A Novel Non-Synonymous Polymorphism (p.Arg240His) in C4b-Binding Protein Is Associated with Atypical Hemolytic Uremic Syndrome and Leads to Impaired Alternative Pathway Cofactor Activity[1]

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Atypical hemolytic uremic syndrome (aHUS) is a disorder characterized by hemolytic anemia, thrombocytopenia, and acute renal failure. Mutations, polymorphisms, and copy number variation in complement factors and inhibitors are associated with aHUS. In this study, we report the first functional non-synonymous polymorphism in the complement inhibitor C4b-binding protein (C4BP) α-chain (c.719G>A; p.Arg240His), which is associated with aHUS. This heterozygous change was found in 6/166 aHUS patients compared with 5/542 normal ($\chi 2 = 6.021$; $p = 0.014$), which was replicated in a second cohort of aHUS patients in which we found 5/170 carriers. The polymorphism does not decrease expression efficiency of C4BP. p.Arg240His is equally efficient as the wild type in binding and supporting degradation of C4BP but its ability to bind C3b and act as cofactor to its degradation both in fluid phase and on surfaces is impaired. This observation supports the hypothesis that dysregulation of the alternative pathway of complement is pivotal for aHUS. Three of the patients carry also mutations in membrane cofactor protein and factor H strengthening the hypothesis that individuals may carry multiple susceptibility factors with an additive effect on the risk of developing aHUS. ***The Journal of Immunology,*** **2008, 180: 6385–6391.**

Atypical hemolytic uraemic syndrome (aHUS)[3] is characterized by the triad of a microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure in the absence of a preceding diarrheal illness. aHUS, although rare, is a severe disease with death rates up to 25% in the acute phase of the disease and with 50% patients requiring kidney transplantation (1). aHUS can be either sporadic or familial and defective complement regulation occurs in both forms. The disease is associated with mutations in complement inhibitors (2) factor H (*CFH*) (3–5), membrane cofactor protein (*MCP*) (6, 7) and factor I (*CFI*) (8, 9) as well as gain of function mutations in both factor B (*FB*) (10) and *C3* (11). Mutations in the genes encoding CD55 (12) and C4b-binding protein (C4BP) (13) have not yet been implicated in aHUS, although they are part of a single nucleotide polymorphism haplotype block strongly associated with severity of the disease (14).

C4BP is a plasma protein consisting of seven identical α-chains and a unique β-chain (15). The α- and β-chains contain eight and three complement control protein (CCP) domains (or short consensus repeats), respectively. CCP domains are characteristic feature of complement inhibitors and consist of ~60 amino acids forming a compact hydrophobic core that is surrounded by five or more β-strands organized into β-sheets (16). C4BP controls mainly C4b-mediated reactions thereby inhibiting the classical and lectin pathways (17) but, it does act as a factor I (FI) cofactor in degradation of C3b and therefore contributes to regulation of the alternative pathway although not as strongly as factor H (FH) (18). Each α-chain of C4BP contains a C4b/C3b binding site, but most likely due to sterical hindrance, only up to four C4b or C3b can bind to one C4BP molecule (19). We have previously localized the binding site for C4b to CCP1–3 (20), whereas binding of C3b in addition requires CCP4 (i.e., CCP1–4) (18). C4BP interacts not only with C4b (21) and C3b (18) but also with anticoagulant protein S (22), serum amyloid P component (23), heparin (24), DNA (25), and a number of bacterial proteins (26).

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[3] Abbreviations used in this paper: aHUS, atypical hemolytic uremic syndrome; C4BP, C4b-binding protein; CCP, complement control protein (domain); FH, factor H; FI, factor I; MCP, membrane cofactor protein; wt, wild type.

Table I. *Primers used for amplification of C4BPA* and *C4BPB* prior to direct sequencing

Exon	Primers 5′–3’	(Mg) mM	Temp (°C)
C4BPA			
1	(F) CAGTGCTGCTTTATTTCTGCTG	1.5	51.2
	(R) TCTGCTGAAAACACCTCCAA		
2	(F) CACTCCAGGCTGTCATTTCC	2.0	62.7
	(R) TTGATGAAGCTGGGTCTCTG		
3	(F) TAAGATGCTGTGTCCCAAGG	1.5	51.2
	(R) TCTGAGACAACTTTCCAAAGATAGG		
4	(F) TGTGTTTCTGGAAGCAGAGGTG	2.0	58.9
	(R) ATCCTTTGCTTGAGGACGGGA		
5	(F) TGGGAAATGATATCCAAGAACA	1.5	54.2
	(R) GGCTTACACTTGACAACTGAGGT		
6	(F) TCCCTGTGCATCTTTACAGGTAT	3.0	58.9
	(R) TCAGGTGGCCAAATAAATGA		
7	(F) AACTGGATTAGCAGTGGCAG	1.5	58.9
	(R) CAGTACGGGGTACCTTTAG		
8	(F) TGATACATCAGGCCTTGCAG	1.5	58.9
	(R) GTCCCCTTCTGGCACTAACA		
9	(F) TCTTCATATAAGTTTAATCC	1.5	50
	(R) ATTCCAAGAATCATGCAAG		
10	(F) TACATGTATTCTGCAATGTGCTAC	2.0	64.7
	(R) GCTCTCAGGACACGTCCAC		
11	(F) CCGAGACTGTTATCATGTCCTTC	2.0	64.7
	(R) ATGCCACCATTTTTAGGGG		
12A	(F) CTTGTTTTCCCAGCCTCAAC	1.5	62.7
	(R) CATGAGCCACACAGAGGATG		
12B	(F) GAAAATGGCCCTGGAGGTAT	1.5	58.9
	(R) TGGCCCAAACAGTAAAACAA		
C4BPB			
1	(F) GACTGGTCAACTGGATTTGCATTC	1.5	58.0
	(R) AGCTCCTATTGAGTGACGAGCAT		
2	(F) GCAAAAAGACATGAGAATGGG	1.5	58.0
	(R) GGATGTGAAGGAGTAAAACAGG		
3	(F) TGGCCTTTGCTGTGTTG	1.5	55.0
	(R) CAGGGATGTGGCAAATGTC		
4	(F) AACAGCTGCAATTAGGGGTG	1.0	55.0
	(R) CCAAAACAATGCTGCCAAG		
5	(F) CCAGTCTCCATTACCCAATTC	1.0	58.0
	(R) AGCATATGCCAGGAGTGATG		
6	(F) TTACTTAAGCTAGCCTGGTTCTGAGC	1.5	60
	(R) ACATGGTGCATTATACAACTG	1.5	

So far, no inherited defects in C4BP were described in man, and in this study, we have examined whether C4BP is an additional susceptibility factor for aHUS.

