Insights into the Effects of Complement Factor H on the Assembly and Decay of the Alternative Pathway C3 Proconvertase and C3 Convertase*

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The activated fragment of C3 (C3b) and factor B form the C3 proconvertase (C3bB), which is cleaved by factor D to C3 convertase (C3bBb). Older studies (Conrad, D. H., Carlo, J. R., and Ruddy, S. (1978) J. Exp. Med. 147, 1792–1805; Pangburn, M. K., and Müller-Eberhard, H. J. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 2416–2420; K. F. (1979) J. Immunol. 122, 75–81) indicated that the complement system is triggered by three activation pathways, the classical, the lectin, and the alternative (AP) pathways, all leading to the formation of unstable protease complexes, named C3 convertases, that cleave the inactive central component C3 into C3a and C3b fragments. C3b molecules deposited on pathogens or damaged self-cells provide a molecular platform for the formation of an active AP C3 convertase, C3bBb, which enzymatically generates many more C3b molecules, resulting in a positive feedback loop. Notably, the AP C3 convertase is crucial within the complement cascade as it also amplifies complement activation after C3b is generated through the other pathways.

Complement can be triggered by three activation pathways, the classical, the lectin, and the alternative (AP) pathways, all leading to the formation of unstable protease complexes, named C3 convertases, that cleave the inactive central component C3 into C3a and C3b fragments. C3b molecules deposited on pathogens or damaged self-cells provide a molecular platform for the formation of an active AP C3 convertase, C3bBb, which enzymatically generates many more C3b molecules, resulting in a positive feedback loop. Notably, the AP C3 convertase is crucial within the complement cascade as it also amplifies complement activation after C3b is generated through the other pathways.

The formation of the AP C3 convertase requires a two-step assembly process, in which factor B (FB) first binds to C3b in a Mg2+-dependent manner to form the labile C3 proconvertase C3bB. Once bound to C3b, the FB becomes susceptible to properdin and C3 nephritic factor did not restore C3bBb formation. FH almost completely inhibited the C3bBb decay-accelerating activity, as reported previously, and also exerted an apparent inhibitory effect on C3b binding. We also found that FH does not dissociate C3bB. We document that FH does not affect C3bB assembly, indicating that FH does not efficiently compete with factor B for C3b binding. We also found that FH does not dissociate C3bB. FH showed a strong C3bBb decay-accelerating activity, as reported previously, and also exerted an apparent inhibitory effect on C3bB formation. The latter effect was not fully attributable to a rapid FH-mediated dissociation of C3bBb complexes, because blocking decay with properdin and C3 nephritic factor did not restore C3bBb formation. FH almost completely prevented release of the smaller cleavage subunit of FB (Ba), without modifying the amount of C3bB complexes, suggesting that FH inhibits the conversion of C3bB to C3bBb. Thus, the inhibitory effect of FH on C3bBb formation is likely the sum of inhibition of C3bB conversion to C3bBb and of C3bBb decay acceleration. Further studies are required to confirm these findings.

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Lanes 1 and 7 of Fig. 4B were duplicated.

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WITHDRAWN

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Lanes 1 and 7 of Fig. 4B were duplicated.
Effects of FH on the Assembly and Decay of C3bB and C3bBb

...teolytic activation by the plasma serine protease factor D (FD), generating the Ba and Bb fragments. Ba dissociates from the complex, whereas the Bb fragment remains bound to C3b, generating the active C3 convertase C3bBb. Properdin (P) binding to C3 convertase stabilizes this otherwise labile complex (17, 18).

To prevent complement activation on host tissues, AP C3 convertase activity is tightly controlled by the set of fluid phase and membrane-bound regulators (5, 6, 19), of which complement factor H (FH) is a prominent member (20–22). Factor H is an abundant plasma glycoprotein that not only modulates fluid-phase complement but also has the ability to inhibit C3b amplification selectively on self-surfaces.

Very old studies (23–25) proposed that FH could prevent C3bB C3 proconvertase assembly by competing with FB for binding to C3b. In these experiments, binding assays performed on C3b-coated sheep erythrocytes showed that FB both displaces bound FH from the cell and inhibits the equilibrium binding of FH to C3b. Competition between FH and FB for C3b binding resulting in an inhibitory effect on C3 proconvertase assembly was supported by more recent surface plasmon resonance studies (26). The authors found that when FB, together with increasing amounts of FH, was flowed over a C3b-coated surface, C3bB C3 proconvertase formation decreased in an FH concentration-dependent manner. Notably, because following FB and FH injections the net mass change was the sum of C3 proconvertase formation and FH binding to the C3b-coated surface, it was actually difficult to dissect between the binding of C3bB C3 proconvertase formation and FH binding to the C3b surface, it was actually difficult to dissect between the binding of FH and the inhibitory effects of FH on C3bB C3 proconvertase formation (26), in keeping with a previous report (27), which does not accelerate decay of the already formed C3bB C3 proconvertase.

Whether FH affects the formation of C3bB was not investigated yet. However, the recent demonstration that the dissociation of already formed C3bB C3 proconvertase (17, 18) and C3bBb C3 convertase (17, 18) is well documented (28–30). Investigations into the kinetics of dissociation of C3bB C3 proconvertase and C3bBb C3 convertase, we performed previously by Hourcade et al. (31) were performed with 3 μg/ml C3b in PBS by incubating C3b-coated wells at 37 °C for 2 h with different concentrations of FB (0–500 ng/ml) in the presence or in the presence of FD (25 ng/ml), respectively, both diluted in assay buffer (8.1 mM Na2HPO4, 1.8 mM NaH2PO4, 4% BSA, 0.1% TWEEN 20, and 25 mM NaCl). FH was added at 0.1% TWEEN 20 and 25 mM NaClO4.

To overcome these shortcomings, in this study we set up a novel user-friendly method based on combined microplate and Western blotting techniques, which specifically detect either C3bB or C3bBb, with the aim of investigating the effect of FH on the following: 1) the assembly and decay of the AP C3 proconvertase C3bB, and 2) the formation and decay of the AP C3 convertase C3bBb.

