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 (C3bb). 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, 756–761) have demonstrated the existence of a complement alternative pathway regulator factor H (FH) that prevents C3bB assembly and decay and C3bBb formation, with the aim of investigating the effect of FH on C3bB assembly and decay and C3bBb formation 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 its inhibitory effect on C3bBb formation and of C3bBb decay acceleration. Further studies are required to confirm these findings.

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dolytic 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 FH and the inhibitory effects of FH on C3bB assembly. Tortajada et al. (26), in keeping with a previous report (27), found that FH increased C3bB C3 proconvertase formation by competing with FB for C3b binding resulting in an inhibitory effect on C3 proconvertase assembly.

Whether FH affects the formation of C3bBb from C3bB has not been investigated yet. However, as the dissociation of already formed C3bB C3 convertase (C3 convertase activity) is well documented (28), the depletion of FH into the formation and dissociation of the AP C3bBb C3 convertase complexes is hampered by the instability of the two complexes and the paucity of sensitive and specific detection assays.

To overcome these shortcomings, in this study we set up user-friendly assays 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-mouse 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-acetylmuraminic acid (sialic acid, A0812), and EGTA (1,10-phenanthroline, A4956) were obtained 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-heparan sulfate monoclonal antibody (catalog no. MA1204) was purchased by Millipore (Temecula, CA). Polyclonal rabbit anti-human FH was obtained from Calbiochem (catalog no. 341276, Bil-lerica, MA). Polyclonal goat antiserum to human FB was obtained from Quidel (catalog no. A311, San Diego, 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 Assays—We set up a novel user-friendly assay based on combined microplate and Western blotting 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 for 2 h at 37 °C or in the presence of FD (25 mg/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). 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 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 M H2SO4, and absorbance was measured at 450 nm. Each reaction was performed in duplicate, and the OD values were averaged.

For the generation of C3bBb(Mn2+) complexes, C3bBb(Mg2+) complexes, C3b-coated microtiter wells were treated as above except that the incubation was performed in the presence of 2 mM MnCl2, 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.

| Stage | Conditions | Reagents | Notes |
|-------|------------|----------|-------|
| A     | C3b-coating | O/n +4°C | MnCl₂ 2 mM, 37°C (1, 2, 4 and 8h) |
|       |            |          | C3-convertase formation + FB +/− FD +/- FH |
|       |            |          | Protein detachment and WB analysis |
| B     | C3b-coating | O/n +4°C | MgCl₂ 10 mM, 25°C (5, 10, 30 and 45 min) |
|       |            |          | C3-convertase assembly + FB |
|       |            |          | Quantification of Bb and Ba supernatant levels by ELISA |
| C     | C3b-coating | O/n +4°C | MnCl₂ 2 mM, 2 h 37°C |
|       |            |          | Protein detachment and WB analysis |
| D     | C3b-coating | O/n +4°C | MgCl₂ 10 mM, 10 min 25°C |
|       |            |          | C3-convertase formation + 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-convertase 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-convertase formation + 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-convertase formation + FB + FD +/− P +/− FH |
|       |            |          | Spontaneous or FH-mediated decay; buffer +/- FH |
|       |            |          | Protein detachment and WB analysis |

*Notes: FH refers to factor H, C3b to complement component C3b, WB to Western Blotting.*
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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, pH 7.5 for 1 h at room temperature, subjected to 10% SDS-PAGE, and transferred by electroblotting to a PVDF membrane. Proteins were detected with polyclonal rabbit anti-human FB antibody (1:1000) followed by HRP-conjugated anti-rabbit antibody (1:10,000) and the ECL chemiluminescence detection system. C3bB and C3bBb formation was evaluated by the visualization of the bands 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 ImageJ (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. 1A).

