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The Tick-Over Theory Revisited: Formation and Regulation of the Soluble Alternative Complement C3 Convertase (C3(H₂O)Bb)

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Abstract

The molecular interactions between the components of the C3 convertase of the alternative pathway (AP) of complement and its regulators, in both surface-bound and fluid-phase form, are still incompletely understood. The fact that the AP convertase is labile makes studies difficult to perform. According to the so called tick-over theory, hydrolyzed C3, called C3(H₂O), forms the initial convertase in fluid phase together with factor B. In the present study, we have applied western blot analysis and ELISA together with fluorescence resonance energy transfer (FRET) to study the formation of the fluid-phase AP convertases C3(H₂O)Bb and C3bBb and their regulation by factor H and factor I at specific time points and, with FRET, in real time. In our hands, factor B showed a higher affinity for C3(H₂O) than for C3b, although in both cases it was readily activated to Bb. However, the convertase activity of C3bBb was approximately twice that of C3(H₂O)Bb, as monitored by the generation of C3a. But in contrast, the C3(H₂O)Bb convertase was more resistant to inactivation by factor H and factor I than was the C3bBb convertase. Under conditions that totally inactivated C3bBb, C3(H₂O)Bb still retained approximately 25% of its initial activity.

Keywords

Alternative pathway convertase; C3b; C3(H₂O); factor B; fluid phase; fluorescence resonance energy transfer (FRET)

1. Introduction

The complement system is activated by three pathways: the classical, lectin, and alternative pathways. Activation of any one of these pathways leads to the activation of the central complement component 3 (C3), a 187-kDa protein consisting of a stable β-chain and a thioester-containing α-chain, linked together by a disulfide bond and non-covalent forces. All three pathways also generate labile enzymatic complexes, the C3 convertases. During cleavage by a C3 convertase to produce its active form, C3b (177 kDa), the molecule undergoes

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conformational changes, exposing the thioester and allowing it to react with either hydroxyl or amine groups exposed on the target surface (Janssen et al., 2005).

The alternative pathway (AP) convertase is formed by an interaction between C3b and factor B, followed by the cleavage of factor B by factor D into Ba and Bb, to form an active enzymatic complex, C3bBb (Fearon et al., 1973). This convertase, which is stabilized by the addition of properdin, the only recognized natural positive regulator of complement (Schwaebler and Reid, 1999), is then able to generate more C3b molecules, initiating a positive feedback loop. The AP complex usually utilizes Mg^{2+} for binding, but studies have shown that Ni^{2+} can create a more stable convertase (Fishelson et al., 1983).

Generation of the initial C3b molecules, which initiates the positive feedback reaction, is described by the tick-over theory (Lachmann and Halbwachs, 1975). According to this theory, C3(H₂O), a form of C3 in which the internal thioester has been hydrolyzed by water, forms the initial convertase with factor B. This reaction constantly takes place at a low rate (tick-over) in blood (Pangburn and Müller-Eberhard, 1983; Pangburn et al., 1981). We have demonstrated the generation of C3(H₂O) at the interface between blood and gas in an *in vitro* model of cardiopulmonary bypass (CPB) and *in vivo* in patients during CPB (Ekdahl et al., 1992; Pekna et al., 1993).

The recently published crystal structures of C3 and C3b have identified potential sites for factor B binding to C3b in the new N-terminal segment α' NT, as well as in the CUB and C345C domains (Janssen et al., 2006; Janssen et al., 2005). In the proenzyme form of factor B, Ba, consisting of three complement control protein (CCP) domains, is linked with Bb, which consists of one metal ion-binding Von Willebrand factor A (VWA) domain and one serine protease domain (Hourcade et al., 1995).