Experimental Procedures

Complement Proteins, Chemicals and Antibodies—Microplates 96-microwell, Nunc-Immuno Maxisorp, and HRP-conjugated goat anti-rabbit antibody (catalog no. 626520) were obtained from Thermo Scientific (VWR International PBI srl, Milano, Italy). Purified native complement proteins C3b, FB, FD and P were from Complement Technology Inc. (Tyler, TX). FH was purchased from Merck (Nottingham, UK). Bovine serum albumin (BSA), rabbit polyclonal anti-human FB antibody (catalog no. HPA001817), peroxidase-labeled polyclonal anti-goat IgG (whole molecule, catalog no. A5420) antibody, heparan sulfate (HS, H7640) sodium salt from bovine kidney, N-acetylneuraminic acid (sialic acid, A0812), and EGTA (34596) were purchased from Sigma-Aldrich. Peroxidase-labeled polyclonal anti-rabbit IgG (H+L, catalog no. PI-1000) antibody was purchased by Vector Laboratories Inc. (Burlingame, CA). 3,3’,5,5’-Tetramethylbenzidine substrate was from Bethyl (Tema Ricerca srl, Bologna, Italy). Mouse anti-heparin sulfates monoclonal antibody (catalog no. AB4100) was purchased from Millipore (Temecula, CA). Polyclonal rabbit anti-human C5/C5b antibody (catalog no. ab46168) was purchased from Abcam (Cambridge, UK). Hybrid-P hydrophobic polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences) and the ECL Select chemiluminescence detection reagent (Amersham Biosciences) were purchased from GE Healthcare (Euroclone Spa, Milano, Italy). Normal human serum (NHS) was a pool obtained from 15 healthy volunteers.

ELISAs for C3bB C3 Proconvertase Assembly and C3bBb C3 Convertase Formation—For the generation of Ni2+-dependent AP C3bB C3 proconvertase and C3bBb C3 convertase, we performed previously by Hourcade et al. (31, 32) were performed with 3 μg/ml C3b in PBS by incubating C3b-coated wells at 37 °C for 2 h with different concentrations of FB (0–500 ng/ml) in the presence or in the presence of FD (25 ng/ml), respectively, both diluted in assay buffer (8.1 mM Na2HPO4, 1.8 mM NaH2PO4, 4% BSA, 0.1% TWEEN 20, and 25 mM NaCl). FH was added at 0.1% TWEEN 20 and 25 mM NaClO4.

For the generation of Ni2+-dependent AP C3bB C3 proconvertase and C3bBb C3 convertase, we performed previously by Hourcade et al. (31, 32) were performed with 3 μg/ml C3b in PBS by incubating C3b-coated wells at 37 °C for 2 h with different concentrations of FB (0–500 ng/ml) in the presence or in the presence of FD (25 ng/ml), respectively, both diluted in assay buffer (8.1 mM Na2HPO4, 1.8 mM NaH2PO4, 4% BSA, 0.1% TWEEN 20, and 75 mM NaCl) containing 2 mM NiCl2. After washes, the C3bB and C3bBb complexes were detected by ELISA using polyclonal goat anti-human FB antibody (1:10,000) followed by HRP-conjugated anti-goat antibody (1:40,000), both diluted in antibody buffer (8.1 mM Na2HPO4, 1.8 mM NaH2PO4, 4% BSA, 0.1% TWEEN 20, and 25 mM NaCl) supplemented with 2 mM NiCl2. Color was developed using 3,3’,5,5’-tetramethylbenzidine substrate and stopped with 2 mM H2SO4, and absorbance was measured at 450 nm. Each reaction was performed in duplicate, and the OD values were averaged.

For the generation of C3bB(Mn2+) or C3bBb(Mg2+) complexes, C3b-coated microtiter wells were treated as above except that the incubation was performed in the presence of 2 mM MnCl2 for 2 h at 37 °C or 10 mM MgCl2 and for 30 min at 25 °C, respectively.

Selective C3bB C3 Proconvertase and C3bBb C3 Convertase Formation Assays—We set up a novel user-friendly method based on combined microplate and Western blotting (WB) techniques to selectively generate and detect C3bB and C3bBb complexes. ELISA plates were coated with C3b, blocked as above, and then washed with wash buffer supplemented with 2 mM MnCl2 (33) or 10 mM MgCl2 (34) for C3bB or C3bBb, respectively. C3bB(Mn2+) complexes were assembled by incubating C3b-coated wells at 37 °C for 1, 2, 4, and 8 h with FB (1000 ng/ml), diluted in assay buffer containing 2 mM MnCl2 (Fig. 1A). C3bBb(Mg2+) complexes were formed by incubating...
**FIGURE 1.** Experimental design of microplate/WB assays.

**A**
C3b-coating

- o/n +4°C
- MnCl₂ 2 mM, 37°C
- C₃-proconvertase assembly + FB
- C₃-convertase formation + FB +/- FD +/- FH
- Protein detachment and WB analysis
- Quantification of Bb and Ba supernatant levels by ELISA

**B**
C3b-coating

- o/n +4°C
- MgCl₂ 10 mM, 25°C
- C₃-proconvertase assembly + FB
- Spontaneous or FH-mediated decay; buffer +/− FH
- Protein detachment and WB analysis

**C**
C3b-coating

- o/n +4°C
- MnCl₂ 2 mM, 2 h 37°C
- C₃-proconvertase assembly + FB
- Wash
- MnCl₂ 2 mM, 37°C
- Spontaneous or FH-mediated decay; buffer +/− FH
- Protein detachment and WB analysis

**D**
C3b-coating

- o/n +4°C
- MgCl₂ 10 mM, 10 min 25°C
- C₃-proconvertase assembly + FB + FD
- Spontaneous or FH-mediated decay; buffer +/− FH
- Protein detachment and WB analysis

**E**
C3b-coating

- o/n +4°C
- MgCl₂ 10 mM, 10 min 25°C
- C₃-proconvertase assembly + FB + FD +/− P +/− FH
- Protein detachment and WB analysis

**F**
C3b-coating

- o/n +4°C
- MgCl₂ 10 mM, 10 min 25°C
- C₃-proconvertase assembly + FB + FD +/− P +/− FH
- Spontaneous or FH-mediated decay; buffer +/− FH
- Protein detachment and WB analysis

**G**
C3b-coating

- o/n +4°C
- MgCl₂ 10 mM, 10 min 25°C
- C₃-proconvertase assembly + C₃-convertase formation + FB + FD +/− FH
- Wash
- MgCl₂ 10 mM, 10 min 25°C
- Spontaneous or FH-mediated decay; buffer +/− FH
- Protein detachment and WB analysis

**H**
C3b +/− HS or SA-coating

- o/n +4°C
- NiCl₂ 2 mM, 37°C
- C₃-proconvertase assembly + C₃-convertase formation + FB + FD
- Protein detachment and WB analysis
- Spontaneous or FH-mediated decay; buffer +/− FH

