THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 13, pp. 11082–11090, April 1, 2011

Disease-associated N-terminal Complement Factor H Mutations Perturb Cofactor and Decay-accelerating Activities*

Received for publication, December 13, 2010, and in revised form, January 25, 2011 Published, JBC Papers in Press, January 26, 2011, DOI 10.1074/jbc.M110.211839

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Many mutations associated with atypical hemolytic uremic syndrome (aHUS) lie within complement control protein modules 19–20 at the C terminus of the complement regulator factor H (FH). This region mediates preferential action of FH on self, as opposed to foreign, membranes and surfaces. Hence, speculation on disease mechanisms has focused on deficiencies in regulation of complement activation on glomerular capillary beds. Here, we investigate the consequences of aHUS-linked mutations (R53H and R78G) within the FH N-terminal complement control protein module that also carries the I62V variation linked to dense-deposit disease and age-related macular degeneration. This module contributes to a four-module C3b-binding site (FH1–4) needed for complement regulation and sufficient for fluid-phase regulatory activity. Recombinant FH1–4^{V62} and FH1–4^{I62} bind immobilized C3b with similar affinities (K_D = 10–14 μM), whereas FH1–4^{I62} is slightly more effective than FH1–4^{V62} as cofactor for factor I-mediated cleavage of C3b. The mutant (R53H)FH1–4^{V62} binds to C3b with comparable affinity (K_D ~12 μM) yet has decreased cofactor activities both in fluid phase and on surface-bound C3b, and exhibits only weak decay-accelerating activity for C3 convertase (C3bBb). The other mutant, (R78G)FH1–4^{V62}, binds poorly to immobilized C3b (K_D >35 μM) and is severely functionally compromised, having decreased cofactor and decay-accelerating activities. Our data support causal links between these mutations and disease; they demonstrate that mutations affecting the N-terminal activities of FH, not just those in the C terminus, can predispose to aHUS. These observations reinforce the notion that deficiency in any one of several FH functional properties can contribute to the pathogenesis of this disease.

Surface deposition and self-propagation of the activation-specific opsonic complement-protein fragment, C3b, is tightly regulated. Effective control minimizes damage to host tissue by the potentially destructive complement system (1–3). At least three diseases, age-related macular degeneration (AMD)[2] (4–7), dense-deposit disease (DDD) (4, 8), and atypical hemolytic uremic syndrome (aHUS) (9–15), are linked to deficiencies in complement regulatory processes. Mutations and polymorphisms associated with one or more of these three pathologies occur in genes for at least six complement proteins. Two of these, C3 (16) (the precursor of C3b) and factor B (FB) (17), participate directly in the C3b-generating amplification cascade of the "tickover" or alternative pathway (AP). The remainder are regulatory proteins that help to down-regulate and inactivate C3b: C4b-binding protein (18), membrane cofactor protein (15, 19, 20), factor I (FI) (21) and complement factor H (FH) (9, 10). In addition to dysregulation of the complement cascade caused by mutations in its components or regulators, autoantibodies to FH have been described in patients with AMD (22) and aHUS (15, 23–25) that prevent association of FH with cell surfaces.

Both AMD and DDD feature tissue-specific accumulations of extracellular deposits in affected tissues. Macular drusen are an early hallmark of AMD, a common cause of blindness among the elderly (26). Complement regulators occur within drusen, as do activation-derived protein fragments from the amplification stages and terminal pathway of complement (26, 27). The dense deposits of DDD, a severe, predominantly pediatric, kidney disorder (28), accumulate in the glomerular basement membrane, and their protein content likewise strongly suggests complement activation. Like DDD, aHUS is a rare inheritable chronic kidney disease caused by complement hyperactivity; but aHUS is characterized by microvascular endothelial cell activation, thrombocytopenia, and hemolytic anemia leading to end-stage renal failure (29).

Sequence variations in the gene for complement regulator FH are linked to all three diseases (30). FH (31) is an abundant plasma glycoprotein (350–600 mg/liter) that regulates the AP of complement. It thereby ensures that soluble C3 levels will not become exhausted by excessive complement activation in the fluid phase and that C3b levels on surfaces are prevented from self-propagating. A single-nucleotide polymorphism

* This work was supported by Medical Research Council United Kingdom Grant G0701298 (to C. L. H.), Wellcome Trust Grant 081179 (to P. N. B.), and the Kidney Research United Kingdom and the Academy of Medical Sciences (D. K.).

⌘ *Author's Choice*—Final version full access.

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[2] The abbreviations used are: AMD, age-related macular degeneration; aHUS, atypical hemolytic uremic syndrome; AP, alternative pathway; CCP, complement control protein module; DDD, dense-deposit disease; FB, factor B; FD, factor D; FH, factor H; FH1–4, factor H modules 1–4; FI, factor I; IH_{50}, 50% inhibition of hemolysis; NHS, normal human serum; SNP, single-nucleotide polymorphism; SPR, surface plasmon resonance.

(SNP) leading to substitution of His for Tyr at FH position 402 (Y402H) confers increased risk of AMD and DDD (4–7); another SNP encoding Ile (rather than Val) at position 62 confers decreased risk of these diseases (4). But the very great majority of variations in FH predisposing to aHUS are single-point missense mutations in the C-terminal segment of the protein (11).