There are several binding sites for C3b on factor B, in both the Ba and Bb domains. The complex formed between C3b and B is fairly stable as demonstrated by ultracentrifugation and electrophoresis (Müller-Eberhard and Schreiber, 1980; Vogt et al., 1977), but once factor D cleaves B into Ba and Bb, the convertase spontaneously dissociates and has a short half-life of 90 s (Fishelson et al., 1984) (Pangburn and Müller-Eberhard, 1986). This reaction may be explained by the notion that factor B docks to C3b via the Ba domain. Support for this hypothesis comes from the observation that site-directed mutagenesis within each of the three CCPs of Ba destroys the hemolytic activity if the binding capacity of the Ba domain is inhibited (Hourcade et al., 1995). Also, purified Ba is able to inhibit AP convertase formation (Prydzial and Isenman, 1987), and factor B binding to erythrocyte-fixed C3b is inhibited by an anti-Ba monoclonal antibody (Ueda et al., 1987). Bb has a lower affinity for C3b and, unlike Ba, cannot rebind to C3b after dissociation (Prydzial and Isenman, 1987). The crystal structure of intact factor B has recently been published. Interpretation of the structure confirms previous conclusions that the CCP domains of the Ba fragment provide an important binding site for C3b. The authors hypothesize that the CCP1 domain may hinder the binding of C3b to the metal ion-binding VWA domain of Bb (Milder et al., 2007).

The AP convertase is regulated at several levels: In the blood plasma, factor I in the presence of a cofactor (e.g., factor H, CR1, MCP) immediately cleaves the α' -chain of C3b at two sites to generate the fragment iC3b, which is unable to associate with factor B (Bokisch et al., 1975). Factor H also accelerates the dissociation of the AP convertase, a property that it shares with DAF and CR1 (Pangburn et al., 1977). The AP complex is stabilized by properdin (Fearon and Austen, 1975b). Data from our laboratory indicate that C3 adsorbed to a polymer surface can form an initiating AP convertase (Andersson et al., 2002) and that this C3(H₂O)-containing convertase is more resistant to cleavage with factor I and H than is the C3b-containing AP convertase (Andersson et al., 2005).

The exact molecular interactions between the components of the AP convertase and its regulators, in surface-bound or fluid-phase form, are still not fully understood, and the fact that the AP C3 convertase is labile makes these studies difficult to perform. In previous studies, indirect techniques such as hemolytic activity assays and indirect fluorescence techniques have been used (Fishelson et al., 1984; Pangburn, 1987; Prydzial and Isenman, 1988). In the present study, we have applied western blot analysis and ELISA together with fluorescence resonance energy transfer (FRET) to study the formation of the fluid-phase AP convertases C3(H₂O)Bb and C3bBb and their regulation by factor H and factor I at specific time points and, with FRET, in real time.

2. Methods

2.1. Commercial Reagents

Bovine serum albumin (BSA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Alexa 546 and Alexa 594 protein labeling kits were purchased from Molecular Probes (Eugene, OR, USA). Sheep anti-human factor B was obtained from Binding Site (Birmingham, UK), and rabbit anti-human C3c-Horse Radish Peroxidase (HRP), anti-human C3d antibodies, and rabbit anti-sheep IgG-HRP were obtained from Dako A/S (Glostrup, Denmark). Immun-Blot PVDF membranes were purchased from Bio-Rad (Hercules, CA, USA).

2.2. Purification of proteins

Human C3 and factor I were prepared according to Hammer et al. and Fearon (Fearon, 1977), respectively. Factor H was prepared from human serum essentially according to Hammer et al., except that the first step consisted of a euglobulin precipitation as described by Nilsson et al. (Nilsson and Müller-Eberhard, 1965). C3b was generated by digesting C3 with 1% (w/w) trypsin (Sigma) for 5 min at 22° C, and the resulting C3a and C3b fragments were separated by gel filtration on Sephadex G-100 (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated in PBS. C3 was digested with 25 % trypsin (w/w) for 10 min at 37°C into C3c and C3d. After addition of soybean trypsin inhibitor (Sigma) in two-fold molar excess, the resulting fragments were separated by gel filtration as described above. C3c was then further purified from uncleaved C3 by incubation with anti-human C3d antibody immobilized on Sepharose gel. C3(H₂O) was produced by repeated freeze-thawing of C3 (Isenman et al., 1981). Since proteins have a tendency to aggregate and to denature during freeze-thawing the size of the C3(H₂O) generated in this way was checked by electrophoresis under non-reducing conditions on 6% SDS-PAGE gels. In addition, C3 in serum and EDTA plasma were analysed by western blot together with the C3b and C3(H₂O) preparations under reducing and non-reducing conditions.

Factor D was purified from peritoneal dialysis fluid of patients with renal failure, as described by Catana and Schifferli (Catana and Schifferli, 1991), while factor B and properdin were purified according to Lambris et al. (Lambris and Müller-Eberhard, 1984). Polyclonal anti human C3a antibodies were raised and characterized as described previously (Nilsson et al., 1988).