**I**
C3b-coating

- o/n +4°C
- NiCl₂ 2 mM, 30 min 37°C
- Protein detachment and WB analysis

**J**
C3b-coating

- o/n +4°C
- NHS 20% +/− EGTA
- MgCl₂ 10 mM, 37°C
- Protein detachment and WB analysis

**FIGURE 1.** Experimental design of microplate/WB assays.
Effects of FH on the Assembly and Decay of C3bB and C3bBb

C3b-coated wells at 25 °C for 5, 10, 30, and 45 min with FB (1000 ng/ml) and FD (5 ng/ml), in assay buffer with 10 mM MgCl₂ (Fig. 1B). After washes, the complexes were detached from microtiter wells by incubation with 10 mM EDTA and 1% SDS for 1 h at room temperature, subjected to 10% SDS-PAGE, and transferred by electrophoretic blotting to a PVDF membrane. Proteins were detected with polyclonal rabbit anti-human FB antibody (1:500) followed by HRP-conjugated anti-rabbit antibody (1:3,000) and the ECL chemiluminescence detection system. C3bB and C3bBb formation was evaluated by the visualization by WB of the B band (93 kDa) and the Bb band (60 kDa), respectively. The intensity of the band detected by WB was estimated by densitometry using Image (National Institutes of Health).

For the generation of C3bB(Ni²⁺) and C3bBb(Ni²⁺) complexes, C3b-coated wells were incubated at 37 °C with FB (1000 ng/ml) and FD (5 ng/ml) in assay buffer containing 2 mM NiCl₂ for different times (5, 10, 20, 30, 60, and 120 min). The Ni²⁺-protein complexes were evaluated by WB as described above (Fig. 1H).

To determine the effect of FH on C3bB and C3bBb formation, FH (2640 ng/ml) was added together with FB and FD to C3b-coated wells by using the selective experimental conditions described above (Fig. 1, A, B, and H), and it was visualized by WB as FH band of 150 kDa by using polyclonal goat anti-human FH antibody (1:1000) followed by HRP-conjugated anti-goat antibody (1:15,000). Physiological molar ratios among complement proteins were used (molar ratios FB/FH = 1:1.6). In the reaction performed in the presence of 2 mM NiCl₂, FH was added together with FB (1000 ng/ml) and FD (5 ng/ml), diluted in assay buffer with 10 mM MgCl₂, for different times (5, 10, 30, 60, and 120 min). The Ni²⁺-protein complexes were evaluated by WB as described above.

In preliminary experiments, the binding efficiency of HS to the plate was verified by using an ELISA. Microtiter plates were coated with 3 or 30 μg/ml HS in PBS, in the presence or in the absence of 3 μg/ml C3b, by overnight incubation at 4 °C, blocked with 1% BSA, 0.1% Tween 20 in PBS for 1 h at 37 °C, and washed with 0.1% Tween 20 in PBS. HS immobilized on the surface were detected with a monoclonal mouse anti-HS antibody (1:100) followed by HRP-conjugated goat anti-mouse antibody (1:2000), both diluted in PBS. Color was developed using 3,3',5,5'-tetramethylbenzidine substrate and stopped with 2 M H₂SO₄, and absorbance was measured at 450 nm. Each reaction was performed in duplicate, and the OD values were averaged.

Selective C3bB C3 Proconvertase and C3bBb C3 Convertase Decay Assays—To study spontaneous or FH-mediated decay of C3 proconvertase and C3 convertase over time, C3bB(Mn²⁺) and C3bBb(Mg²⁺) were allowed to form for 2 h at 37 °C and 10 min at 37 °C, respectively, then were washed and incubated with selective assay buffers in the presence or in the absence of FH (2640 ng/ml; molar ratio FB/FH = 1:1.6) for the following time periods: C3bB(Mn²⁺), 30, 60, 120, and 240 min; C3bBb(Mg²⁺), 2, 4, 8, and 16 min (Fig. 1, C and D). For C3bB(Mn²⁺) and C3bBb(Mg²⁺) formed together during a 30-min period at 37 °C, both spontaneous and FH-mediated decay were monitored by incubating the complexes at 37 °C with assay buffer alone or with FH (2640 ng/ml) for 5, 30, 60, and 120 min (Fig. 1F). Following washes, the remaining complexes were detached from microtiter wells by incubation with 10 mM EDTA and 1% SDS and subjected to WB analyses as described above. The amount of C3bB and C3bBb formed before decay was also evaluated as baseline. The percentage of residual Bb band was calculated as the ratio of the densities (in pixel²) of the Bb bands after decay and the corresponding baseline Bb band density before decay × 100.

C3bBb(Mg²⁺) C3 Convertase Formation and Decay in the Presence of Properdin—To evaluate the ability of P to stabilize C3bBb(Mg²⁺) formation and decay, the complexes were generated by incubating C3b-coated wells with FB (1000 ng/ml), FD (5 ng/ml), and P (1145 ng/ml; molar ratio FB/P = 5:1) (35) in the presence or in the absence of FH (2640 ng/ml; molar ratio FB/FH = 1:1.6) at 37 °C (Fig. 1E). Following washes, spontaneous and FH-mediated decay was performed for a further 10 min with assay buffer alone or FH (2640 ng/ml). The complexes were detached from microtiter wells and subjected to WB analyses as described above.

Human plasma was obtained from 5 normal volunteers and 11 patients presenting histological and clinical evidence of C3 glomerulopathies (C3G), and the fraction was purified as described (36).

C3NeF-IgG hemolytic activity was quantified by determining the ability of the IgG fraction purified from plasma to stabilize the cell-bound C3bB convertase in the hemolytic assay (HA) (36). Under these experimental conditions, the residual convertase activity (expressed in %) in control experiments performed in the presence of normal IgG (400 μg) was below 20%. The presence of C3NeF activity in the assay resulted in increased residual convertase activity. The study was approved by the Bioethical Committee of the Azienda Sanitaria Locale in Bergamo (Number 00148858/III). Informed consent was obtained from patients and controls.