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-FH antibody (1:1000) followed by HRP-conjugated anti-goat antibody (1:15,000). Physiological molar ratios of complement proteins were used (molar ratios FB/FH = 1:2 and PB/FH = 1:1.6). In the reaction performed in the presence of 2 mM NiCl₂, FH was added together with FB and FD to C3b-coated wells, at different molar ratios (1:1.6; 1:5; 1:10). In the reaction performed in the presence of SA, C3bB(Ni²⁺) and C3bBb(Ni²⁺) were generated by incubating FB (1000 ng/ml) and FD (5 ng/ml) diluted in assay buffer with 2 mM NiCl₂, MgCl₂ and HS-coated (3 and 30 μg/ml) or SA-coated (3 and 30 μg/ml) wells at 37 °C for 30 min, in the presence or absence of FH (Fig. 1H). C3bB(Ni²⁺) and C3bBb(Ni²⁺) 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. Microwell 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 25 °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(Ni²⁺) and C3bBb(Ni²⁺) 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. 1I). 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 (114.5 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) for 10 min at 25 °C (Fig. 1E). Following washes, spontaneous or FH-mediated decay was performed for a further 10 min at 25 °C with assay buffer alone or FH (2640 ng/ml). C3bBb complexes were detached from microtiter wells and subjected to WB analyses as described above.

C3NeF-IgG hemolytic activity was quantified by determining the ability of the IgG fraction purified from plasma to stabilize the cell-bound C3bBb 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 both 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 the Bb bands after decay and...
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the corresponding baseline Bb band density before decay × 100.

To evaluate the effect of FH on C3NeF-stabilized C3 convertase formation, C3bBb(Mn²⁺) 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 Ba—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 by WB and ELISA as described previously. C5b formation was evaluated by WB using the corresponding 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⁺, in addition the amount of the complexes formed was too small to be detected by ELISA, likely due to degradation of the C3bBb(Mn²⁺) complexes during post-reaction incubation with the secondary antibodies. Results

ELISA with Ni²⁺ Stabilization Limiting the Ability to 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 FB 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 C3bB, 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 C3B 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. As shown in Fig. 2, B and C, in either condition the amount of the complexes formed was too small to be detected by ELISA, likely due to degradation of the C3bBb(Mn²⁺) or C3bBb(Mg²⁺) during post-reaction incubation with the secondary antibodies.

We then analyzed the complexes immediately after the incubation reaction with the antibody. As shown in Fig. 3A, the complexes immediately after formed Bb bands of C3bBb(Mn²⁺) and C3bBb(Mg²⁺) could be visualized well (Fig. 2, B and C). The amount that only the B band of C3bB was visualized in the product of the reaction with Mn²⁺, even when FD was added to the incubation mixture (Fig. 2B, right panel). Similarly, only the Bb band of C3bBb was detected in the reaction with Mg²⁺ and FD (Fig. 2C, right panel). Neither B nor Bb bands were observed in the reaction with Mg²⁺ in the absence of FD.

We then analyzed the time course of C3bB(Mn²⁺) and C3bBb(Mg²⁺) formation. As shown in Fig. 3A, the B band of C3bBb(Mn²⁺) was clearly detected after 2 h of incubation and was still evident at 8 h independently of the presence of FD, further confirming the specificity of the test for C3bB assembly. The kinetics of C3bBb(Mg²⁺) (Fig. 3B) formation was faster than that of C3bBb(Mn²⁺), and indeed the Bb band was already well detected after 10 min of incubation and the intensity further increased at 45 min of incubation. For the subsequent experiments, we used the new combined microplate/WB assays for specifically studying C3bBb(Mn²⁺) and C3bBb(Mg²⁺) complexes.

Effect of FH on C3bBb(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 C3bBb(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
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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 results in inhibition of C3bBb convertase formation. Thus, we investigated the effect of FH on C3bBb(Mg²⁺) formation by incubating C3b-coated wells with FB and FD in the presence of FH.
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In the presence of FH, the amount of C3bBb(Mg\textsuperscript{2+}/H\textsubscript{11001}) 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\textsuperscript{2+}) decay. C3bBb(Mg\textsuperscript{2+}) was allowed to form for 10 min and then incubated with buffer alone or with buffer containing FH (Fig. 5B). As shown in Fig. 5B, in the absence of FH, C3bBb(Mg\textsuperscript{2+}) dissociated in a time-dependent manner, but a faint band was still detectable at 16 min. The decay of C3bBb(Mg\textsuperscript{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\textsuperscript{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