2.3. Labeling of proteins with fluorophores and quality control

The FRET pair Alexa 546/Alexa 594 was conjugated according to the manufacturer's specifications. Following these protocols, C3(H₂O), C3b, and C3c were conjugated with Alexa 594, and factor B was conjugated with Alexa 546. The degree of labeling was determined by both spectrophotometry and by matrix-assisted laser desorption/ionization (MALDI) mass spectrometer with time-of-flight (TOF; MALDI Micro MX, Waters Micromass, Milford, MA). The instrument is equipped with a N₂ UV laser emitting at 337 nm, a pulsed ion extraction source, an electrostatic reflectron of 2.3 m effective path length, a 2 GHz 8-bit transient analog

to digital converter with real time peak display and fast dual micro-channel plate detectors. The acceleration voltage is 20 kV. Typically, 50 single-shot mass spectra were summed to give a composite spectrum. All data were reprocessed using Waters MassLynx software.

In order to determine the fluorophore distribution, samples of the labeled proteins were separated by SDS-PAGE and transferred to PVDF-membranes, which were then illuminated on a UV table to allow observation of fluorophore emission. Aliquots of C3(H₂O) and C3b were digested with trypsin and aliquots of factor B were activated with factor D in the presence of C3b prior to SDS-PAGE.

The labeled C3(H₂O), C3b, and factor B were tested for activation by factor D and the susceptibility of the labeled C3(H₂O) and C3b to cleavage by factor I, with factor H as cofactor.

2.4. Activity analyses

2.4.1. Convertase formation—The activity of labeled C3(H₂O), C3b, and factor B was checked by incubating 2.0 μ M labeled or unlabeled C3 C3(H₂O) or C3b, 0.7 μ M labeled or unlabeled factor B, and 68 nM factor D in 50 μ l veronal buffered saline (VBS: 1.8 mM Na-5,5 diethyl-barbital and 3.12 mM barbituric acid (Sigma), with 0.145 M NaCl; VBS-Mg²⁺: VBS supplemented with 1 mM Mg²⁺) for up to 60 min. at 37°C. Samples were analyzed by SDS-PAGE, followed by transfer to PVDF membranes. The membranes were developed using sheep anti-human factor B antibody with rabbit anti-sheep IgG-HRP or rabbit anti-human C3c-HRP antibody for detection.

2.4.2. Cleavage by factor I and factor H—Additional tests were performed to evaluate the activity of labeled C3(H₂O) and of C3b with factor H. Labeled C3(H₂O) and C3b at 1 μ M were incubated with 0.13 μ M factor H and 13 nM factor I for up to 30 min. at 37°C in VBS. Activity was measured in terms of the ability of factor I to cleave the α -chain of C3(H₂O) and the α' -chain C3b, as detected by western blotting using rabbit anti-human C3c-HRP. To specifically mimic the conditions in the fluorescence experiments, 1.3 μ M C3(H₂O) or C3b was incubated with 0.42 μ M factor B, 0.74 μ M factor H, and 78 nM factor I at 30-s intervals for up to 2 min., followed by western blot analysis.

2.4.3. Convertase activity—To determine whether the labeled proteins displayed convertase activity, 1.0 μ M labeled C3(H₂O) or C3b was incubated for up to 30 min in VBS-Mg²⁺ with 0.9 μ M labeled factor B, 1.0 μ M unmodified native C3, and 48 nM factor D at 37°C. Tests were run with and without a 1-min preincubation with factor H and factor I (0.74 μ M and 78 nM, respectively). Generation of C3a was detected by western blot analysis using biotinylated polyclonal rabbit anti-C3a antibody, and the production of the 9-kDa C3a fragment was quantified using public domain software (Java-based ImageJ software) from the NIH. In addition, the generation of C3a was measured after precipitation of high molecular proteins with 11% (w/v) PEG 6000 by ELISA as described previously (Ekdahl et al., 1992).