No definitive explanation of why particular mutations or SNPs predispose to one disease rather than another exists despite significant knowledge of structure-function relationships of FH (32). FH (see Fig. 1*a*) is composed entirely of 20 CCP modules (33, 34) that although similar in sequences and structures (35) are diverse in function (36–38). The N-terminal four CCPs (that encompass the V62I SNP) are necessary and sufficient to perform two principal FH activities: cofactor for FI-mediated destruction of fluid-phase C3b, and accelerated decay of the C3b-containing complexes that enzymatically activate C3 to C3b (39, 40) in the AP. The seventh CCP (wherein lies the Y402H SNP) may, through binding to glycosaminoglycans or other polyanions (38, 41, 42), assist FH to dock onto self-surfaces (as opposed to foreign ones) requiring protection from complement. CCP modules 19 and 20, the site of nearly all aHUS-linked missense mutations, encompass additional strong C3b-binding and surface-binding sites. The C terminus likely helps FH distinguish self from foreign surfaces on the basis of polyanionic carbohydrates (43–45). It is vital for controlling the AP of complement on cell surfaces but is not a requirement of fluid-phase AP regulation.

Mice in which wild-type FH was genetically replaced with a truncated version (missing CCP modules 16–20) developed aHUS-like symptoms (46). Notably, mice in which FH had been knocked out altogether and in which plasma C3 levels are consequently severely depleted due to lack of fluid-phase regulation, develop DDD-like pathology (47). It is also notable that those disease-linked FB and C3 mutations that differentially affect susceptibility to regulation by fluid-phase regulators or cell surface regulators, predispose to DDD, or to aHUS, respectively (17, 48). Together, these findings suggest that aHUS could arise when defective host surface protection coincides with a level of fluid-phase complement regulation that is sufficient to avoid significant depletion of plasma C3 and factor B levels. In such a scenario, sufficient C3 and FB are available to activate the complement cascade on inadequately protected host surfaces, with pathogenic consequences.

We set out to characterize aHUS-linked mutations R53H (49) and R78G (50) that occur within the N-terminal CCP of FH and are not involved in self-surface recognition. We show that these mutations disrupt function but that they do so by different mechanisms because R53H, but not R78G, bind normally to C3b.

EXPERIMENTAL PROCEDURES

Preparation of Plasmids (Including DNA)—Native DNA coding for human FH^{V62} was amplified from the human universal QUICK-Clone cDNA library (Clontech) and cloned into pCR4Blunt-TOPO vector (Invitrogen). The DNA coding for FH CCP modules $1–4^{V62}$ (*i.e.* residues 19–263) was amplified from this FH cDNA and inserted into the yeast expression vector pPICZαB (Invitrogen). In protein production trials, the resultant recombinant protein underwent proteolytic degradation before or during purification; this difficulty was circumvented by fusion with tags as follows. DNA coding for a C-terminal His_6 tag and an N-terminal myc tag (EQKLISEEDL) were fused to the DNA encoding FH residues 19–263 cDNA in pPICZαB using the QuikChange site-directed mutagenesis kit (Stratagene) with the following forward primers: GGATGGCGTCCGTTGCCTTCATGTGAACATCATCATCATCATCATCATTAGTCTAGAACA (for C-terminal His_6 tag) and GAGGCTGAAGCTGCAGGAGAACAAAAACTCATCTCAGAAGAGGATCTGGAAGATTGCAATGAACTTCCTCCA (for N-terminal myc tag). This yielded the more stable protein product, $FH1–4^{V62}$. Single-site mutations were subsequently introduced (QuikChange) to create (R53H)$FH1–4^{V62}$, $FH1–4^{I62}$, and (R78G)$FH1–4^{V62}$; thus, identical N-terminal and C-terminal modifications were present in all versions of CFH1–4 in the present study.

Following plasmid preparation, KM71H *Pichia pastoris* cells (Invitrogen) were transformed using electroporation (Bio-Rad GenePulser II), and 1 ml of ice-cold 1 M sorbitol was added. Cells were incubated at 30 °C for 2–3 h without shaking. Selection of *P. pastoris* clones containing the expression plasmid was achieved by streaking transformed yeast onto YPDS (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 1 M sorbitol, 2% (w/v) agar) plates containing 100–300 $\mu g \cdot ml^{-1}$ zeocin. Cell colonies that grew on 300 $\mu g \cdot ml^{-1}$ zeocin plates, consistent with the presence of multiple copies of the gene in the transformed cells, were screened for protein expression.

Protein Production and Purification—Expression was carried out at 30 °C in either 2-liter baffled flasks or a fermentor. For flask expression, 5-ml starter cultures of buffered minimal glycerol enriched with casein amino acids (1% w/v) were transferred to 500 ml of buffered minimal glycerol (with casein amino acids) and incubated for 48 h. For the fermentor, starter cultures (500 ml of buffered minimal glycerol) were transferred into 3.8 liters of buffered minimal glycerol enriched with 1% (w/v) casein amino acids and containing antifoam and 0.5% (w/v) PTM1 salts in a 5-liter fermentation vessel. Following incubation for 16–18 h, recombinant expression was induced with buffered minimal methanol, enriched with 1% (w/v) casein amino acids (for baffled flasks and fermentor growths) and 0.5% (w/v) PTM1 salts (fermentor growths only) as before. After 3–4 days of further incubation with methanol feeds (to 0.5% v/v) every 24 h, cells were pelleted by centrifugation, and the supernatant containing the secreted recombinant protein was filtered (0.22 μm) and its pH adjusted to 7.5. The supernatant was applied manually to an XK 16/20 column packed with 25 ml of IMAC-Sepharose 6 Fast Flow resin (GE Healthcare), charged with $NiSO_4$. His-tagged target proteins were eluted (3 ml/min) with an imidazole gradient, emerging at ~210 mM imidazole. Size-exclusion chromatography on a HiLoad 16/60 Superdex 75 chromatography column (GE Healthcare) was used as a second purification step. Further purification was accomplished using a 1.7-ml Tricorn MonoQ (GE Healthcare) anion-exchange chromatography column in 20 mM sodium carbonate buffer, pH 9.0, containing 1 mM EDTA. The column was eluted with a gradient to 1 M NaCl; the target proteins emerged at 400