Materials and Methods

Subjects

The genes encoding C4BP α- and β-chains (*C4BPA* and *C4BPB*) were sequenced in 40 aHUS patients. In a further 126 aHUS patients and 542 normal controls (from European Collection of Cell Cultures, Health Protection Agency, Porton Down), genotyping of a novel nonsynonymous *C4BPA* single nucleotide polymorphism (c.719G>A, p.Arg240His) was undertaken either by direct sequencing or SnaPshot procedure. The amino acid numbering includes the signal sequence and nucleotide numbering was according to the published cDNA sequence (NM_000715.3) with the first nucleotide of the first methionine assigned as 1. The study was approved by the Northern and Yorkshire Multicentre Research Ethics Committee.

Genotyping of C4BPA *c.719G>A, p.Arg240His*

Genomic DNA was prepared, amplified using primers for *C4BPA* and *C4BPB* (Table I), and sequenced (6). Alternatively genotyping was performed with the ABI PRISIM SnaPshot dNTP Primer extension kit (Applied Biosystems) with subsequent detection with an ABI PRISIM 3100 Genetic analyser. SnaPshot reactions were performed using a primer 5′TCTTTAGAAATCACCTGTC at 0.4 μM concentration. The difference in genotype frequency between patients and controls was tested using the χ^2 test. The findings were replicated in an independent cohort of 170 aHUS patients using direct sequencing.

cDNA clones for recombinant proteins, their expression, and purification

cDNA coding for human C4BP α-chain was in pcDNA3 vector (Invitrogen) (20), and the mutation was introduced using the QuikChange kit (Stratagene): Arg240His (primers: 5′GAA AAA ATC ACC TGT *CAC* AAG CCA GAT GTT TCA3′ and the corresponding antisense primer). Changed nucleotides are italic. The mutation was confirmed by sequencing. Both wild type (wt) and p.Arg240His were expressed in eukaryotic cells and purified by affinity chromatography (20). For circular dichroism analysis, both proteins were dialyzed extensively against 150 mM NaF. Approximately 120 μg of each mutant were analyzed in the far UV-region (185–250 nm) using Jasco J-720 spectropolarimeter. The resolution was 1 nm, the sensitivity 20 mdeg, the speed was 10 nm/min, and every presented spectrum is the average of eight measurements.

Proteins

Human C4BP (27) and FI (28) were purified from human plasma as described in the references. C3b, C4b, C3, and factors B and D were from Complement Technology. Protein concentrations were determined from absorbance at 280 nm using known extinction coefficients or from amino acid analysis following 24-h hydrolysis in 6 M HCl (for recombinant C4BP). C4b, C3b, and C4BP were labeled with ^{125}I at 0.4–0.5 MBq/μg of protein using the chloramine T method.

Competition binding assay (C4b and C3b)

Microtiter plates (Maxisorp, Nunc) were incubated overnight at 4°C with 50 μl of solution containing 10 μg/ml C4b or C3b in 75 mM Na-carbonate (pH 9.6). The wells were washed three times with washing buffer (50 mM Tris-HCl, 0.15 M NaCl, and 0.1% Tween (pH 7.5)) and then incubated at

Table II. *Clinical information on the aHUS patients carrying p.Arg240His*

Patient ID	Sex	Age at Presentation	Renal Function	*CFH*, *MCP*, *CFI, C3*, and *FB* Mutations	*CFHR1* and *CFHR3* Copy Number	C3 (g/L)	C4 (g/L)	FH (g/L)	FI (mg/L)	C4BP (mg/L)
1	M	8	Recovered	*MCP* p.Ser206Pro *CFH, CFI, FB, C3* - nil	1	1.40	0.24	0.68	70	235 ± 27
2	M	16	Recovered	*MCP* p.Ser206Pro *CFH, CFI, FB, C3* - nil	1	0.98	0.31	n/a	n/a	64 ± 9 265 ± 47
3	F	6	Recovered	*CFH* – p.Gln950His *MCP, CFI, FB, C3* - nil	1	0.82	0.19	0.49	61	202 ± 42
4	M	8	Recovered	Nil	2	1.31	0.41	0.70	n/a	n/a
5	F	23	Recovered	Nil	2	1.65	0.28	0.58	47	286 ± 40
6	F	61	Recovered	Nil	1	1.12	0.18	0.70	66	213 ± 29

[a] Samples were taken for analysis upon first arrival to the hospital. C4BP concentration was measured using ELISA (42), C3 and C4 by rate nephelometry (Beckman Array 360), and FH and FI by radioimmunodiffusion (Binding Site). Patient 2 had low C4BP level at first analysis upon arrival to hospital but subsequent sample revealed normal level of C4BP. Normal range of concentrations: C3 0.68–1.80 g/L, C4 0.18–0.60 g/L, FH 0.35–0.59 g/L, FI 38–58 (mg/L), and C4BP 114–345 (mg/L). CFH (6), MCP (6), CFI (9), C3 (11), and FB (10) genes were sequenced as described previously. n/a: Not available.

room temperature with 200 μl of quench (washing buffer supplemented with 3% fish gelatin). After another three washes, the ^{125}I-labeled plasma purified C4BP was added (20 kcpm/well for C4b and 50 kcp/well for C3b) together with unlabeled wt or the p.Arg240His mutant diluted in 50 mM Tris-HCl and 150 mM NaCl (pH 7.5) for C4b and in 25 mM Tris-HCl and 10 mM NaCl (pH 8.0) for C3b. The samples were incubated for 4 h at room temperature, washed three times with cold washing buffer, and the amount of radioactivity associated with each well was measured in a γ-counter (18, 20).