FIGURE 3. Time course of selective C3bB(Mn\textsuperscript{2+}) C3 proconvertase assembly (A) and C3bBb(Mg\textsuperscript{2+}) C3 convertase formation (B) detected by microplate/WB assays. C3bB(Mn\textsuperscript{2+}) and C3bBb(Mg\textsuperscript{2+}) complexes were obtained by incubating C3b-coated wells at time points indicated with 1000 ng/ml FB in the presence or in the absence of 5 ng/ml FD and 2 mM MnCl\textsubscript{2} at 37 °C or 10 mM MgCl\textsubscript{2} at 25 °C, respectively. The amount of C3bB assembled and of C3bBb formed was calculated as the intensity of B band (93 kDa) and Bb band (60 kDa), respectively, and results are reported in the bottom graphs as pixel\textsuperscript{2}/10\textsuperscript{6}. Results of a representative microplate/WB experiment of n = 3 are shown.

FIGURE 4. Effect of FH on C3bB(Mn\textsuperscript{2+}) C3 proconvertase assembly (A) and C3bB(Mn\textsuperscript{2+}) C3 proconvertase decay (B) detected by microplate/WB assays. A, time course of C3bB(Mn\textsuperscript{2+}) assembly. The complexes were obtained by incubating C3b-coated wells at 37 °C for 1, 2, 4, and 8 h with 1000 ng/ml FB and 2 mM MnCl\textsubscript{2} in the presence or in the absence of 2640 ng/ml FH. FH band (150 kDa) could be visualized in the WB, and a representative image is reported on the top. B, time course of spontaneous and FH-mediated decay of C3bB(Mn\textsuperscript{2+}) assembled in 2 h at 37 °C was evaluated by further incubation with buffer alone or with 2640 ng/ml FH for 30, 60, 120, and 240 min at 37 °C. The amount of C3 proconvertase (B band, 93 kDa) was quantified, and results of corresponding densitometries are reported in the graphs below as pixel\textsuperscript{2}/10\textsuperscript{6}. The percentage of residual B band was calculated as the ratio of the densities (in pixel\textsuperscript{2}) of each B band after decay and the corresponding baseline B band density before decay × 100. Results of a representative microplate/WB experiment of n = 3 are shown.
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FIGURE 5. Effect of FH on C3bBb(Mg$^{2+}$) C3 convertase formation (A) and decay (B) detected by microplate/WB assays. A, time course of C3bBb(Mg$^{2+}$) 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(Mg$^{2+}$). 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 FH. The amount of C3bBb formed was calculated as the densitometry of the Bb band (60 kDa), and results are reported in the bottom graph as pixel$^2/10^6$. The percentage of residual Bb band was calculated as the ratio of the densities (in pixel$^2$) of each Bb band after decay and the corresponding baseline Bb band density before decay ($\times 100$). Results of a representative microplate/WB experiment of $n = 3$ are shown.

FIGURE 6. Effect of two different concentrations of FH on C3bBb(Mg$^{2+}$) C3 convertase formation (A) and decay (B) by microplate/WB assays. A, time course of C3bBb(Mg$^{2+}$) formation. The complexes were obtained by incubating C3b-coated wells with FB (1000 ng/ml), FD (5 ng/ml), and 10 mM MgCl$_2$ 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(Mg$^{2+}$) 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 pixel$^2/10^6$. The percentage of residual Bb band was calculated as the ratio of the pixel$^2$ of each Bb band densitometry after decay and the corresponding baseline Bb band densitometry before the decay ($\times 100$). 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(Mg$^{2+}$) C3 Convertase Formation and Decay in the Presence of Properdin or C3NeF-IgG—In the attempt to prevent FH-mediated C3bBb decay, the above
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Experiments were repeated in the presence of 100 μg/ml C3NeF-IgG (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**

FH(12) stabilized 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 presence of FD, the formation of C3bBb(Mn$^{2+}$) could be detected indirectly by measuring Bb and Ba bands released in the supernatant of the incubation mixture in the absence of FH. However, the results showed that in the supernatant of the reaction with the same reaction