mM NaCl. Protein identity was confirmed by Fourier transform ion-cyclotron resonance mass spectrometry (Bruker) or on a quadrupole time-of-flight mass spectrometer (Q-TOF; Micromass). Mass spectrometry-derived masses agreed with theoretical masses taking into account disulfide formation, the myc tag at the N terminus and the His_6 tag at the C terminus, and the erratic presence of an N-terminal Asp-Gly dipeptide that is an artifact of incomplete cleavage of the signal sequence used to promote secretion of recombinant protein. Protein concentrations were estimated using absorbance at 280 nM and calculated extinction coefficients (47870 $M \cdot cm^{-1}$).

Measuring Decay Acceleration Activity on Sheep Erythrocytes—C3b-coated sheep erythrocytes were prepared as described previously (51). Cells were resuspended to 1% (v/v) in AP buffer (5 mM sodium barbitone, pH 7.4, 150 mM NaCl, 7 mM $MgCl_2$, 10 mM EGTA), and the AP convertase (C3bBb) was formed on the cell surface by incubating with FB (7 μg/ml) and FD (0.2 μg/ml; Complement Technologies) at 37 °C for 15 min. Cells (100 μl) were incubated with 50 μl of the FH1–4 samples in PBS/10 mM EDTA for 20 min. Lysis was developed by adding 50 μl of normal human serum (NHS) depleted of FB and FH (4%, v/v NHSΔBΔH) (51) in PBS/10 mM EDTA and incubating at 37 °C for 60 min. To determine the amount of lysis, cells were pelleted by centrifugation, and hemoglobin release was measured at 410 nm. Controls included 0% lysis (buffer only) and 100% lysis (0.1% (v/v) Nonidet P-40), and the observed cell lysis was calculated as a percentage as described previously (51).

Measuring Cofactor Activity on Sheep Erythrocytes—To test FH1–4 cofactor activity, washed EA-C3b cells were resuspended to 2% (v/v) in AP buffer and incubated with an equal volume of a range of concentrations of each of the CFH1–4 samples and 2.5 μg/ml FI (Complement Technology) for 15 min at 25 °C. After three washes in AP buffer, a 50-μl aliquot of cells (2%) was mixed with 50 μl of FB (14 μg/ml) and FD (0.2 μg/ml) and then incubated for 15 min at 25 °C to form AP convertase on the remaining C3b. Lysis was developed by adding 50 μl of NHSΔBΔH (4%, v/v) in PBS/10 mM EDTA and incubating at 37 °C for 30 min. Percentage lysis was calculated as above.

Binding Affinity for C3b by Surface Plasmon Resonance—The binding affinity of the disease-associated CFH1–4 mutants was monitored by surface plasmon resonance (SPR) using a Biacore T100 instrument (GE Healthcare). A Biacore series *S*-carboxymethylated dextran (CM5) sensor chip (GE Healthcare) was prepared by immobilizing on it human C3b (Complement Technology) using standard amine coupling. The reference surface of the chip was prepared by performing a mock coupling in the absence of any protein. Experiments were performed at 25 °C and 30 μl/min flow rate. Duplicate injections were performed for selected samples (concentrations 0.5–20 μM) in 10 mM HEPES-buffered saline with 3 mM EDTA and 0.05% (v/v) surfactant p20 (*i.e.* HBSEP+) (GE Healthcare). A contact time of 90 s was used (this achieved steady-state conditions for most samples) followed by dissociation using running buffer for 600 s. Between sample injections, chips were regenerated by two 45-s injections of 1 M NaCl. Data were processed using the BIAevaluation software (GE Healthcare). Data from the reference cell and a blank (buffer) injection were subtracted and dissociation constants calculated using steady-state fitted model and background-subtracted traces of the individual injections fitting.

Measurement of Decay Acceleration Activity by SPR—Decay-accelerating activity was measured in real-time using a Biacore T100 instrument as described previously (17, 52). All proteins were gel-filtered into the running buffer (HEPES-buffered saline containing 0.5% (v/v) surfactant P20 and 1 mM $MgCl_2$) prior to analysis. Briefly, 900 resonance units of C3b were thiocoupled to the CM5 sensor chip (52). Subsequently, a mixture of FB (110 μg/ml) and FD (1 μg/ml) was flowed (20 μl/min) over the surface for 120 s to form C3 convertase. After allowing the convertase to decay naturally for 120 s, the FH1–4 sample (0.5 μM or 0.17 μM) was flowed across the surface for 90 s, and convertase decay was visualized in real time. Between injections, surfaces were regenerated using 10 mM sodium acetate buffer, pH 4, containing 1 M NaCl. Data were evaluated using BIAevaluation 4.1 (GE Healthcare). As a control, construct was flowed over the bare surface and binding data subtracted from the decay histogram to account for CFH1–4 binding to the C3b surface.