C3NeF-stabilized C3bBb(Mg²⁺) C3 Convertase Formation and Decay—To evaluate the ability of IgG fractions to stabilize C3bBb(Mg²⁺) complexes in spontaneous or FH-mediated decay, C3NeF-stabilized C3bBb(Mg²⁺) complexes were generated by incubating C3b-coated wells for 10 min at 25 °C with purified IgG (50 and 100 μg/ml), immediately followed by FB (1000 ng/ml) and FD (5 ng/ml) diluted in assay buffer with 10 mM MgCl₂. The complexes formed were detached from wells and detected by WB as above. The amount of C3 convertase formation before decay was evaluated as baseline. Spontaneous or FH-mediated decay of the complexes was monitored by further incubation for 10 min at 25 °C with buffer alone or FH (2640 ng/ml), respectively. Following washes, the remaining complexes were detached from microtiter wells and quantified as above (Fig. 1F). The % of residual Bb band was quantified as the ratio of the densities (in pixel²) of Bb bands after decay and
Effects of FH on the Assembly and Decay of C3bB and C3bBb

the corresponding baseline Bb band density before decay × 100.

To evaluate the effect of FH on C3NeF-stabilized C3 convertase formation, C3bBb(Mg²⁺) was generated in the presence of C3NeF-IgG or control IgG (50 or 100 µg/ml) and P (114.5 ng/ml; molar ratio FB/P = 5:1) for 10 min at 25 °C with assay buffer alone or FH (2640 ng/ml; molar ratio FB/FH = 1:1.6). Spontaneous or FH-mediated decay was performed for a further 10 min at 25 °C, and % of residual Bb band was evaluated as described above (Fig. 1G).

Quantification of Complement Fragments Bb and Bb—Bb and Ba fragment levels were measured in the supernatant of the reactions of C3bB(Mn²⁺) and C3bBb(Mn²⁺) complexes in the presence or in the absence of FH (Fig. 1A), by commercial ELISA kit following the protocol procedure (Quidel, San Diego).

C3bBb(Mg²⁺) C3 Convertase and C5b Formation in the Presence of Normal Human Serum—To obtain C3 convertase formation by using a physiological mixture of complement components, microtiter wells were coated with 3 µg/ml C3b in PBS by overnight incubation at 4 °C, blocked with 0.5% BSA in PBS for 1 h at 25 °C, and washed with wash buffer supplemented with 5 mM MgCl₂. C3bBb(Mg²⁺) complexes were formed by incubating C3b-coated wells at 37 °C for 30 min with 20% NHS diluted in PBS containing 5 mM MgCl₂, in the presence or in the absence of 1 mM EGTA (Fig. 1). After washes, formed C3bBb(Mg²⁺) complexes and bound FH were detected in the wells and detected by WB as described previously. C5b formation was evaluated by WB using a polyclonal rabbit anti-human C5/C5b (1:300) antibody, followed by HRP-conjugated anti-rabbit antibody (1:30,000). C5b was visualized as the product of the reaction with Mn²⁺ (31, 32), and for this reason Ni²⁺ stabilized C3bB in a form susceptible to FD cleavage, but the C3bBb(Mn²⁺), once formed, is highly unstable and dissociates immediately, so that only the C3bBb(Mn²⁺) complex could be detected. At variance, Mg²⁺ stabilizes C3bBb but not the proenzyme C3bB (34).

On the basis of this evidence, the ELISA was repeated replacing Ni²⁺ with Mn²⁺ or Mg²⁺ ions to stabilize either the C3bB or the C3bBb, respectively. For C3bB assembly, C3b-coated wells were incubated with FB at 37 °C for 2 h in the presence of 2 mM MnCl₂, whereas C3bBb formation was obtained by incubating C3b-coated wells with FB and FD at 25 °C for 30 min in the presence of 10 mM MgCl₂ (Fig. 1, A and B), based on published data (33, 34). As shown in Fig. 2, B and C, in either condition the amount of Bb complexes formed was too small to be detected by WB, likely due to degradation of the C3bBb(Mg²⁺) (31) during post-reaction incubation with secondary antibodies.

We then analyzed them on WB with an anti-FB antibody, the B and Bb bands of C3bB(Mn²⁺) and C3bBb(Mg²⁺) complexes immediately revealed and identified by WB, compared with values of the reaction without FH (Fig. 4). Furthermore, the ELISA was repeated replacing Ni²⁺ with Mn²⁺ or Mg²⁺ ions to stabilize either the C3bB or the C3bBb, respectively, could be visualized well (Fig. 2, B and C).

Results

ELISA with Ni²⁺ Stabilization Does Not Specifically Discriminate between C3bB C3 Proconvertase and C3bBb C3 Convertase Formation—To study the in vitro assembly of the AP C3bB C3 proconvertase and C3bBb C3 convertase, we first tried the ELISA previously reported by Hourcade et al. (31, 32). Several studies have documented that Ni²⁺ cation prolongs the half-life of both C3bB and C3bBb (27, 32, 37), and for this reason Ni²⁺ has been widely used, instead of the physiological Mg²⁺, to generate complexes stable enough to allow satisfactory measurements by ELISA or to perform structural studies. In a typical assay, increasing amounts of FB or FD plus FD are added to C3b-coated wells in the presence of Ni²⁺ to generate C3bB(Ni²⁺) or C3bBb(Ni²⁺), respectively, and the products are detected using an anti-FB antibody. As shown in Fig. 2A, the ELISA curves from the reaction of coated C3b with either FB alone or FB plus FD showed dose-dependent superimposable profiles. However, when the complexes that formed in the presence or in the absence of FD were detached from the wells and analyzed by WB, both B (93 kDa) and Bb (60 kDa) bands were found in both conditions (Fig. 2A, right side) indicating that the ELISA cannot selectively discriminate between C3bB and C3bBb formation. Indeed, in the presence of FD only a portion of C3bB was converted to C3bBb. Conversely, in the absence of FD, some C3bBb also formed besides C3bBb, possibly due to FD contamination in commercial FB plasma-purified protein (27, 38).

New Assays to Specifically Detect C3bB(Mn²⁺) C3 Proconvertase and C3bBb(Mg²⁺) C3 Convertase Formation—In an effort to specifically generate either C3bB or C3bBb complexes, we exploited the selective stabilization ability of different divalent cations. Hourcade and Mitchell (33) documented that Mn²⁺ stabilizes C3bB in a form susceptible to FD cleavage, but the C3bBb(Mn²⁺), once formed, is highly unstable and dissociates immediately, so that only the C3bBb(Mn²⁺) complex could be detected. At variance, Mg²⁺ stabilizes C3bBb but not the proenzyme C3bB (34).