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**

C3bBb(Mg$^{2+}$) complexes were generated by incubating at 25 °C for 10 min C3b-coated wells with 1000 ng/ml FB, 5 ng/ml FD, and 10 mM MgCl$_2$ in the presence or in the absence of 114.5 ng/ml P and 2640 ng/ml FH. Spontaneous or FH-mediated decay was evaluated by further incubation at 25 °C for 10 min with buffer alone or added with 2640 ng/ml FH. The amount of C3bBb formed was calculated as the intensity of the Bb (60 kDa) band and reported in the bottom graphs as pixel$^{-2}$×10$^6$. Results of a representative microplate/WB experiment of n = 3 are shown.
of recovered C3bBb(Ni\(^{2+}\)) at all time points (Fig. 12, A and B), confirming the results obtained in the ion-selective conditions with Mg\(^{2+}\)/H\(_{11001}\).

Next, to assess the effect of FH on C3bB(Ni\(^{2+}\))/H\(_{11001}\) and C3bBb(Ni\(^{2+}\))/H\(_{11001}\) decay, the complexes obtained after 30 min at 37 °C were incubated with buffer alone or with FH (Fig. 1I).

C3bB(Ni\(^{2+}\))/H\(_{11001}\) did not undergo either spontaneous or FH-mediated decay at each time point compared with baseline (Fig. 13).

At variance, C3bBb(Ni\(^{2+}\))/H\(_{11001}\) spontaneously dissociated in a time-
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**Figure 11. Time course of selective C3bBb(Mn^{2+}) 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, C3bB(Mn^{2+}) and C3bBb(Mn^{2+}) were obtained by incubating C3b-coated wells at time points indicated with 1000 ng/ml FB, 5 ng/ml FD, and 2 mM MnCl_{2}, 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 pixel²-10⁶. 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.

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(Ni^{2+}) C3 Proconvertase and C3bBb(Ni^{2+}) 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 the binding of FH to C3b on C3 proconvertase and C3 convertase assembly, we mimicked FH interaction with C3b in the presence of HS. C3bB(Ni^{2+}) and C3bBb(Ni^{2+}) were obtained by incubating C3b-precoated wells at time points indicated with 1000 ng/ml FB, 5 ng/ml FD, and 2 mM NiCl_{2}, in the presence or in the absence of 30 μg/ml of coated HS (Fig. 1A, B). In the presence of HS, FH did not affect C3 proconvertase assembly (Fig. 1A, B). Also in the presence of HS, FH substantially affected C3 convertase formation (Fig. 1C, D).

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(Ni^{2+}) assembly, although it completely prevented C3bBb(Ni^{2+}) formation (Fig. 1C). C3bBb(Mg^{2+}) 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(Mg^{2+}) 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 initial assembly and dissociation of a functional C3 convertase, C3bBb, by binding to additional C3b molecules forming the C3 convertase (Fig. 15B).