C4b/C3b-degradation assay in a fluid phase

C4BP at 25–200 nM was mixed with 750 nM C3b (or 250 nM C4b), 60 nM FI, and trace amounts of ^{125}I-labeled C3b (or ^{125}I-C4b) in 50 μl of 50 mM Tris-HCl (pH 7.4) supplemented with 150 mM NaCl. The samples were incubated for 1.5 h at 37°C, and the reaction was terminated by the addition of SDS/PAGE sample buffer with reducing agent (dithiothreitol). The samples were then incubated at 95°C for 3 min and applied on 7.5–10% gradient SDS/PAGE gels. The separated proteins were visualized by autoradiography or using Phosphorimager (Molecular Dynamics/GE Healthcare).

Cofactor activity toward C3b deposited on surface (18, 29)

Sheep erythrocytes (0.5×10^9 cells/ml) were washed three times in GVB-Ni (1 mM veronal buffer (pH 7.3), containing 100 mM NaCl, 0.1% gelatin, and 2.5 mM $NiCl_2$). Erythrocytes were then incubated in a thermomixer (Eppendorf Scientific) at 800 rpm for 30 min at 30°C with C3 (0.5 mg/ml), factor B (0.02 mg/ml), and factor D (0.4 μg/ml). After two washes with GVB-Ni, the cells were treated with factors B and D for 5 min at 30°C, washed twice with GVB-EDTA (1 mM veronal buffer (pH 7.3), containing 100 mM NaCl, 0.1% gelatin, and 2 mM EDTA) and incubated with C3 for 20 min at 30°C. After the third and last cycle of incubation with factors B, D, and C3, the cells were washed with $DGVB^{2+}$ (2.5 mM veronal buffer (pH 7.3), containing 70 mM NaCl, 140 mM dextrose, 0.1% gelatin, 1 mM $MgCl_2$, and 0.25 mM $CaCl_2$) and incubated for 30 min at 37°C with 20 μg/ml FI and 0–400 nM of recombinant C4BP. To control the amount of deposited C3b as well as generated iC3b, the cells were incubated with mouse mAbs directed against human C3d or iC3b Ab (3.33 μg/ml each Ab; Quidel) in PBS supplemented with 1% BSA, 15 mM EDTA, and 30 mM NaN_3 followed by matched FITC-labeled secondary Abs (10 μg/ml; DakoCytomation) and analysis by flow cytometry (FACS Calibur, BD Biosciences).

Alternatively, a hemolytic assay was used in which sheep erythrocytes were coated with C3b by sequential incubation with amboceptor, C1, C4, C2 and C3 to obtain EAC3b (30). Approximately 10^7 EAC3b were incubated in Mg-EGTA (2.5 mM veronal buffered saline containing 140 mM dextrose, 0.1% gelatin, 7 mM $MgCl_2$ and 40 mM EGTA) with 70 nM C4BP and 80 nM FI at 37°C. Under these experimental conditions, 20–50% EC3b cells were converted to EC3bi. At indicated time intervals, 120 μl samples were removed, centrifuged and 120 μl of Mg-EGTA containing Factor B (25 ng/ml), Factor D (50 ng/ml) and properdin (0.5 μg/ml) were added to the pellet for 20 min at 30°C to form the C3bBb convertase. Lytic sites were developed by removing 50 μl of samples and adding 150 μl of guinea-pig serum diluted at 1:45 in GVB-EDTA (2.5 mM veronal buffered saline, 0.1% gelatin, 40 mM EDTA). After 1h incubation the samples were centrifuged and the amount of lysis was determined spectrophotometrically.

Results

Prevalence of C4BPA *c.719G>A, p.Arg240His*

All exons of *C4BPA* and *C4BPB* were sequenced in 40 aHUS patients and lead to identification of a novel heterozygous nonsynonymous change (c.719G>A, p.Arg240His) in exon 7 of *C4BPA* (encodes CCP4 of the α-chain of C4BP). To analyze prevalence of this change, a larger group of patients and healthy controls was analyzed. We found this heterozygous change in 6/166 aHUS patients vs 5/542 normal controls ($\chi 2 = 6.021$, $p = 0.014$). The clinical details of the six aHUS patients carrying p.Arg240His are given in Table II. Three of six patients carry other known susceptibility factors for aHUS such as p.Ser206Pro in MCP and p.Gln950His in FH. Patient 4 presented with an associated diarrheal illness at the time of his HUS but his stool culture was clearly negative and therefore he was diagnosed as the aHUS case. However, a subsequent convalescent serum sample was positive for *Escherichia coli* O157 Abs, but it is not possible to know whether the Abs were generated during the infection associated with the current illness or perhaps previously in life. All patients recovered without need for renal replacement therapy. This is in contrast to patients carrying mutations in other complement inhibitors and who most often require transplantation. For instance, in our cohort, as in others, patients known to have a factor H mutation have a poor long-term outcome with ~40% requiring renal replacement therapy (2).

To confirm our findings in and independent cohort of aHUS patients, we determined by direct sequencing the frequency of the Arg240His change in the French aHUS cohort in collaboration with Dr. Fremeaux-Bacchi. We found that 5 of 170 (3%) aHUS patients carried the Arg240His change ($\chi 2 = 3.808$, $p = 0.051$). Of the five carriers in that cohort, one also had a *C3* mutation, one a heterozygous *MCP* mutation, one a *CFI* mutation, and one autoantibodies against FH while in one patient we could not identify any known susceptibility risk.

p.Arg240His does not decrease expression level of C4BP

To determine whether C4BP expression was affected by the polymorphism, we have expressed p.Arg240His and wt in a transient manner in a human HEK293 cell line. We found that both the p.Arg240His and the wt expressed well (Fig. 1*A*). We have also observed that stably transfected clones used for protein purification