2.5. FRET experiments

FRET utilizes two fluorophores, termed “donor” and “acceptor.” The acceptor fluorophore has an absorption that spectrally overlaps the donor’s emission spectrum. Thus, upon excitation of the donor, some of the energy is non-radiatively transferred to the acceptor, rather than being emitted as light. The transferred energy causes excitation in the acceptor, and light is emitted at the acceptor’s wavelength rather than the donor’s. This transfer only takes place when the fluorophores are in close proximity; the Förster distance (R_0), at which the transfer efficiency is at 50%, is generally <100 Å. Here we have utilized Alexa 546/Alexa 594 ($R_0 = 71$ Å), a FRET pair that has been previously evaluated (Lichlyter et al., 2003). For comparison, the size

of C3b and iC3 are reported to be approximately $100 \times 150 \text{ \AA}$ and the radius of factor B to be approximately $80 \text{ \AA}$ (Nishida et al., 2006; Smith et al., 1984).

Fluorescence measurements were performed using a Fluorolog 3–22 (Jobin Yvon Inc., Edison, NJ) with a 450 W xenon lamp, in a quartz 0.5-ml cuvette. All experiments were performed with slit spectral bandwidths of 2 (exc) and 4 (em) nm and with an integration time of 1 s. Protein reactions took place in VBS containing 1 mM Mg^{2+} (VBS- Mg^{2+}) or, in some cases, 1 mM Ni^{2+} (VBS- Ni^{2+}), at a constant temperature of 37°C . In each case, similar results were obtained when the reaction took place in the presence of VBS- Mg^{2+} and VBS- Ni^{2+} . Typical reaction volumes were 200 μL , with 20- μL additions made over time using glass capillaries (Vitrex, Herlev, Denmark); all reagents were warmed to 37°C before being added. The data in each figure shows one representative experiment out of at least 4 performed with similar results.

2.5.1. Formation of C3b/C3(H_2O) and factor B complexes—In order to verify complex formation, the emission spectra of 2 or 4 nM labeled C3(H_2O) or C3b (collectively referred to as C3b/C3(H_2O)) and C3c were collected before and after the addition of labeled factor B (2 nM). Alternatively, changes in fluorescence intensity at the maximum emission wavelength of labeled C3b/C3(H_2O) (611 nm) were measured over time, during which labeled factor B was added. In all the time-resolved reactions, 2 nM of labeled factor B and 4 nM of labeled C3b/C3(H_2O) were used. We deduced experimentally that the addition of 0.4 μM unlabeled factor B and 1.3 μM unlabeled C3b/C3(H_2O), corresponding to approximately 20% of the normal plasma concentrations of factor B and total C3, respectively, was required for optimal protein interactions, and these concentrations were therefore included in all time-resolved measurements.

Attempts were made to perform measurements of convertase formation under more physiological conditions in the presence of properdin (80 nM). However, in our hands, properdin was found to have a significant quenching effect on labeled complement proteins, and therefore it was impossible to study its effect on the soluble AP convertase using the FRET technique.

In addition, attempts were made to dissociate the protein complex using EDTA, since the complex is dependent on ions. However, the EDTA itself caused a significant increase in the base fluorescence of C3b/C3(H_2O), presumably as a result of conformational changes caused by the chelation of C3-bound Ca^{2+} (data not shown).

2.5.2. Generation of AP convertases—In order to monitor the activation of the AP convertase complexes in real time, we performed time-resolved experiments in which factor D (8 nM) was added either simultaneously with labeled factor B or at 120 s after the addition of labeled factor B. Factor B and factor D (separately or together, depending on the experimental setting) were prewarmed to 37°C prior to addition to the cuvette. In each analysis, volume changes were controlled by adding the corresponding amount of buffer to samples without added proteins. Western blot analysis showed that factor D did not cleave factor B-Alexa 546 during incubation at 37°C for up to 1 h (not shown). Hence, it must be concluded that the factor B added to the reaction chamber did not contain any significant amount of Bb or Ba fragments.

2.5.3. Regulation of AP convertases by factor I and factor H—Complement regulation reactions were also observed in real time using time-resolved experiments involving the addition of 0.74 μM factor H and/or 78 nM factor I, (approximately 20% of the plasma concentrations) performed in the same manner as those involving factor D. The reagents were added together or individually, and in some experiments factor D was also added after incubation with factor H and factor I. Again, buffer was added for control of volume changes.