On the basis of this evidence, the ELISA was repeated replacing Ni²⁺ with Mn²⁺ or Mg²⁺ ions to stabilize either the C3bB or the C3bBb, respectively. For C3bB assembly, C3b-coated wells were incubated with FB at 37 °C for 2 h in the presence of 2 mM MnCl₂, whereas C3bB formation was obtained by incubating C3b-coated wells with FB and FD at 25 °C for 30 min in the presence of 10 mM MgCl₂ (Fig. 1, A and B), based on published data (33, 34). As shown in Fig. 2, B and C, in either condition the amount of Bb complexes formed was too small to be detected by WB, likely due to degradation of the C3bBb(Mg²⁺) during post-reaction incubation with secondary antibodies.

We then analyzed them on WB with an anti-FB antibody, the B and Bb bands of C3bB(Mn²⁺) and C3bBb(Mg²⁺) complexes immediately revealed and identified by WB, compared with values of the reaction without FH (Fig. 4). Furthermore, the ELISA was repeated replacing Ni²⁺ with Mn²⁺ or Mg²⁺ ions to stabilize either the C3bB or the C3bBb, respectively, could be visualized well (Fig. 2, B and C).

Effect of FH on C3bB(Mn²⁺) C3 Proconvertase Assembly and Decay—To investigate whether FH prevents C3bB assembly by competing with FB binding to C3b (23–25), C3b-coated wells were incubated with FB in the presence or absence of FH added at a physiological molar ratio with FB (Fig. 1A). FH did not affect C3bB(Mn²⁺) assembly after 1, 2, 4, and 8 h of incubation, as documented by no change in densitometry of the B bands on WB, compared with values of the reaction without FH (Fig. 4A). As reported in Fig. 4A, top, FH band could already be visualized in the WB of the products detached from the wells 1 h of incubation, before C3 proconvertase formation, and FH
band increased at 2 and 4 h in parallel with C3bB(Mn²⁺) assembly. These results would indicate that FH binds both C3b and C3bB.

To evaluate whether binding of FH to C3bB affects C3 proconvertase dissociation, C3bB(Mn²⁺) was allowed to form over 2 h, and the complexes were subsequently incubated for different time intervals with buffer alone or with buffer containing FH (Fig. 1C). The intensity of the B bands did not change over 240 min decay versus baseline, either in the absence or in the presence of FH, indicating that in our conditions FH did not displace FB from C3b (Fig. 4B). Altogether the above results reveal that FH, at the physiological FH/FB molar ratio, did not affect either C3bB proconvertase assembly or its decay.

**Effect of FH on C3bBb(Mg²⁺) C3 Convertase Formation and Decay**—We then wondered whether the capability of FH to bind C3bB affects C3 convertase formation. Thus, we investigated the effect of FH on C3bBb(Mg²⁺) formation by incubating C3b-coated wells with FB and FD.
presence or in the absence of FH (Fig. 1B). In the presence of FH, the amount of C3bBb(Mg\(^{2+}\)) complexes, detected as Bb band density at each time point, was greatly reduced compared with the reaction without FH (Fig. 5A). We also analyzed spontaneous and FH-mediated C3bBb(Mg\(^{2+}\)) decay. C3bBb(Mg\(^{2+}\)) was allowed to form for 10 min and then incubated with buffer alone or with buffer containing FH (Fig. 1D). As shown in Fig. 5B, in the absence of FH, C3bBb(Mg\(^{2+}\)) dissociated in a time-dependent manner, but a faint band was still detectable at 16 min. The decay of C3bBb(Mg\(^{2+}\)) was strongly accelerated in the presence of FH at a physiological molar ratio with FB. Indeed, no Bb band could be detected after 4 min of decay with FH, confirming that FH is very efficient in Bb displacement from C3b. Notably, the effect of FH on C3bBb(Mg\(^{2+}\)) formation and decay was concentration-dependent (Fig. 6). Because the decay of C3bBb in the presence of FH was very rapid, on the basis of the above results we could not dissect whether the apparent inhibitory effect of FH on C3bBb formation was due
Effects of FH on the Assembly and Decay of C3bB and C3bBb

Effects of FH on the Assembly and Decay of C3bB and C3bBb

FIGURE 5. Effect of FH on C3bBb(Mg2+/H) C3 convertase formation (A) and decay (B) detected by microplate/WB assays. A, time course of C3bBb(Mg2+/H) formation. The complexes were obtained by incubating C3b-coated wells at 25 °C for 5, 10, 30, and 45 min in the presence or in the absence of 2640 ng/ml FH. B, time course of spontaneous and FH-mediated decay of C3bBb(Mg2+/H). The complexes formed during 10 min at 25 °C were further incubated for 2, 4, 8, and 16 min in the presence or in the absence of 2640 ng/ml FH. The amount of C3bBb formed was calculated as the densitometry of the Bb band (60 kDa), and results are reported in the bottom graphs as pixel²/10⁶. The percentage of residual Bb band was calculated as the ratio of the densities (in pixel²) of each Bb band after decay and the corresponding baseline Bb band density before decay/100. Results of a representative microplate/WB experiment of n = 3 are shown.

FIGURE 6. Effect of two different concentrations of FH on C3bBb(Mg2+/H) C3 convertase formation (A) and decay (B) by microplate/WB assays. A, time course of C3bBb(Mg2+/H) formation. The complexes were obtained by the incubation at 25 °C for 5, 10, 30, and 45 min of C3b-coated wells with FB (1000 ng/ml), FD (5 ng/ml), and 10 mM MgCl₂ in the presence or in the absence of FH at two different final molar ratios of FB/FH (1:0.8, 1320 ng/ml FH, or 1:1.63, 2640 ng/ml FH). B, time course of spontaneous and FH-mediated decay of C3bBb(Mg2+/H) originated in 10 min at 25 °C was evaluated by further incubation at 25 °C for 2, 4, 8, and 16 min in the presence or in the absence of FH at final molar ratios of FB/FH 1:0.8, 1320 ng/ml FH, or 1:1.63, 2640 ng/ml FH (physiological ratio). The amount of C3bBb (Bb band, 60 kDa) was quantified, and corresponding densitometry is reported in the graph below as pixel²/10⁶. The percentage of residual Bb band was calculated as the ratio of the pixel² of each Bb band densitometry after decay and the corresponding baseline Bb band densitometry before the decay/100. A representative microplate/WB analysis of n = 2 experiments is shown.

to FH-mediated dissociation of C3bBb molecules as soon as they formed or to FH-mediated blocking of C3bB conversion to C3bBb.