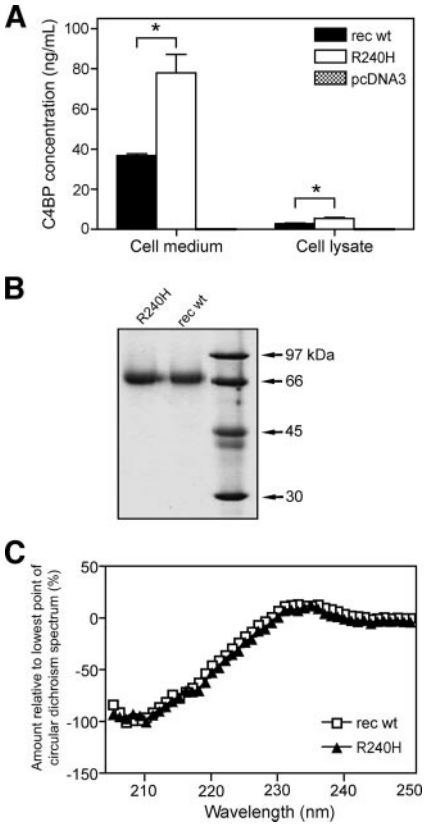

FIGURE 1. The p.Arg240His expresses well and is not affected by folding problems. *A*, Expression of C4BP variants after transient transfection. HEK293 cells were transiently transfected with C4BP variants and an empty vector using lipofectin. C4BP level was measured by ELISA in cell lysates and the conditioned media collected during 48-h incubation. Results of three independent tranfections are shown as means with bars indicating SD. Statistical analysis was performed using Student's *t* test. $*$, $p < 0.05$. *B*, SDS/PAGE analysis of recombinant wt C4BP and the p.Arg240His mutant which were expressed in eucaryotic cells and purified by affinity chromatography. The proteins were separated by 10% SDS/PAGE under reducing conditions and stained with Coomassie. m.w. markers are shown to the right. *C*, Circular dichroism analysis of wt C4BP and the p.Arg240His mutant. Approximately 120 μg of each mutant were analyzed in the far UV-region (185–250 nm), and every presented spectrum is the average of eight measurements. In all figures the p.Arg240His mutant is simplified as R240H.

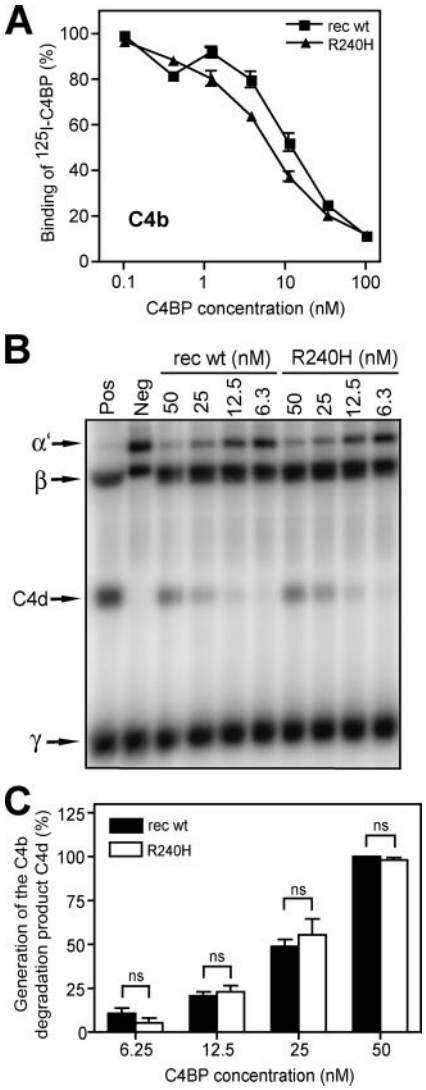

FIGURE 2. The p.Arg240His polymorphism does not affect binding and degradation of C4b. *A*, Competition assay: increasing concentrations of fluid phase wt C4BP or the p.Arg240His mutant competed with trace amounts of ^{125}I-labeled C4BP for binding of immobilized C4b. The 100% binding was estimated in the absence of fluid phase competitor. Results of two independent experiments performed in triplicates are shown. *B*, C4b-degradation assay: C4BP variants (6–50 nM) were incubated with 250 nM C4b 200 nM FI and trace amounts of ^{125}I-labeled C4b for 1.5 h at 37°C. Immediately afterward, a sample buffer with reducing agent was added, samples were heated at 95°C, and the proteins separated by SDS/PAGE electrophoresis (7.5–10% gradient gel). The gel was dried and subjected to autoradiography. As a control, FI was omitted in the incubation mixture. *C*, Results of densitometric analysis (the C4d fragment) of three independent C4b-degradation assays presented as means ± SD. Statistical analysis was performed using Student's *t* test; ns-Not significant.

expressed comparable amounts of p.Arg240His and the wt. Accordingly, the plasma level of C4BP was normal in the five tested patients (Table II).

Expression and characterization of recombinant C4BP variants

To analyze whether the polymorphism affects any function of C4BP, both recombinant C4BP molecules were expressed in a stable manner in a human HEK293 cell line and purified from culture media by affinity chromatography using mAb mAb104 directed against CCP1 of α-chain. Both forms of C4BP were analyzed by SDS/PAGE under both reducing and nonreducing conditions (Fig. 1*B*). Both proteins migrated with the same apparent velocity and both were correctly assembled into high m.w. complexes composed of six α-chains. Furthermore, both proteins had similar folding as assessed by circular dichroism (Fig. 1*C*). Taken together, it does not appear that the mutation cause any gross folding problems.

p.Arg240His binds C4b and acts as a FI cofactor in degradation of C4b as well as the wt

The ability of C4BP variants to bind C4b was tested using a direct binding assay in which microtiter plates were covered with C4b and the ^{125}I-C4BP was added together with increasing concentrations of the wt and the p.Arg240His mutant. We found that purified wt and p.Arg240His C4BP bound C4b well with the p.Arg240His showing significant 1.6-fold increase in the binding (Fig. 2*A*). The difference between the two curves was statistically significant at a concentration of C4BP yielding 50% binding as determined by