Cofactor Assay in Fluid Phase—A dilution series fluid-phase assay (53) was used to measure cofactor activity for FI-mediated proteolytic cleavage of C3b. FI, FH, and C3b were all purchased from Complement Technology. For the positive control, FI (0.045 μg) and of C3b (3 μg) were mixed with 0.3 μg of FH (*i.e.* 0.1 μM) in a final reaction volume of 20 μl of PBS; the negative control was prepared in the same way but replacing FH1–4 with PBS. Experimental reaction mixtures contained a range of FH1–4 concentrations in place of FH. Reaction mixtures were vortexed and incubated in a water bath (37 °C) for 15 min. An aliquot of 2 × reducing SDS buffer (7.5 μl NuPage sample buffer (4×) (Invitrogen), 3 μl of NuPage reducing agent (1×) (Invitrogen) in a final volume of 30 μl of ddH_2O) was added to stop the reaction, and samples were heated prior to SDS-PAGE. The FI/FH-catalyzed α'-chain cleavage was followed by visualizing the products and unused substrates using Coomassie Blue staining.

RESULTS

Truncated Human FH Constructs Were Produced in P. pastoris—The preparation from patients' plasma of purified FH mutants, in a form free of other sequence variants, presents logistical and technical difficulties. In the current work, we avoided these issues by examining functional consequences of disease-linked N-terminal FH sequence variations in the context of recombinant, truncated FH constructs consisting only of CCPs 1–4 (with an N-terminal myc tag and a C-terminal His_6 tag; see Fig. 1, *b* and *c*) (FH1–4). The allotypic variants FH1–4^{V62} and FH1–4^{I62} were successfully produced in *P. pastoris* (Fig. 1*c*). The availability of both of these "wild-type" variants allowed comparison of their functional activities (see below) to establish a benchmark for subsequent studies of (R53H)FH1–4^{V62} and (R78F)FH1–4^{V62} that were also produced in *P. pastoris* (Fig. 1*c*). Not only was recombinant FH1–4 produced and purified in very much higher yields than recombinant FH (54, 55), but also its use in the current study avoided the complicat-

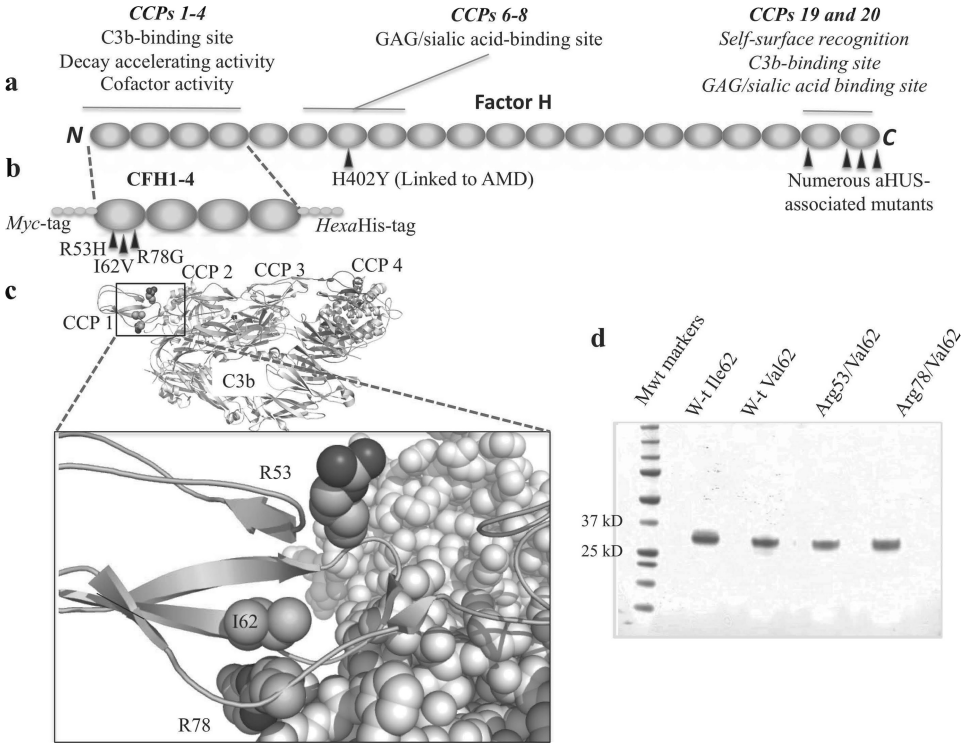

FIGURE 1. **Sequence variants of FH analyzed in the current study.** *a*, modular composition of full-length human FH (each *oval* corresponds to a CCP module) highlighting functional sites and disease-associated mutations and polymorphic variations. *b*, constructs, bearing myc and His_6 tags, produced in *P. pastoris* and analyzed for functional activity in the current study. *c*, location of Arg^{53}, Ile^{62}, and Arg^{78} within the co-crystal structure (57) of a CFH1–4·C3b complex (Protein Data Base ID code 2WII). Note that Arg^{53} is surface-exposed in this complex, Ile^{62} is buried within CCP 1 of CFH1–4, and Arg^{78} participates in an intermolecular H-bonded contact. *d*, reducing SDS-PAGE of variants used in this study.