Effect of FH on C3bBb(Mg2+)/C3 Convertase Formation and Decay in the Presence of Properdin or C3NeF-IgG—In the attempt to prevent FH-mediated C3bBb decay, the above
Effects of FH on the Assembly and Decay of C3bB and C3bBb

![Diagram showing the effects of FH on C3bB and C3bBb formation and decay](image)

As an alternative strategy, we took advantage from patients with C3G who develop C3NeF, circulating IgG autoantibodies targeting C3bBb, which stabilize the complex and inhibit FH-mediated decay (41, 42). We isolated IgGs from 11 C3G patients and selected two preparations that were strongly positive for C3NeF by hemolytic assay (C3NeF-IgG activity ≥100%). No hemolytic activity was found in the purified IgG sample from a plasma pool of three healthy subjects.

C3bBb(Mg$^{2+}$) complexes were formed for 10 min in the presence of two different concentrations (50 and 100 μg/ml) of patient or control IgG, and they were then allowed to dissociate for an additional 10 min in the presence or in the absence of FH (Fig. 1F). As shown in Fig. 8, A and B, C3NeF-IgG from patient 5 (Fig. 8A) was the most potent and substantially limited FH-mediated C3bBb decay in a dose-dependent fashion, so that in the presence of 100 μg/ml C3NeF-IgG a Bb band with 64% intensity compared with baseline (before decay) was recovered at the end of decay with FH, although no Bb band was detected at the end of FH-mediated decay of C3bBb formed in the presence of control IgG. When the above experiments were repeated in the presence of both P and 100 μg/ml C3NeF-IgG from patient 5 (Fig. 1G), FH-mediated C3bBb decay was almost completely prevented (81% stabilization of the C3bBb(Mg$^{2+}$) complexes) (Fig. 9). Even though FH-mediated decay was efficiently blocked in this condition, C3bBb(Mg$^{2+}$) formation in the presence of FH was not fully restored; FH, added together with FB and FD to C3b-coated wells, caused a 56% reduction in the amount of C3bBb(Mg$^{2+}$) formed over 10 min in the presence of P and 100 μg/ml C3NeF-IgG, compared with the same reaction without FH (Fig. 9). For comparison, in samples with control IgG and P (in which FH-mediated decay was not prevented), FH reduced the amount of C3bBb(Mg$^{2+}$) formed by 97%. These results would suggest that FH may have a direct effect on C3bBb formation from C3bB proconvertase.

Effect of FH on C3bBb C3 Convertase Formation from C3bB—Mn$^{2+}$ stabilizes C3bB in a form susceptible to FD cleavage, but the C3bBb(Mn$^{2+}$) complex, once formed, is highly unstable and rapidly dissociates (33). Consistently, as shown in Fig. 3A, the time course of C3bBb(Mn$^{2+}$) assembly revealed that independently of the presence of FD, no Bb band of C3bBb(Mn$^{2+}$) could be detected. We wondered whether in this condition, in the absence of FD, the formation of C3bBb(Mn$^{2+}$) could be detected indirectly by measuring Bb bands released in the supernatant of the experiment. As shown in the supernatant of the reaction with the same reaction without FH (Fig. 11A), Bb release in the supernatant of the presence of FH was inhibited compared with the same reaction in the absence of FH (with FH, Bb 88–109 ng/ml and Ba 30–51 ng/ml versus without FH: Bb 200–500 ng/ml and Ba 53–165 ng/ml) (Fig. 11B). Altogether these results support the hypothesis that FH does prevent the conversion of C3bB to C3bBb.

Effect of FH on C3bBb(Ni$^{2+}$) C3 Proconvertase and C3bBb(Ni$^{2+}$) C3 Convertase Formation and Decay—To evaluate whether the diverse effect of FH on C3bB(Mn$^{2+}$) compared with C3bBb(Mg$^{2+}$) formation and decay was attributable to the different ions used to stabilize either complex (Mn$^{2+}$ versus Mg$^{2+}$), we repeated the microplate/WB experiments by incubating surface-bound C3b with FB and FD in the presence of Ni$^{2+}$, which stabilized both C3bB and C3bBb and yielded both B and Bb bands on WB (Figs. 1H and 12, A and B). The formation of C3bB and C3bBb complexes peaked at 30 min. To evaluate the kinetics of C3bB and C3bBb formation at shorter time scales, we repeated the experiments using 5, 10, 20, and 30 min of incubation. B and Bb bands were already well detected after 5 min. The intensity of B band remained stable thereafter, whereas Bb band intensity further increased at 10, 20, and 30 min of incubation (Fig. 12B).

As shown in Fig. 12, A and B, C3bBb(Ni$^{2+}$) assembly was not affected by FH. Dose-response studies showed that FH decreased C3bBb(Ni$^{2+}$) formation only when added at FH/FB molar ratios 3–6-fold higher than physiological levels (Fig. 12C). FH, at physiological levels, strongly reduced the amount...
of recovered C3bBb(Ni²⁺) at all time points (Fig. 12, A and B), confirming the results obtained in the ion-selective conditions with Mg²⁺/HCl.

Next, to assess the effect of FH on C3bBb(Ni²⁺) decay, the complexes obtained after 30 min at 37 °C were incubated with buffer alone or with FH (Fig. 1I).

C3bBb(Ni²⁺) did not undergo either spontaneous or FH-mediated decay at each time point compared with baseline (Fig. 13). At variance, C3bBb(Ni²⁺) spontaneously dissociated in a time-
Effects of FH on the Assembly and Decay of C3bB and C3bBb

Dependent manner and FH accelerated the decay (Fig. 13). Therefore, independently from the ion used, FH had no effect on C3bB, although it reduced C3bBb formation and accelerated its decay.

Effect of FH on C3bB(Ni²⁺) C3 Proconvertase and C3bBb(Ni²⁺) C3 Convertase Formation in the Presence of Heparan Sulfate or Sialic Acid—The binding of FH to C3b has been shown to be favored at the cell and tissue level by FH interaction with polyanions such as glycosaminoglycans on cell surfaces (39, 43). Thus, to study the effect of FH on proconvertase and C3 convertase assembly and dissociation, we mimicked FH interaction with C3bB(Ni²⁺) and C3bBb(Ni²⁺) coating on microtiter plates with SA and SA coated with mixtures of SA and FH (39, 43).