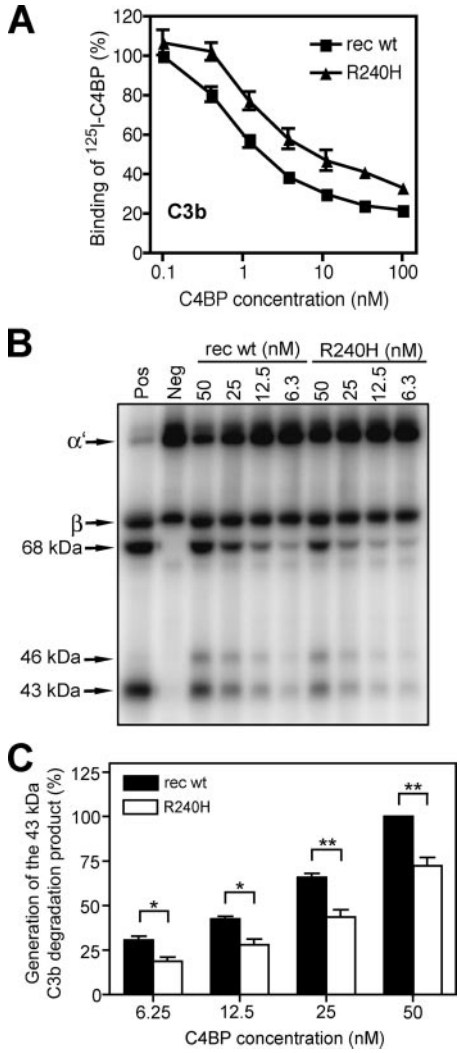

FIGURE 3. The p.Arg240His mutant of C4BP serves poorly as cofactor in degradation of C3b in a solution. *A*, Competition assay: increasing concentrations of fluid phase wt C4BP or the p.Arg240His mutant competed with trace amounts of ^{125}I-labeled C4BP for binding of immobilized C3b. The 100% binding was estimated in the absence of fluid phase competitor. Results of two independent experiments performed in triplicates are shown. *B*, C3b-degradation assay was performed as in Fig. 2*B* except that 750 mM C3b was used instead of C4b. *C*, Results of densitometric analysis of three independent C3b-degradation assays (43 kDa fragment of iC3b).

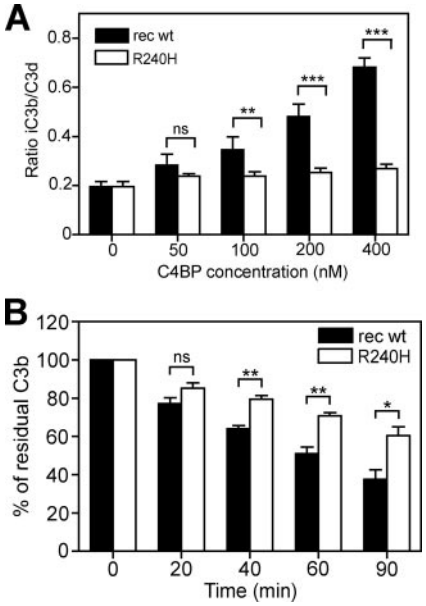

FIGURE 4. The p.Arg240His mutant of C4BP serves poorly as cofactor in degradation of C3b in a solution. *A*, Cofactor activity of C4BP variants toward C3b deposited on a surface. Sheep erythrocytes were coated with C3b and incubated with C4BP and FI for 1 h, upon which generated C3b and iC3b were assessed by flow cytometry using specific Abs. The experiments were performed on three independent occasions in duplicates. *B*, Hemolytic assay measuring cofactor activity of C4BP variants toward C3b deposited on a surface. Sheep erythrocytes were coated with C3b by sequential incubation with amboceptor, C1, C4, C2, and C3. EAC3b were incubated with 70 nM C4BP and 80 nM FI at 37°C. At indicated time intervals, aliquots were removed, centrifuged, and incubated with Factor B, D, and properdin to form the C3bBb convertases. Lytic sites were developed by adding guinea-pig serum for 1 h, the samples were centrifuged, and the amount of lysis was determined spectrophotometrically. The experiment was performed on three independent occasions in duplicates. *C–E* statistical analysis was performed using Student's *t* test. Ns-Not significant; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$; bars indicate SD.

Student's *t* test. To assess the FI cofactor activity of C4BP in a fluid phase, C4BP variants were incubated with ^{125}I-labeled C4b and FI. Proteins were then separated and visualized by autoradiography (Fig. 2*B*) followed by densitometric detection of C4d product. The p.Arg240His mutant was equally as good a cofactor in the cleavage of C4b as wt (Fig. 2*C*).

The p.Arg240His mutant binds C3b with lower affinity than the wt and shows impaired cofactor activity in degradation of C3b

C4BP interacts with C3b and is an efficient cofactor in the degradation of C3b by FI (18, 31). CCP4, in which p.Arg240His resides, is required for binding of C3b (18). We found that p.Arg240His bound C3b with a significantly 4-fold lower affinity than wt (Fig. 3*A*). We have used Student's *t* test at a concentration of C4BP yielding 50% binding to assess significance of the difference. Three assays were used to test whether this impaired affinity for C3b had functional consequences. First, C4BP, FI and ^{125}I-C3b were incubated in a fluid phase and in the presence of the wt C4BP, α-chain subunit of C3b disappeared and several degradation products of 68, 46, and 3 kDa became apparent (Fig. 3*B*). We have performed densitometric analysis of α-chain and 43 kDa degradation products and found that there was a significant difference in cofactor activity between wt and p.Arg240His at all concentrations tested (Fig. 3, *B* and *C*).

C4BP acts also as a cofactor in degradation of deposited C3b. Erythrocytes coated with C3b were incubated with FI and C4BP followed by detection of C3b and degradation product iC3b with specific Abs. Although wt C4BP degraded C3b into iC3b in a dose-dependent manner, p.Arg240His was severely impaired (Fig. 4*A*). This was confirmed by independent hemolytic assay in which the mutant could not support degradation of C3b as well as the wt (Fig. 4*B*). Thus, p.Arg240His clearly has impaired ability to regulate the alternative pathway of complement.