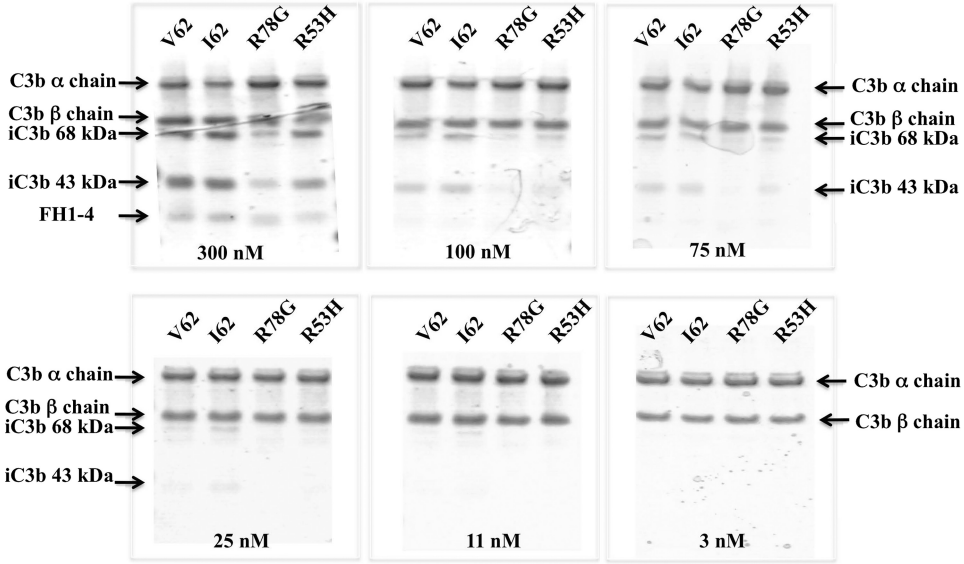

FIGURE 2. **Fluid-phase cofactor activity.** FI and C3b were incubated for 10 min with a series of concentrations of the two wild-type FH1–4 variants and the two aHUS-linked mutants. Subsequently, the loss of the intact C3b α′-chain and appearance of its FI cleavage products (43-kDa and 68-kDa fragments of iC3b α′-chain) were visualized by SDS-PAGE. Gels are labeled according to the concentrations of FH1–4 variant/mutant in the incubation mixture. It is apparent that in the range 75–300 nM, (R78G)FH1–4^{V62} promotes production of less 68-kDa iC3b α′-chain fragment than (R78G)FH1–4^{V62} or the wild-type variants. The presence of 25 nM FH1–4^{I62}, but not of 25 nM FH1–4^{V62} (or the mutants), produces detectable 68-kDa iC3b α′-chain fragment.

ing presence of the second, stronger, C3b-binding site of FH in CCPs 19 and 20.

FH1–4^{I62} Is Slightly More Active than FH1–4^{V62} although Both Bind C3b Equally Well—In fluid-phase cofactor assays (Fig. 2) both wild-type variants of FH1–4 promoted cleavage of the C3 α-chain cleavage by FI, although FH1–4^{V62} was less efficient in this respect than FH1–4^{I62}. An assay for cofactor activity for FI-mediated cleavage of C3b on cell surfaces similarly demonstrated that both variants are active although FH1–4^{V62} is less efficient than FH1–4^{I62} (Fig. 3). SPR studies demonstrated that FH1–4^{I62} (K_D = 14 μM) binds no better to C3b (amine-coupled to a Biacore CM5 sensor chip) than does FH1–

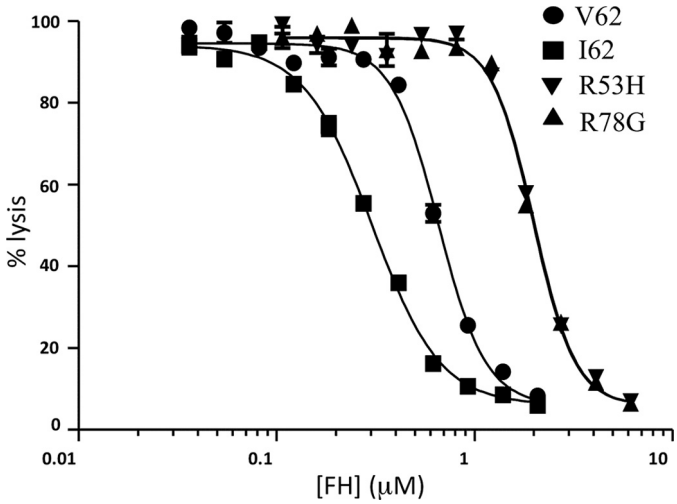

FIGURE 3. **Cofactor activity for C3b cleavage on sheep erythrocytes.** Each FH1–4 variant was tested for its ability to act as a cofactor for FI-catalyzed inactivation of C3b deposited on the surfaces of sheep erythrocytes. To achieve this, convertase (C3bBb) was formed on residual C3b whereupon lysis commences. The extent of hemolysis (*y* axis) therefore reflects the amount of C3b remaining after incubation with FI and each FH1–4 variant. FH1–4^{V62} (*circles*), FH1–4^{I62} (*squares*), (R53H)FH1–4^{V62} (*upside-down triangles*), and (R78G)FH1–4^{V62} (*triangles*) are shown.