We confirmed HS coating on the microtiter plates by ELISA (Fig. 1A). Also in the presence of HS, FH did not substantially affect C3 proconvertase assembly, because it had a strong inhibitory effect on C3 convertase formation (Fig. 1B).

Because SA glycans, along with glycosaminoglycans, also are thought to act as self-markers (44), we repeated the above experiment using wells coated with SA and C3b (Fig. 1H). As observed for HS, in the presence of two different concentrations (3 or 30 μg/ml) of coated SA, FH did not affect C3Bb(Ni²⁺) assembly, although it completely prevented C3bBb(Ni²⁺) formation (Fig. 1C).

C3bBb(Mg²⁺) C3 Convertase and C5b Formation in the Presence of Normal Human Serum—A previous study reported that C3 adsorbed to a polystyrene surface can form C3 convertase and mediate the AP of complement activation in the presence of pig serum (45). Thus, we wondered whether immobilized C3b in our assay could originate a C3 convertase functionally active in the presence of human serum. To this purpose, we incubated C3b-coated wells with NHS as source of complement proteins for 30 min at 37°C (Fig. 1). In this condition, we confirmed C3bBb(Mg²⁺) formation (Bb 60-kDa bands; Fig. 15), which was not affected by EGTA, that inhibits the classical and lectin pathways without affecting the AP (46). Notably, we also found formation of C5b, as clearly visualized by the detection on WB of an α'-chain (104 kDa) band (Fig. 15A). These results support evidence that the microplate-based method presented here can mimic the assembly and dissociation of a functional C3 convertase, C3bBb, and its decay.

In vivo APRIL 8, 2016

FIGURE 1. Time course of selective C3bBb(Mn²⁺) C3 convertase formation in the absence or in the presence of FH, by microplate/WB assay (A) and results of Bb and Ba fragments ELISA in the supernatant (B). A, C3bBb(Mn²⁺) and C3bBb(Mg²⁺) were obtained by incubating C3b-coated wells at time points indicated with 1000 ng/ml FB, 5 ng/ml FD, and 2 mM MnCl₂, in the presence or in the absence of 2640 ng/ml FH. The intensity of the B band (93 kDa) as index of C3 proconvertase assembly was quantified, and the results expressed as pixel²/cm². B, FH band (150 kDa) could be visualized in the WB, and a representative image is reported on the top. Results of a representative microplate/WB experiment of n = 3 are shown. B, Bb and Ba fragment levels (nanograms/ml) were measured, by ELISA, in the supernatant of the same reactions.
added FD could be attributable to protease contaminants in the commercial plasma-purified FB protein (27, 38).

With the aim of specifically generating C3bB and C3bBb complexes, we combined microplate and WB techniques and exploited the selective stabilization properties of Mn$^{2+}$/H11001 and Mg$^{2+}$/H11001 on C3bB and C3bBb (33, 34, 52), respectively. A previous study (33) documented that Mn$^{2+}$/H11001 stabilizes C3bB in a form susceptible to FD cleavage, but C3bBb(Mn$^{2+}$/H11001), once formed, is highly unstable and dissociates immediately. FB binding to C3b depends on elements in fragment Ba and on the metal ion-de-
Effects of FH on the Assembly and Decay of C3bB and C3bBb

FIGURE 13. Effect of FH on C3bB(Ni²⁺) C3 proconvertase and C3bBb C3 convertase decay detected by microplate/WB assays. Time-dependent spontaneous and FH-mediated decay of C3bB(Ni²⁺) and C3bFb formed during 30 min at 37 °C by incubation of C3b-coated wells with FB and NiCl₂, respectively, and reported in the microplate/WB methods with ion-based selective stabilization, further validated ion-based stabilization. C3b coated on plastic wells, as in the context via its thioester moiety, which suggests caution in interpreting these data. However, the experiments performed in the presence of human serum indicate that in this condition C3b binds to FH and the corresponding baseline decay and the corresponding baseline decay. Results of representative microplate/WB experiments are shown.

FH does not affect C3bB assembly. The finding here that the C3bB complexes formed in the same amounts in the presence or in the absence of FH would suggest that FH does not compete enough with FB for binding to C3b to prevent C3bB assembly. A plausible explanation of our results derives from biophysical studies in the literature, showing that the binding affinity between C3b and FH molecules is lower compared with the affinity between C3b and FB (Kₐ C3b-FB, 1 μM, versus Kₐ C3b-FH, 73 nM) (29, 33, 56), which would indicate that C3b-FB interaction is favored toward that between C3b and FH. Finding that FH band was detected before C3bB(Mn²⁺) complex formation would indicate that the interaction between C3b and FH might have faster kinetics than that between C3b and FB. However, C3b-FH binding is weaker than C3b-FB interaction hence C3b proconvertase formation does not significantly affect C3bB assembly. Notably, lack of a FH effect on spontaneous C3bB decay is not independent of the ions used (Mn²⁺ and Ni²⁺), which is validated by data that gene mutations resulting in reduced capacity of FH to bind cell surfaces result in uncontrolled complement activation and aHUS (58, 59). Our present results of C3bB and C3bBb assembly in the presence of HS or SA show that even in this more physiological pattern, FH does not substantially affect C3 proconvertase assembly but has a strong inhibitory effect on C3 convertase formation. However, we found that FH at molar ratios with FB higher than those present in normal blood was able to partially prevent C3bB proconvertase assembly. The finding here that the C3bB and C3bBb are formed and regulated.

Assembly and regulation of the AP C3 convertase C3bBb is strictly modulated, and alterations in C3bBb control lead to tissue damage (4). Consistently, genetic abnormalities of the two components of AP C3 convertase, FB and C3, or of the main AP regulator FH, which result in increased C3 convertase formation and resistance to dissociation, lead to complement-mediated severe tissue and organ injury and dysfunction, as observed in patients with aHUS, a rare thrombotic microangiopathy that targets the microvasculature of the kidney and other organs (4–6, 46), or in patients with C3G (7, 8, 55). Understanding how the AP C3 proconvertase and C3 convertase are assembled and modulated by FH is therefore of great relevance.