Discussion

The involvement of the complement system in the pathogenesis of aHUS was suggested in the early observation that aHUS patients exhibited complement activation as reflected by low plasma C3 levels and/or C3 deposition in the kidney (32). Accordingly, genetic abnormalities in several complement inhibitors have been shown to predispose to aHUS and are found in ~50% cases. Often more than one mutation or a predisposing polymorphism is found

in the same patient. The complement regulator most extensively studied has been FH, in which aHUS-associated mutations affect binding to polyanionic glycosaminoglycans (33) and C3b (34), which decreases the protective effect of FH on endothelial cells. Mutations in MCP reduce its surface expression or/and ability to inhibit complement yet again predisposing renal endothelial cells vulnerable (35, 36). FH and MCP are both cofactors to FI, and therefore, it was not surprising that alterations in FI are also found in aHUS. The outcome of transplantation is poor in patients with either a *CFH* or *FI* mutation while patients known to have only a *MCP* mutation have a satisfactory transplantation outcome. This is expected because MCP is a transmembrane regulator and allografts will therefore be protected by wt MCP. Taken together, only two complement inhibitors have not been implicated in aHUS so far–decay accelerating factor (CD55) and C4BP. The present report is the first one to describe an alteration in the C4BP α-chain associated with aHUS. This novel non-synonymous polymorphism p.Arg240His was found in 6 of 166 investigated aHUS patients and in 5 of 177 patients in independent cohort. The p.Arg240His polymorphism was, however, also identified in 5 of 542 healthy controls. In two (siblings) of the aHUS patients carrying p.Arg240His polymorphism, we had previously identified a change in *MCP* (S206P), which severely impairs binding to C3b (36). In another patient, we found additional FH polymorphism that was in some (37) but not all studies (38) associated with aHUS. However, in the three remaining, not related, patients no additional predisposing factors were identified yet. We, therefore, suggest that *C4BPA* and in particular p.Arg240His may be another susceptibility factor for the development of aHUS. The phenotype of the six aHUS patients carrying this change is unusual in that all recovered renal function and suggests that the effect of p.Arg240His in C4BP is not as pronounced as in many patients carrying a *CFH* or *CFI* mutation.

The polymorphism does not appear to affect C4BP level in serum as observed by measurement of C4BP in the carriers and normal controls. Furthermore, both wt and p.Arg240His variant expressed well in eucaryotic cells that were transfected in a stable or transient manner. We have also analyzed whether p.Arg240His is equally stable in human serum as the wt. To this end equal, physiological concentrations of the two variants were added to serum depleted from C4BP and incubated for up to 24 h. The functional activity of the two variants was then assessed in a C4b-degradation assay and no differences were found (not shown).

Because p.Arg240His was also found in ~1% of normal subjects, we have assessed the functional significance of this change. Therefore, recombinant p.Arg240His was expressed, purified, and compared with the wt. Interestingly, we did not detect any defect in binding of C4b and its ensuing degradation implying that p.Arg240His does not affect the ability of C4BP to regulate the classical and the lectin pathways of complement. This is in full agreement with our previous data showing that only CCP1–3 of the C4BP α-chain are needed for binding and degradation of C4b (20). The p.Arg240His polymorphism is located in CCP4 and should not affect interaction with C4b. However, the p.Arg240His mutant displayed statistically significant decreased binding to C3b and supported C3b degradation both in solution and on surface significantly less efficient than the wt. This observation is in good agreement with the fact that CCP1–4 are known to be required for full binding of C3b and its degradation (18). Analysis of 3D model of C4BP (39) shows that Arg240 is solvent exposed and forms a salt bridge with Asp284. The mutation, found in a domain interacting with C3b, could thus impede C3b binding either directly or indirectly, through minor local structural changes. Both Arg and His are classified as positively charged amino acids but there are significant differences between these two: His is a much shorter amino acid and it has much lower pKa value than Arg. Therefore, His may not always be positively charged depending on surrounding amino acids. Interestingly, arginines are often part of hot spots on protein surfaces, i.e., areas that are involved in interactions (40). In fact Arg240 forms the crucial part of an epitope for a mAb (MK67) directed against C4BP CCP4 (data not shown). This suggests that this Arg240 could definitively contribute to binding of C3b as its side chain is accessible for protein-protein interaction.

The association of C4BP with aHUS is not as strong as for most defects in *CFH*/*MCP*/*CFI*, and our findings suggest that C4BP is unlikely to be as significant a predisposing factor but they do support the hypothesis that C4BP is an additional susceptibility factor for the development of aHUS and that uncontrolled activation of the alternative pathway on cellular surfaces is pivotal for the disease (41). The relative importance of C4BP for the regulation of the alternative pathway in comparison with FH is not clear. In the quantitative in vitro comparison, we found that C4BP appears to be a comparable cofactor in degradation of C3b in a solution to FH but that C4BP is much weaker when C3b is to be degraded on surfaces (18). Considering the abundance of FH, the polymorphism in C4BP is unlikely to be a primary predisposing factor but a rather a secondary modifier which has an additive effect. Moreover, that this polymorphism was found in three patients with mutations in *MCP* and *CFH* adds weight to the hypothesis that individuals may carry multiple susceptibility factors in the complement system and that these have an additive effect on the risk of developing the disease.

Disclosures

The authors have no financial conflict of interest.