2.6. Statistics

Emission spectra are presented as percentages of the emission of 2 nM samples at 571 nm. Emission results from time-resolved analyses are presented as percentages of the baseline fluorescence, i.e., the emission of labeled C3b/C3(H₂O) before the addition of other proteins. Differences in the emission changes at given time points were analyzed with single-factor analysis of variance (ANOVA), and *p*-values less than 0.05 were considered significant. Experiments were performed at least four times, and data are presented as representative curves.

3. Results

3.1 Reagent preparation and quality control

In order to ascertain that the freeze-thawing procedure used to generate the C3(H₂O) had not led to aggregation, the protein size of C3(H₂O) was checked by electrophoresis under non-reducing and reducing conditions. When analysed by non-reducing SDS-PAGE, the C3(H₂O) and C3b preparations contained low amounts of dimers visible on Coomassie stained gels (Fig. 1A). Similar proportion of high molecular weight aggregates were visualized by western blot in these preparations and in serum and EDTA-plasma under non-reduced conditions (not shown). In contrast, no aggregates were found when the same samples were analyzed under reducing conditions, indicating that the interaction was due to noncovalent forces since amide- and ester-bound C3 activation products are resistant to reducing agents (Law and Levine, 1977). The C3(H₂O) also contained minute amounts of C3b as detected by MALDI.

The degrees of Alexa labeling were a fluorophore-to-protein (F/P) ratio of 3–4 for factor B, 3–5 for C3(H₂O), and approximately 5 for C3b as determined by both spectrophotometry and MALDI mass spectrometry. The fluorophore distribution was approximately equal on the α - and β -chains of C3 (Fig. 1B), and digestion with trypsin prior to SDS-PAGE showed that the label was evenly distributed in the α -chain. Since the fluorophores are amine-reactive, it was assumed that they were distributed randomly. For factor B the labeling was negligible on the Ba fragment, as compared to the Bb fragment (Fig. 1B).

Activity tests showed that the labeled C3(H₂O), C3b, and factor B retained their biological activity, measured as activation by factor D, and the labeled C3(H₂O) and C3b also displayed unaltered susceptibility to cleavage by factor I, with factor H as cofactor.

3.2. C3b/C3(H₂O) and factor B complex formation

3.2.1. Spectrum analyses—Factor B-Alexa 546 was mixed with 2 or 4 nM C3(H₂O)-, C3b-, and C3c-Alexa 594 in VBS-Mg²⁺. Emission spectra were collected by exciting Alexa-546 at 553 nm (the observed peak excitation in this system) and measuring emission between 565 and 620 nm. A decrease in factor B-Alexa-546 (donor) peak emission at 571 nm was observed when the amount of C3(H₂O)-Alexa 594 or C3b-Alexa 594 (acceptor) was increased from 2 to 4 nM (Fig. 2A and B, emission differences indicated by arrows). This intensity loss was not seen for the control system C3c-Alexa 594, the inactive form of C3 (Fig. 2C), where no complex formation is expected since it is proteolytically inactivated. Therefore, this change in emission seems to be dependent on FRET (Lichlyter et al., 2003) and thus reflects the binding event. The 571 nm intensity was diminished about 16 and 12 % for C3(H₂O) and C3b respectively. The observed difference in amplitude indicates that C3(H₂O) bound factor B more efficiently than did C3b. At longer wavelengths around 611 nm an increase in fluorescence is apparent for all three molecular systems. This is due to the increased amount of Alexa 594, which absorbs a fraction of the excitation light.

3.2.2. Time-resolved analyses—When measuring the peak emission of C3b/C3(H₂O)-Alexa 594 at 611 nm over time, we found that the interactions between labeled C3b/C3(H₂O) and factor B were enhanced by the addition of unlabeled factor B and C3b/C3(H₂O) (0.4 and 1.2 μ M, respectively); this observation is consistent with previous reports that complement proteins of the AP require a minimum concentration to function (Nilsson et al., 1993). Therefore, all time-resolved analyses were performed in the presence of the added proteins. Concentrations of the other added proteins (factors D, H, and I) were also adjusted to 20% of the normal serum concentration.