An intriguing but poorly investigated aspect regarding AP complement regulation is whether FH has any effect on C3b assembly (23, 24). Here, for the first time, we document that FH does not affect C3bB assembly. The finding here that the C3bB complexes formed in the same amounts in the presence or in the absence of FH would suggest that FH does not compete enough with FB for binding to C3b to prevent C3bB assembly. A plausible explanation of our results derives from biophysical studies in the literature, showing that the binding affinity between C3b and FH molecules is lower compared with the affinity between C3b and FB (Kₐ C3b-FB, 1 μM, versus Kₐ C3b-FH, 73 nM) (29, 33, 56), which would indicate that C3b-FB interaction is favored toward that between C3b and FH. Finding that FH band was detected before C3bB(Mn²⁺) complex formation would indicate that the interaction between C3b and FH might have faster kinetics than that between C3b and FB. However, C3b-FH binding is weaker than C3b-FB interaction hence C3b proconvertase formation does not significantly affect C3bB assembly. Notably, lack of a FH effect on spontaneous C3bB decay is not independent of the ions used (Mn²⁺ and Ni²⁺), which is validated by data that gene mutations resulting in reduced capacity of FH to bind cell surfaces result in uncontrolled complement activation and aHUS (58, 59). Our present results of C3bB and C3bBb assembly in the presence of HS or SA show that even in this more physiological pattern, FH does not substantially affect C3 proconvertase assembly but has a strong inhibitory effect on C3 convertase formation. However, we found that FH at molar ratios with FB higher than those present in normal blood was able to partially prevent C3bB proconvertase assembly. In addition, coating of HS or SA on a plastic well may not be sufficient to emulate a cell surface. Thus, we cannot exclude the possibility that in a cell-based context, SA and glycosaminoglycans present in high amounts in the glyocalyx and the cell surface, could favor FH clustering and influence the...
interactions of C3b with FB or FH in favor of the latter (25), resulting in efficient antagonistic effects of FH on C3bB formation.

The few available data in the literature indicated that FH does not induce decay of assembled C3bB proconvertase (26, 27), and this was confirmed by our finding here that C3bB was not dissociated by FH.

In contrast and in harmony with consolidated evidence (27, 32), FH was very efficient at dissociating the C3bBb C3 convertase at a physiological molar ratio with FB, and P did not prevent FH-mediated C3bBb decay. Co-crystal structural comparison of C3bBb with the complex between C3b and the regulatory N-terminal four complement control protein domains of FH (C3b/FH1–4) (56) indicated that FH-induced C3bBb dissociation is mediated by the first two complement control proteins of FH, which bind the α’NT, MG2, and MG6–MG7 domains of C3b (56). Because the Ba fragment binds the α’NT domain of C3b (37, 52, 60) and Bb binds the MG domains, we speculate that FH cannot displace FB from C3b in C3bB because FB limits the accessibility of FH-binding sites on C3b. Once Ba is released from C3bB to form C3bBb, the α’NT region of C3b would become available to the interaction with FH, which in turn dislocates Bb from the C3bBb complex through steric hindrance (51, 56). In addition, because the complementary surfaces of FH and Bb are both negatively charged, they could contribute to the destabilization of the C3bBb complex through electrostatic repulsion (56, 61). More structural studies by NMR or x-ray crystallography are needed to better understand the underlying mechanisms.

It is relevant that we found an apparent strong inhibitory effect of FH on C3bBb C3 convertase formation, as documented by a failure to detect significant Bb band on WB from

FIGURE 14. HS-coated ELISA (A) and effect of FH on C3bB(Ni2⁺) C3 proconvertase and C3bBb(Ni2⁺) C3 convertase formation in the presence of HS (B) or SA (C) detected by microplate/WB assays. Microtiter plates were coated with 3 or 30 μg/ml HS in PBS, in the presence or in the absence of 3 μg/ml C3b. Immobilized HS was detected with a monoclonal mouse anti-HS antibody (1:100) followed by HRP-conjugated goat anti-mouse antibody (1:2000). Values are given as the OD averages with standard deviation (n = 3 each). B and C, C3bB(Ni2⁺) and C3bBb(Ni2⁺) complexes were obtained by incubating C3b and HS-coated (B) or SA-coated (C) (3 or 30 μg/ml) wells with FB (1000 ng/ml), FD (5 ng/ml), and NiCl₂ (2 mM) at 37 °C for 30 min, in the presence or in the absence of FH (2640 ng/ml). The amount of C3bB or C3bBb formed was calculated as the intensity of the B (93 kDa) or Bb (60 kDa) bands, respectively, and reported in the bottom graphs as pixel² · 10⁶. FH band (150 kDa) could be visualized in the WB. Results of a representative microplate/WB experiment of n = 3 are shown.
Effects of FH on the Assembly and Decay of C3bB and C3bBb

The reaction of C3b with FB and FD in the presence of FH. Based on the strong decay accelerating activity of FH on C3b, we first argued that this FH effect could be attributable to a rapid FH-accelerated dissociation of newly formed complexes. Finding that the addition of C3NeF and P, which almost completely prevented C3 convertase FH-mediated decay, did not fully restore C3bBb(Mg²⁺) formation suggested that FH could not affect C3bB generation. Data that in the reaction of C3b with FB and FD in the presence of Mn²⁺, completely prevented the release of Bb and C5b from the supernatant, without modifying the assembly of complexes, would indeed support the hypothesis that the conversion of C3bB to C3bBb would suggest that the inhibitory effect of FH on C3bBb formation is likely the sum of inhibition of C3bB conversion to C3bBb and of C3bBb decay acceleration.

In summary, taking advantage of the developed assay-friendly assays based on combined microplate and WB techniques that specifically detect either C3bB or C3bBb, we shed new light on mechanisms underlying the regulation of the AP C3 proconvertase and C3 convertase by FH as follows. 1) We document that at the physiological molar ratio with FB, FH does not affect C3bB formation and dissociation even in the presence of HS or SA that favors FH-C3b interaction. 2) We confirm that FH blocks C3bB formation and accelerates its decay. 3) We found that FH inhibits the conversion of C3bB to C3bBb. However, additional structural studies are needed to better understand the interaction between FH and the C3bB and C3bBb complexes, and cell-based studies are warranted to confirm such mechanisms in a more physiological context in which C3b is attached via its thioester to a surface nucleophile.

The assays presented here could be a useful tool for studying the effect of other complement regulators on C3bB and C3bBb assembly and decay, as well as for a rapid screening of the effect of C3NeFs isolated from patients with C3G on C3bBb stabilization. A further application of the assays could be to analyze the functional consequences of FB, C3, or FH mutants/variants from patients with aHUS or C3G on C3bB and C3bBb formation and decay.

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March 20, 2017

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Insights into the Effects of Complement Factor H on the Assembly and Decay of the Alternative Pathway C3 Proconvertase and C3 Convertase
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