References

1. Kavanagh, D., T. H. Goodship, and A. Richards. 2006. Atypical haemolytic uraemic syndrome. *Br. Med. Bull.* 77–78: 5–22.
2. Caprioli, J., M. Noris, S. Brioschi, G. Pianetti, F. Castelletti, P. Bettinaglio, C. Mele, E. Bresin, L. Cassis, S. Gamba, et al. 2006. Genetics of HUS: the impact of MCP, CFH, and IF mutations on clinical presentation, response to treatment, and outcome. *Blood* 108: 1267–1279.
3. Warwicker, P., T. H. Goodship, R. L. Donne, Y. Pirson, A. Nicholls, R. M. Ward, P. Turnpenny, and J. A. Goodship. 1998. Genetic studies into inherited and sporadic hemolytic uremic syndrome. *Kidney Int.* 53: 836–844.
4. Perez-Caballero, D., C. Gonzalez-Rubio, M. E. Gallardo, M. Vera, M. Lopez-Trascasa, S. Rodriguez de Cordoba, and P. Sanchez-Corral. 2001. Clustering of missense mutations in the C-terminal region of factor H in atypical hemolytic uremic syndrome. *Am. J. Hum. Genet.* 68: 478–484.
5. Caprioli, J., P. Bettinaglio, P. F. Zipfel, B. Amadei, E. Daina, S. Gamba, C. Skerka, N. Marziliano, G. Remuzzi, and M. Noris. 2001. The molecular basis of familial hemolytic uremic syndrome: mutation analysis of factor H gene reveals a hot spot in short consensus repeat 20. *J. Am. Soc. Nephrol.* 12: 297–307.
6. Fremeaux-Bacchi, V., E. J. Kemp, J. A. Goodship, M. A. Dragon-Durey, L. Strain, C. Loirat, H. W. Deng, and T. H. Goodship. 2005. The development of atypical haemolytic-uraemic syndrome is influenced by susceptibility factors in factor H and membrane cofactor protein: evidence from two independent cohorts. *J. Med. Genet.* 42: 852–856.
7. Richards, A., E. J. Kemp, M. K. Liszewski, J. A. Goodship, A. K. Lampe, R. Decorte, M. H. Muslumanoglu, S. Kavukcu, G. Filler, Y. Pirson, et al. 2003. Mutations in human complement regulator, membrane cofactor protein (CD46), predispose to development of familial hemolytic uremic syndrome. *Proc. Natl. Acad. Sci. USA* 100: 12966–12971.
8. Fremeaux-Bacchi, V., M. A. Dragon-Durey, J. Blouin, C. Vigneau, D. Kuypers, B. Boudailliez, C. Loirat, E. Rondeau, and W. H. Fridman. 2004. Complement factor I: a susceptibility gene for atypical haemolytic uraemic syndrome. *J. Med. Genet.* 41: e84.
9. Kavanagh, D., E. J. Kemp, E. Mayland, R. J. Winney, J. S. Duffield, G. Warwick, A. Richards, R. Ward, J. A. Goodship, and T. H. Goodship. 2005. Mutations in complement factor I predispose to development of atypical hemolytic uremic syndrome. *J. Am. Soc. Nephrol.* 16: 2150–2155.
10. Goicoechea de Jorge, E., C. L. Harris, J. Esparza-Gordillo, L. Carreras, E. A. Arranz, C. A. Garrido, M. Lopez-Trascasa, P. Sanchez-Corral, B. P. Morgan, and S. Rodriguez de Cordoba. 2007. Gain-of-function mutations in complement factor B are associated with atypical hemolytic uremic syndrome. *Proc. Natl. Acad. Sci. USA* 104: 240–245.