3.3. Generation of AP convertases

The peak emission of C3(H₂O) and C3b-Alexa 594 at 611 nm in VBS-Mg²⁺, as measured over time, was significantly ($p=0.04$ for both) lower when 8 nM unlabeled factor D was added together with factor B-Alexa 546 than when labeled factor B was added alone (Fig. 3). This is indicative of less interaction between C3(H₂O)/C3b and factor B as a result of the presence of factor D. No activity was observed for labeled C3c compared to labeled C3(H₂O) indicating that C3c did not interact with factor B (not shown). Similar results were obtained using VBS-Ni²⁺. Western blot analysis of samples incubated under similar conditions for 1–5 min and detected with anti-factor B antibodies showed that factor B was rapidly, and almost quantitatively, cleaved by factor D under these conditions (data not shown).

3.4. Regulation of AP convertases by factor I and factor H

The emission of C3(H₂O)- and C3b-Alexa 594 at 611 nm was decreased when factor H was added together with factor B-Alexa 546, as compared to the addition of buffer alone, and this difference was significant for C3b ($p=0.02$) (Fig. 4). Factor I alone did not affect the emission. When factor H and factor I were present together, however, a significant ($p=0.007$) increase in the emission of C3b-Alexa 594 occurred over time, an effect that was not seen for C3(H₂O). When factor H and factor I were added simultaneously together with factor B-Alexa 546, the change in emission of C3b-Alexa 594 was significantly ($p=0.02$) greater than that for the addition of labeled factor B alone (Fig. 5). It should be noted that there was a greater dilution effect in the experiments shown in Fig. 4, as compared to those in Fig. 5. Addition of factor H and factor I to labeled C3b without the presence of labeled factor B (not shown) did not cause an increase in emission to the same degree as in Fig. 5.

Western blot analysis of samples of C3(H₂O) and C3b that had been incubated with factors B, H, and I for 30–120 s revealed that both C3 forms were degraded within <30 s (not shown), a process that mirrored the rapid reactions observed in the fluorescence measurements.

In addition, convertase complexes were formed when labeled C3(H₂O) or C3b was incubated with factor B and factor D, with or without preincubation with factors H and I. Substrate C3 was added, and the activity of the formed convertase complexes was monitored as generation of C3a detected by western blot analysis (Fig. 6). The activity of C3(H₂O) convertase was shown to be less susceptible to factor I-mediated cleavage than was that of the C3b convertase; approximately 25% activity was retained, while there was no observable activity for the C3b convertase. These results were corroborated by ELISA of generated C3a fragments; the C3(H₂O) convertase generated 84 ± 27 ng/ml C3a after factor I-mediated cleavage, as opposed to 354 ± 55 ng/ml without ($p<10^{-6}$). C3b convertase generated 871 ± 137 ng/ml C3a without factor I preincubation, but did not generate detectable results with factor I (Fig. 6 inserts).

4. Discussion

Nucleophilic attack or similar modification of the internal thiolester of C3 generates C3(H₂O). This form of C3 has C3b-like properties, in that it is capable of binding factor B and factor H

and of forming a fluid-phase AP convertase (Pangburn and Müller-Eberhard, 1983; Pangburn et al., 1981). We have previously demonstrated the generation of C3(H₂O) *in vitro* when gas is bubbled through whole blood (Ekdahl et al., 1992), and *in vivo* during CPB to a maximal level of 100 nM, which corresponds to hydrolysis of approximately 1% of the total pool of C3 (Pekna et al., 1993). This result indicates that the molecular ratio between C3(H₂O) and factor B *in vivo* would be 1:20 (0.1:2 μ M), and it is thus reasonable to postulate that the equilibrium in that situation would favor the formation of C3(H₂O)/Bb convertase in the fluid phase. In addition, we have shown that C3, when adsorbed to a biomaterial surface, is able to form an initial AP convertase because it is conformationally altered to a C3(H₂O)-like conformation (Andersson et al., 2002). Furthermore, this surface-bound C3(H₂O)/Bb convertase has been found to be less susceptible to inactivation by factor I and factor H than is the C3b/Bb convertase (Andersson et al., 2005).

These earlier results prompted us to further analyze and compare the formation and regulation of fluid-phase AP convertase complexes containing C3b and C3(H₂O), by studying the generation of C3a fragments using western blotting and ELISA. In both methods, we observed that the C3bBb complex was totally inactivated after preincubation with factor H and factor I, while C3(H₂O)Bb showed a residual activity of approximately 25%.