4^{V62} (K_D = 10 μM) (Fig. 4). The ability of wild-type constructs to decay the convertase, C3bBb, was assessed by hemolysis assay (Fig. 5*a*) and in real time by SPR (Fig. 5*b*). Both assays demonstrated that FH1–4^{V62} and FH1–4^{I62} variants accelerate decay of the C3bBb complex with approximately equivalent activity (IH_{50} 8.7 nM and 7.9 nM, respectively). Subsequent work focused on aHUS-linked mutants in the FH1–4^{V62} setting.

R78G Mutant Has Severely Compromised Regulatory Function—Based on SPR results (Fig. 4) it is apparent that the affinity of (R78G)FH1–4^{V62} for C3b is weak compared with that of wild-type FH1–4^{V62} (or FH1–4^{I62}). It proved impossible to ascertain an accurate K_D using the quantity of (R78G)FH1–4^{V62} (20 μM in the solution flowed over the chip) available. It may be estimated, however, that the K_D of the mutant-C3b interaction exceeds 35 μM under the same conditions used to measure a K_D of 10–11 μM for the complex of wild-type FH19–20 and C3b. To investigate the functional consequences of this weak binding the complement-regulating activity of (R78G)FH1–4^{V62} was assessed.

Using fluid-phase assays of cofactor activity, the (R78G)FH1–4^{V62} mutant was shown to be much less efficient compared with wild-type FH1–4^{V62} (Fig. 2). This finding was reproduced in cell-based cofactor activity assays (Fig. 3) in which nearly 3-fold more (R78G)FH1–4^{V62}, with respect to wild-type FH1–4^{V62}, was needed to achieve 50% inhibition of complement-mediated hemolysis (IH_{50} 1.65 μM *versus* 0.64 μM, respectively). Assays of C3bBb decay-accelerating activity on the surface of sheep erythrocytes also showed that (R78G)FH1–4^{V62} is much less effective (IH_{50} 218 nM) than wild-type FH1–4^{V62} (IH_{50} 8.7 nM) (Fig. 5*a*). These data were supported by SPR-based decay acceleration assays (Fig. 5*b*).

R53H Mutant Binds Well to C3b but Is Functionally Defective—A second aHUS-linked mutant, (R53H)CFH1–4^{V62}, was found by SPR to bind to immobilized C3b with affinity comparable with that of the wild-type CFH1–4^{V62} (Fig. 4). Despite this unimpaired affinity for C3b, (R53H)FH1–4^{V62}, like (R78G)FH1–4^{V62}, proved nearly 3-fold less active than wild-type FH1–4^{V62} as a cofactor for FI-catalyzed C3b cleavage on sheep erythrocyte surfaces (IH_{50} 1.67 μM *versus* 0.64 μM; Figs. 2 and 3). The (R53H)CFH1–4^{V62} mutant was also less active in fluid-phase cofactor assays. The ability to decay C3bBb of (R53H)CFH1–4^{V62} was also severely impaired compared with wild-type protein as measured both by hemolysis assay (IH_{50} 346 nM; Fig. 5*a*) and in real time by SPR (Fig. 5*b*).

DISCUSSION

The life-threatening kidney disease aHUS has been discussed extensively with respect to mutations that prevent the C terminus of complement FH from mediating selective regulatory action on self (rather than foreign) surfaces. We set out to discover whether a causal link could also be established between aHUS and mutations in the N-terminal module of FH, which is not involved in binding directly to surfaces.

It was important to first consider the widespread V62I polymorphism of FH. Ile^{62} has been reported to be protective (*versus* Val^{62}) for aHUS (46) as well as AMD (4) and DDD (4, 46), an effect ascribed to its superior cofactor activity (51). Indeed our results, obtained using FH1–4^{V62} and FH1–4^{I62}, agree with previous findings showing that twice the concentration of full-length FH^{V62}, relative to full-length FH^{I62}, was needed to achieve 50% inactivation of C3b by FI in fluid-phase and on cell surfaces (51). These authors also found that FH^{I62} binds slightly more tightly to C3b (K_D = 1.0 μM) than does FH^{V62} (K_D = 1.3 μM), consistent with higher cofactor activity. We, however, observed no difference in C3b binding between FH1–4^{V62} and FH1–4^{I62}. Thus, the effect of I62V on C3b binding by FH is not exaggerated in the FH1–4 setting despite FH1–4 having only one C3b-binding site whereas full-length FH contains an additional, stronger, C3b-binding site.

We additionally showed that both FH1–4^{V62} and FH1–4^{I62} variants accelerate decay of the C3bBb complex with equivalent activity. This also agrees with data from Tortajada *et al.* (51), demonstrating similar relative decay accelerating activities for both wild-type variants of full-length FH. In summary, the new data establish that the CFH1–4^{I62} and CFH1–4^{V62} variants function efficiently in complement regulation with the CFH1–4^{I62} variant showing slightly better cofactor activity. Subtle differences between the two versions are consistent with their minor structural differences (56) and their relatively weak associations with disease propensity at least until old age. These studies provided a useful base line for functional analyses of the mutants.