Traditionally, to evaluate formation and/or decay of the AP C3 convertase, hemolytic assays have been widely used (46, 47). These assays, performed on the surface of C3b-coated sheep erythrocytes, make it possible to determine the functional activity of the cell surface-bound C3bBb by measuring the degree of erythrocyte lysis, but they do not provide a direct estimation of C3bBb complex formation and decay. In addition, surface plasmon resonance has largely been used to study C3bB and C3bBb formation and dissociation (33, 38, 48). Nevertheless, surface plasmon resonance is not universally available, and the method is not the most suitable for exploring the impact of FH on C3bB and C3bBb assembly and decay, because following FH injection the net mass change on the chip is a balance between FH binding to C3b and B/Bb release (26, 49). Alternatively, an ELISA has been used, in which purified C3b-coated microplate wells are incubated with FB or FB plus FD to selectively generate C3bB or C3bBb, respectively (27, 50). In most studies the ELISA was done in the presence of Ni^{2+} instead of the physiological Mg^{2+}, because Ni^{2+} is well known to prolong the half-life of both C3bB and C3bBb (27, 37, 51). However, the above method cannot discriminate between C3bB and C3bBb, because WB analysis of the ELISA products showed the formation of both B and Bb bands of the C3bB and C3bBb complexes, independently of the presence of FD in the reaction mixture. The simultaneous detection of C3bB and C3bBb in the presence of FD is consistent with evidence that Ni^{2+} strongly stabilizes C3bB (37). Moreover, C3bBb formation in the absence of...
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-
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FIGURE 13. Effect of FH on C3bB(Ni^{2+}) C3 proconvertase and C3bBb(Mn^{2+}) C3 convertase decay detected by microplate/WB assays. Time-dependent spontaneous and FH-mediated decay of C3bB(Ni^{2+}) and C3bBb(Mn^{2+}) formed during 30 min at 37 °C by incubation of C3b-coated wells (1000 ng/ml), FD (5 ng/ml), and NiCl_{2} (2 μM) or MnCl_{2} (600 ng/ml), with buffer alone or added with 2640 μg/ml FH. The amount of C3bB or C3bBb for the respective condition of the B (93 kDa) or Bb (60 kDa) band density before decay and the corresponding baseline B or Bb band density after decay were calculated as the ratio of the densities of each B or Bb band after decay and baseline B or Bb band density before decay. Results of a representative microplate/WB experiment of % residual band density after 15 min (HB) or 30 min (FB) decay are shown.

FH band was detected before C3bB(Mn^{2+}) complex formation was observed here independently of the ions used (Mn^{2+} and Ni^{2+}). In the reaction mixture, further validation of C3b coated on plastic wells, as in the experimental setting here, is not oriented as in physiological cell 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 provides a suitable molecular platform for the formation of functionally active C3 and C5 convertases.

FH binds to the host cell surface through interaction with oligosaccharides, such as glycosaminoglycans and sialic acid, bearing clusters of negative charges, and FH binding to such polyanions shifts FH from a latent to an active conformation, which significantly increases the affinity for C3b (39). This interaction prevents inadvertent action of the AP of complement on host cells and mediates its ability to distinguish between host cells and pathogens that do not have such glycans (57). The relevance of FH-glycan interaction in protecting cells and tissues from complement-activating products is highlighted by data that gene mutations resulting in reduced capability 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 that FH at molar ratios with FB higher than those present in normal blood was able to partially prevent C3bB proconvertase 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 (K_{D} C3b-FH, 1 μM, versus K_{D} C3b-FB, 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(Mn^{2+}) complex formation would indicate that the interaction between C3b and FH might have faster kinetics than that between C3b and FB. However, C3b-FH interaction is weaker than C3b-FB interaction because of C3 proconvertase formation 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 C3bB 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 (K_{D} C3b-FH, 1 μM, versus K_{D} C3b-FB, 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(Mn^{2+}) complex formation would indicate that the interaction between C3b and FH might have faster kinetics than that between C3b and FB. However, C3b-FH interaction is weaker than C3b-FB interaction because of C3 proconvertase formation 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.

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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](https://example.com/figure14.png)

**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 assay. 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.
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FIGURE 15. C3bBb(Mg$^{2+}$) C3 convertase and C5b formation by using normal human serum detected by microplate/WB assays. A, C3bBb(Mg$^{2+}$) complexes were obtained by incubating C3b-coated wells with 20% NHS at 37 °C for 30 min, in the presence 5 mM MgCl$_2$. C3bB and C5b formation was evaluated as the presence of Bb (60 kDa) and α′-chain C5b (104 kDa) bands, respectively. FH could be visualized in the WB as a 150-kDa band. Results of a representative microplate/WB experiment of n = 3 are shown. B, schematic describing C3 and C5 convertase formation and C5b deposition on C3b-coated wells with 20% NHS.

In summary, taking advantage of newly developed user-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 C3bBb formation and accelerates its decay, without modifying the amount of C3bB complexes, would indeed support the hypothesis that FH inhibits the conversion of C3bB to C3bBb. However, additional structural studies are needed 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 easy tools 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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