In order to dissect the mechanisms that are responsible for these differences, we used western blot analyses in combination with the FRET technique. We sequentially monitored 1) complex formation between C3b or C3(H₂O) and factor B, 2) the generation of active AP convertases, and 3) the regulation of these AP convertases by factor I and factor H.

We found an indication that C3(H₂O) bound slightly more factor B than did C3b, as demonstrated by a decreased donor emission with an increase of acceptor concentration (Fig. 2A–B). In contrast, control samples with C3c did not show a drop in donor emission, supporting a specific interaction (Fig. 2C). Pryzdial and Isenman have reported that the interaction in the fluid phase between C3(H₂O) and factor B is of lower affinity than that between C3b and factor B (Pryzdial and Isenman, 1988), whereas our results here suggest a higher affinity between C3(H₂O) and factor B. However, it is not possible to make a direct comparison between these two studies, since the previous study used an indirect fluorescence technique in which binding of the C3b/C3(H₂O) and factor B was monitored by the quenching of a fluorescent competitive inhibitor for factor B, whereas we have used the direct FRET technique. Furthermore, the ratio between C3b/C3(H₂O) and factor B differed in the two studies. The study by Pryzdial and Isenman was conducted using C3b/C3(H₂O) in molecular deficit when compared to factor B (1:3, or 1.15 and up to 4.5 μ M), versus 1:0.3 (1.3:0.4 μ M) in the present study, which was designed to mimic the physiological ratio between the total amount of C3/C3 fragments and factor B.

The cleavage of factor B in complex with C3b/C3(H₂O) into Ba and Bb was demonstrated by western blot analysis after the addition of factor D. Factor D, when added to mixtures of C3(H₂O) and factor B in the FRET cuvette, induced a decrease in emission when compared to the mixture devoid of factor D. A similar pattern was seen for C3b, although the corresponding signals were lower. The fact that the change in acceptor emission (611 nm) seemed to be instantaneous and was followed by a relatively stable emission suggests a rapid reaction between factors B and D (Fig. 3). This interaction between factor B and C3b/C3(H₂O) in the fluid phase is more rapid than that monitored between surface immobilized C3/C3-fragments and factor B by Biacore or quartz crystal microbalance (QCM) techniques (Andersson et al., 2002; Jokiranta et al., 2001). The discrepancy does not seem surprising since the rate of binding measured by these techniques rely on the diffusion rate of the protein in solution while in this study both proteins are able to diffuse.

Fishelson et al. have previously observed a functional half-life of approximately 90 s for the AP complex using an indirect immunofluorescence technique (Fishelson et al., 1984). Our results suggest that the uncleaved components of the AP complex remain associated.

It is not likely that the significant loss of fluorophore that we observed was due to the cleavage of factor B, since the Ba fragment of factor B was very sparsely labeled. The decreased emission can best be explained by the loss of Bb that occurs in factor B after cleavage or conformational change and leads to less optimal interactions between the fluorophores (Hinshelwood and Perkins, 2000; Milder et al., 2007). The decreased acceptor emission is most likely caused by loss of FRET induced by dissociation of factor B from C3b/C3(H₂O), since Bb has a much lower affinity for active C3 and will not readily reassociate (Fishelson et al., 1984), whereas uncleaved factor B can continuously dissociate and reassociate with C3b/C3(H₂O). Also, the Ba fragment, which consists of a trimer of CCP regions (Hinshelwood and Perkins, 2000; Milder et al., 2007), has been shown to bind to C3b and C3(H₂O) with low affinity (Prydzial and Isenman, 1987), a reaction that could cause further dissociation and hinder the binding of uncleaved factor B (see Fig. 7 for the hypothesized model).

The result of our western blot analyses led us to use FRET to study the effects of factor H and factor I, both independently and simultaneously, on the interaction between C3b/C3(H₂O) and factor B. As expected, factor H caused a dissociation of the complexes, resulting in a lower acceptor emission at 611 nm, whereas factor I had no effect in the absence of factor H (Fig. 4). However, in the presence of both factors H and I, a significant increase was seen in the emission of C3b but not of C3(H₂O). In addition to the initial increase, the reactions also showed a gradual increase in emission over time. To determine whether this increase was dependent on factor I activity, we incubated C3(H₂O) and C3b in parallel in the presence of factors B, H and I, then detected the generation of iC3b and C3(H₂O) by western blotting. The results strengthened the hypothesis that the gradual change in emission was due to factor I activity, although the same effect was not seen for C3(H₂O). The most likely explanation for these findings may be related to the conformational changes produced in C3b during its degradation to iC3b and C3c, which result in increased emission and more accessible fluorophores. This finding would suggest that factor B is still able to bind to C3b that has been cleaved by factor I, since the emission increase effect was much less pronounced when factor B was not present, perhaps because the binding sites for factor B are distinct from the binding sites for factor H and the cleavage sites of factor I (Janssen et al., 2006).