The poor affinity we observed between (R78G)FH1–4^{V62} and C3b agrees with inferences based on a co-crystal structure of C3b and CFH1–4 (57) in which Arg^{78} in the N-terminal CCP forms an H-bonded interaction with Glu^{732} in the α′-N-terminal domain of C3b (Fig. 1*c*). *In vivo*, loss of affinity of the (R78G)FH N terminus for C3b could impair its ability to control AP convertases both in fluid phase and on cell surfaces. Therefore, the complement-regulating function of (R78G)FH1–4^{V62} was assessed and found to be very poor both in terms of cofactor and decay-accelerating activities. Taken together, these data support a disease mechanism in which (R78G)FH is function-

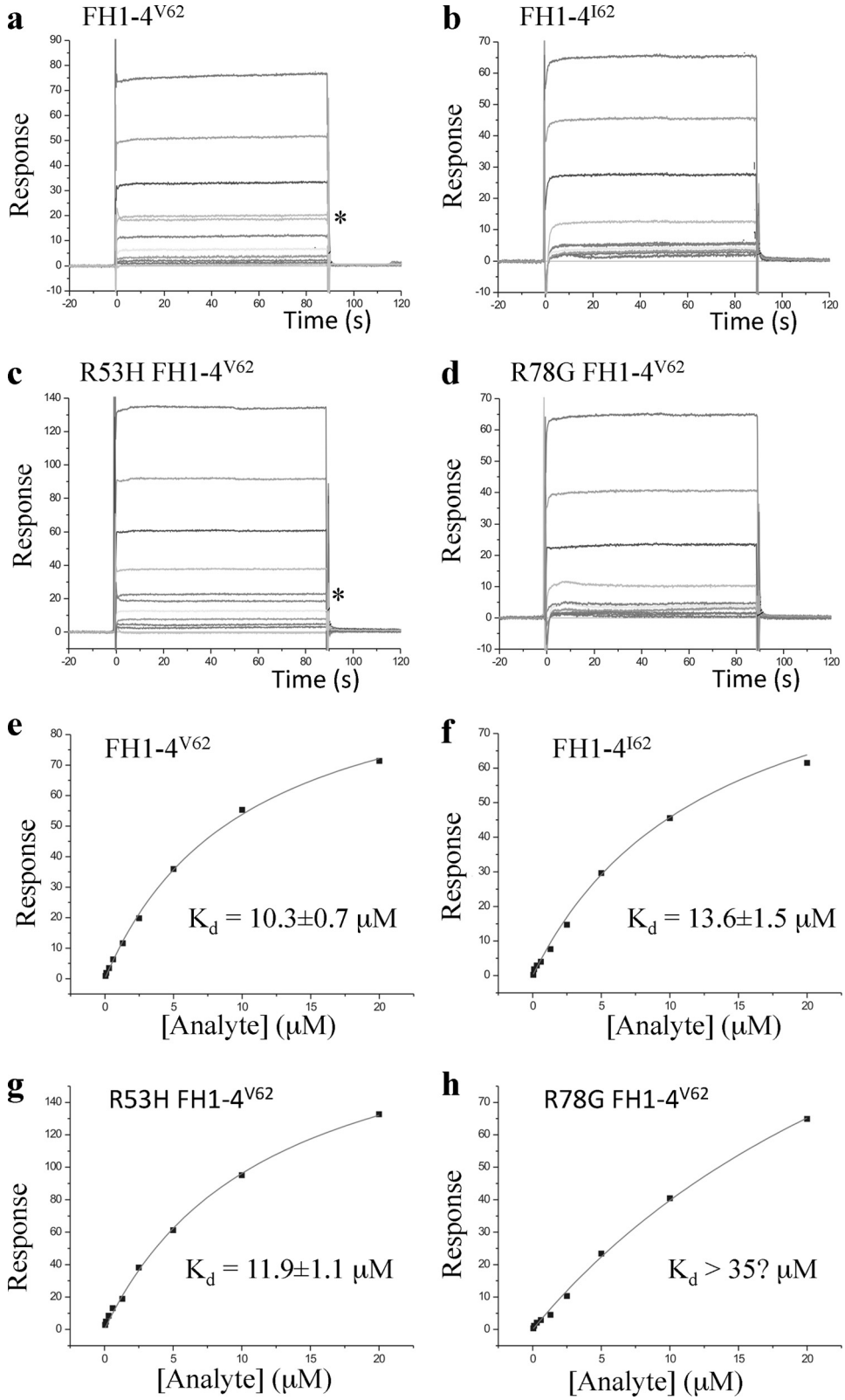

FIGURE 4. **Measurements of affinity for C3b.** SPR response curves for concentration series (0.05, 0.1. 0.3, 0.6, 1.3, 2.5, 5.0, 10, and 20 μM; duplicate traces that did not overlay marked with *) of FH1–4^{V62} (*a*), FH1–4^{I62} (*b*), (R53H)FH1–4^{V62} (*c*), and (R78G)FH1–4^{V62} (*d*), respectively, flowed over C3b (2,000 response units) immobilized by amine coupling on a CM5 sensor chip. Plots of response (units) *versus* concentration, used to estimate K_D values, are shown for FH1–4^{V62} (*e*), FH1–4^{I62} (*f*), (R53H)FH1–4^{V62} (*g*), and (R78G)FH1–4^{V62} (*h*).

ally compromised by poor affinity for C3b, potentially resulting in severely impaired complement regulatory function and explaining low C3 levels in this patient (50). The physiological significance and consequences of these observations depend on the levels of the various proteins and whether or not they are locally present in sub-saturating concentrations. Very often an