11. Fremeaux-Bacchi, V., C. Regnier, J. Blouin, M. A. Dragon-Durey, W. H. Fridman, B. Janssenc, and C. Loirat. 2007. Protective or aggressive: paradoxical role of C3 in atypical hemolytic uremic syndrome. *Mol. Immunol.* 44: 172.
12. Kavanagh, D., R. Burgess, D. Spitzer, A. Richards, M. L. Diaz-Torres, J. A. Goodship, D. E. Hourcade, J. P. Atkinson, and T. H. Goodship. 2007. The decay accelerating factor mutation I197V found in hemolytic uraemic syndrome does not impair complement regulation. *Mol. Immunol.* 44: 3162–3167.
13. Sellier-Leclerc, A. L., V. Fremeaux-Bacchi, M. A. Dragon-Durey, M. A. Macher, P. Niaudet, G. Guest, B. Boudailliez, F. Bouissou, G. Deschenes, S. Gie, et al. 2007. Differential impact of complement mutations on clinical characteristics in atypical hemolytic uremic syndrome. *J. Am. Soc. Nephrol.* 18: 2392–2400.
14. Esparza-Gordillo, J., E. Goicoechea de Jorge, A. Buil, L. Carreras Berges, M. Lopez-Trascasa, P. Sanchez-Corral, and S. Rodriguez de Cordoba. 2005. Predisposition to atypical hemolytic uremic syndrome involves the concurrence of different susceptibility alleles in the regulators of complement activation gene cluster in 1q32. *Hum. Mol. Genet.* 14: 703–712.
15. Kask, L., A. Hillarp, B. Ramesh, B. Dahlbäck, and A. M. Blom. 2002. Structural requirements for the intra-cellular subunit polymerization of the complement inhibitor C4b-binding protein. *Biochemistry* 41: 9349–9357.
16. Kirkitadze, M. D., and P. N. Barlow. 2001. Structure and flexibility of the multiple domain proteins that regulate complement activation. *Immunol. Rev.* 180: 146–161.
17. Blom, A. M., B. O. Villoutreix, and B. Dahlback. 2004. Functions of human complement inhibitor C4b-binding protein in relation to its structure. *Arch. Immunol. Ther. Exp.* 52: 83–95.
18. Blom, A. M., L. Kask, and B. Dahlbäck. 2003. CCP1–4 of the C4b-binding protein α-chain are required for factor I mediated cleavage of C3b. *Mol. Immunol.* 39: 547–556.
19. Ziccardi, R. J., B. Dahlbäck, and H. J. Müller Eberhard. 1984. Characterization of the interaction of human C4b-binding protein with physiological ligands. *J. Biol. Chem.* 259: 13674–13679.
20. Blom, A. M., L. Kask, and B. Dahlbäck. 2001. Structural requirements for the complement regulatory activities of C4BP. *J. Biol. Chem.* 276: 27136–27144.
21. Scharfstein, J., E. B. Correa, and G. R. Gallo. 1978. Human C4b-binding protein: association with immune complexes in vitro and in vivo. *J. Clin. Invest.* : 437–442.
22. Dahlbäck, B., and J. Stenflo. 1981. High molecular weight complex in human plasma between vitamin K-dependent protein S and complement component C4b-binding protein. *Proc. Natl. Acad. Sci. USA* 78: 2512–2516.
23. Schwalbe, R., B. Dahlbäck, A. Hillarp, and G. Nelsestuen. 1990. Assembly of protein S and C4b-binding protein on membranes. *J. Biol. Chem.* 265: 16074–16081.
24. Hessing, M., R. A. Vlooswijk, T. M. Hackeng, D. Kanters, and B. N. Bouma. 1990. The localization of heparin-binding fragments on human C4b-binding protein. *J. Immunol.* 144: 204–208.
25. Trouw, L. A., S. C. Nilsson, I. Goncalves, G. Landberg, and A. M. Blom. 2005. C4b-binding protein binds to necrotic cells and DNA, limiting DNA release and inhibiting complement activation. *J. Exp. Med.* 201: 1937–1948.
26. Blom, A. M., B. O. Villoutreix, and B. Dahlbäck. 2004. Complement inhibitor C4b-binding protein - friend or foe in the innate immune system? *Mol. Immunol.* 40: 1333–1346.
27. Dahlbäck, B. 1983. Purification of human C4b-binding protein and formation of its complex with vitamin K-dependent protein S. *Biochem. J.* 209: 847–856.
28. Crossley, L., and R. Porter. 1980. Purification of the human complement control protein C3b inactivator. *Biochem. J.* 191: 173–182.
29. Nilsson, S. C., D. Karpman, F. Vaziri-Sani, A. C. Kristoffersson, R. Salomon, F. Provot, V. Fremeaux-Bacchi, L. A. Trouw, and A. M. Blom. 2007. A mutation in factor I that is associated with atypical hemolytic uremic syndrome does not affect the function of factor I in complement regulation. *Mol. Immunol.* 44: 1845–1854.
30. Fremeaux-Bacchi, V., L. Weiss, C. Demouchy, A. May, S. Palomera, and M. D. Kazatchkine. 1994. Hypocomplementaemia of poststreptococcal acute glomerulonephritis is associated with C3 nephritic factor (C3NeF) IgG autoantibody activity. *Nephrol. Dial. Transplant.* 9: 1747–1750.
31. Nagasawa, S., and R. M. Stroud. 1977. Mechanism of action of the C3b inactivator: requirement for a high molecular weight cofactor (C3b-C4bINA cofactor) and production of a new C3b derivative. *Immunochemistry* 14: 749–756.
32. Thompson, R. A., and M. H. Winterborn. 1981. Hypocomplementaemia due to a genetic deficiency of β 1H globulin. *Clin. Exp. Immunol.* 46: 110–119.
33. Jokiranta, T. S., Z. Z. Cheng, H. Seeberger, M. Jozsi, S. Heinen, M. Noris, G. Remuzzi, R. Ormsby, D. L. Gordon, S. Meri, et al. 2005. Binding of complement factor H to endothelial cells is mediated by the carboxy-terminal glycosaminoglycan binding site. *Am. J. Pathol.* 167: 1173–1181.
34. Jokiranta, T. S., V. P. Jaakola, M. J. Lehtinen, M. Parepalo, S. Meri, and A. Goldman. 2006. Structure of complement factor H carboxyl-terminus reveals molecular basis of atypical haemolytic uremic syndrome. *EMBO J.* 25: 1784–1794.
35. Fremeaux-Bacchi, V., E. A. Moulton, D. Kavanagh, M. A. Dragon-Durey, J. Blouin, A. Caudy, N. Arzouk, R. Cleper, M. Francois, G. Guest, et al. 2006. Genetic and functional analyses of membrane cofactor protein (CD46) mutations in atypical hemolytic uremic syndrome. *J. Am. Soc. Nephrol.* 17: 2017–2025.
36. Liszewski, M. K., M. K. Leung, B. Schraml, T. H. Goodship, and J. P. Atkinson. 2007. Modeling how CD46 deficiency predisposes to atypical hemolytic uremic syndrome. *Mol. Immunol.* 44: 1570–1579.
37. Caprioli, J., F. Castelletti, S. Bucchioni, P. Bettinaglio, E. Bresin, G. Pianetti, S. Gamba, S. Brioschi, E. Daina, G. Remuzzi, and M. Noris. 2003. Complement factor H mutations and gene polymorphisms in haemolytic uraemic syndrome: the C-257T, the A2089G, and the G2881T polymorphisms are strongly associated with the disease. *Hum. Mol. Genet.* 12: 3385–3395.
38. Neumann, H. P., M. Salzmann, B. Bohnert-Iwan, T. Mannuelian, C. Skerka, D. Lenk, B. U. Bender, M. Cybulla, P. Riegler, A. Konigsrainer, et al. 2003. Haemolytic uraemic syndrome and mutations of the factor H gene: a registry-based study of German speaking countries. *J. Med. Genet.* 40: 676–681.
39. Blom, A. M., A. Foltyn-Zadura, B. O. Villoutreix, and B. Dahlbäck. 2000. Positively charged amino acids at the interface between α-chain CCP1 and CCP2 of C4BP are required for regulation of the classical C3-convertase. *Mol. Immunol.* 37: 445–453.
40. Ma, B., T. Elkayam, H. Wolfson, and R. Nussinov. 2003. Protein-protein interactions: structurally conserved residues distinguish between binding sites and exposed protein surfaces. *Proc. Natl. Acad. Sci. USA* 100: 5772–5777.
41. Pickering, M. C., E. G. de Jorge, R. Martinez-Barricarte, S. Recalde, A. Garcia-Layana, K. L. Rose, J. Moss, M. J. Walport, H. T. Cook, S. R. de Cordoba, and M. Botto. 2007. Spontaneous hemolytic uremic syndrome triggered by complement factor H lacking surface recognition domains. *J. Exp. Med.* 204: 1249–1256.
42. Kask, L., L. Trouw, B. Dahlbäck, and A. M. Blom. 2004. C4b-binding protein-protein S complex inhibits phagocytosis of apoptotic cells. *J. Biol. Chem.* 279: 23869–23873.