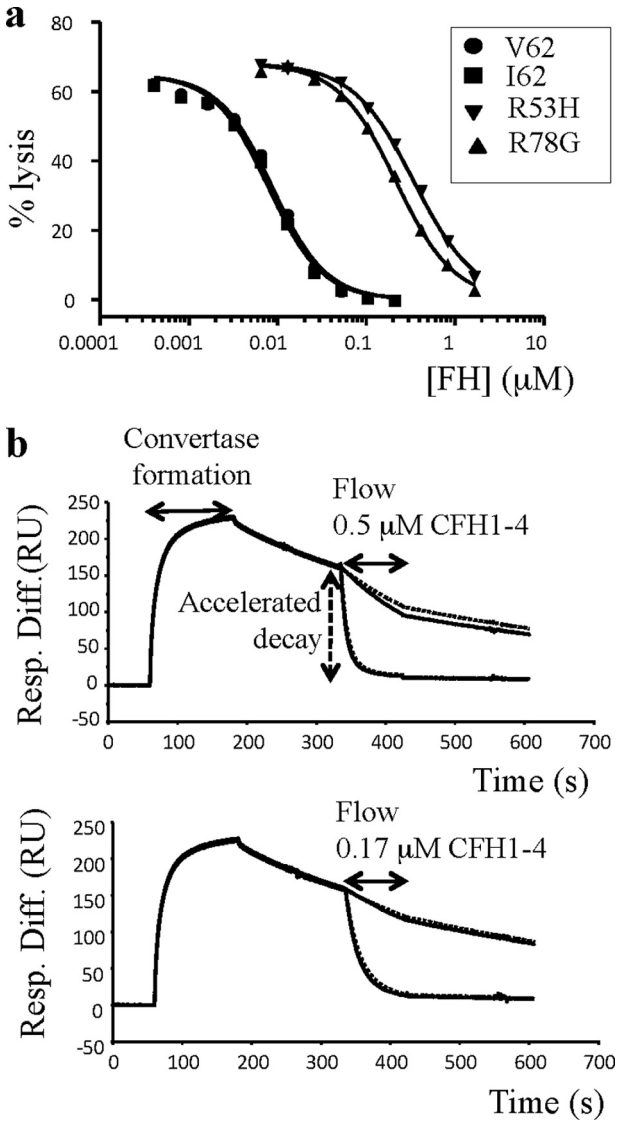

FIGURE 5. **Decay acceleration activity for the alternative pathway C3 convertase.** *a*, alternative pathway convertase, C3bBb, was formed on the surface of sheep erythrocytes. Cells were incubated for 15 min with dilutions of each FH1–4 construct to decay C3bBb before instigating lysis with NHSΔBΔH. Both FH1–4^{V62} (*circles*) and FH1–4^{I62} (*squares*) decayed the convertase efficiently whereas (R53H)FH1–4^{V62} (*upside-down triangles*) and (R78G)FH1–4^{V62} (*triangles*) were up to 40-fold less efficient. *b*, decay of the convertase, C3bBb, by the FH1–4 constructs at 0.17 μM and 0.5 μM was also analyzed in real time by SPR. Both FH1–4^{V62} (*black dotted line*) and FH1–4^{I62} (*black line*) decayed the convertase equally as efficiently whereas (R53H)FH1–4^{V62} (*gray dotted line*) and (R78G)FH1–4^{V62} (*gray line*) had virtually no activity at 0.17 μM.

aHUS patient with a disease-linked *CFH* gene also has a normal version of the *CFH* gene; haploinsufficiency, caused by missense or nonsense mutations in just one allele, has previously been associated with aHUS in many individuals (14, 58), illustrating the delicate balance between complement activation and regulation required for maintenance of homeostasis and health.

The unperturbed C3b-binding ability of a second aHUS-linked mutant, (R53H)FH1–4^{V62}, is consistent with the structural integrity of this mutant as reported previously on the basis of an NMR study (56), and with the location of Arg^{53}, within the crystallized C3b·CFH1–4 complex, on an exposed face of FH rather than adjacent to C3b (Fig. 1*c*) (57). Hence, the diminished cofactor activity of (R53H)FH1–4^{V62} cannot be explained by the lower affinity for C3b as was the case with the R78G mutant. An alternative explanation is that Arg^{53} lies within a putative interaction site for FI, consistent with a previous suggestion that FI binds to CCPs 1–3 within FH as well as to the C345C domain of C3b (57). The decay accelerating activity of (R53H)FH1–4^{V62} is also very poor. Given the wild-type-like affinity of (R53H)FH1–4^{V62} for C3b, these data suggest that impaired ability to accelerate decay of the convertase components may be due to weakened association with Bb. Thus, the side chain of Arg^{53} may participate in interactions with both Bb and FI, implying overlap between binding sites for these ligands within CCP module 1 of FH. The functionally deficient (R53H)FH mutant would likely compete with wild-type FH for binding C3b in heterozygous individuals and could thereby further diminish complement regulatory capacity.

In summary, both N-terminal FH mutants examined in the current study are defective in their ability to control the AP C3 convertase in the fluid phase and on cell surfaces, but the molecular mechanisms underlying the dysregulation caused by these mutant proteins are distinct. Thus, despite differences in their C3b-binding properties, both mutations have similar outcomes with respect to complement regulation and are associated with similar disease symptoms. It follows that a causal link between mutations and disease is the simplest explanation of these data. Previous functional analyses of C-terminal FH mutants have suggested that multiple mechanisms are responsible for defective cell surface regulation in aHUS (59–64); these include decreased binding to C3b/C3d and/or to glycosaminoglycans and altered oligomerization of FH on the surface. Thus, the current observations greatly strengthen the hypothesis that a wide range of defects in FH activity can contribute to a similar disease phenotype.

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