Our fluorescence data and the observation that C3(H₂O)Bb was less susceptible than C3bBb to factor I- and factor H-mediated inactivation (as monitored by the generation of C3a) suggest that although C3b binds to factor B, C3b's inactivation depends on conformational changes that do not occur in C3(H₂O). This observation is in agreement with previous work demonstrating that C3 that is merely adsorbed to a surface has C3 convertase activity with factor B but is resistant to factor I-mediated inactivation (Andersson et al., 2002).

The physiological relevance of our observation regarding the generation of C3b-containing fluid-phase AP convertases is subject to debate, since the concentration of C3b in plasma *in vivo* is expected to be negligible because any generated C3b is immediately converted into iC3b by factors I and H. As shown here, this treatment completely inhibited the activity of the C3b AP convertase. However, *in vivo* properdin is expected to bind to the AP convertase to form C3bBbP (Asghar et al., 1987) and increase its stability up to 10-fold as demonstrated by *in vitro* studies (Fearon and Austen, 1975a). In contrast, not much work of how the stability of C3(H₂O)Bb is affected by properdin is to be found in the literature. Our data presented here suggest that properdin may have a less essential role to retain the activity of C3(H₂O)Bb convertases since these complexes were noticeably resistant to inhibition by factors I and H compared to C3bBb. The interaction between C3(H₂O) and factor B in the fluid phase and the

subsequent generation of active AP convertase complexes may have physiological consequences in clinical settings that are associated with generation of C3(H₂O) due to the interaction of plasma C3 with both the gas surface and the biomaterial surface, such as CPB (Andersson et al., 2002; Ekdahl et al., 1992; Pekna et al., 1993).

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FIGURE 1.

(A) Non-reducing SDS-PAGE gel of C3b (left lane), C3(H₂O) (right lane). (B) Western blot analysis under reducing conditions of (from left to right) C3(H₂O), C3b, EDTA-plasma, and serum using polyclonal anti C3c-antibodies (C) Western blot analysis of Alexa-labeled proteins (reducing SDS-PAGE) under UV illumination. In the sample in the far left lane, factor B (fB Act) had been incubated with factor D and C3b for 60 min to generate Ba and Bb. The other lanes show uncleaved factor B (fB), C3(H₂O), and C3b, respectively. The position of uncleaved factor B (fB (whole)), Bb and Ba, and the alpha and beta chains of C3, and the molecular weights of the markers are indicated.

FIGURE 2.

Binding of C3(H₂O) and C3b to factor B in solution. Normalized emission spectra of C3 (H₂O)-Alexa 594 (A), C3b-Alexa 594 (B), and C3c-Alexa 594 (C) at 2 nM (squares) and 4 nM (diamonds) with 2 nM factor B-Alexa 546 when excited at 553 nm (the excitation peak for A546). The decrease at 571 nm (A546) and increase at 611 nm (A594) (arrows) indicates an energy transfer that occurs when factor B binds C3(H₂O) or C3b in solution (n=3).

FIGURE 3.

Generation of AP convertases, as detected by FRET. Normalized 611 nm peak emission of labeled C3b/C3(H₂O) over time, after excitation at 553 nm (the peak excitation for Alexa 546). After 60 s, labeled factor B with or without unlabeled factor D (fD) was added to C3(H₂O)-Alexa 594 (top) or C3b-Alexa 594 (bottom) in VBS-Mg²⁺. The immediate increase in emission at 60 s is indicative of an interaction between C3b/C3(H₂O) and factor B, which is dampened by the presence of factor D. Similar results were seen in VBS- Ni²⁺ (not shown).

FIGURE 4.

Regulation of AP convertases by factor I and factor H. Normalized 611 nm peak emission of labeled C3b/C3(H₂O) over time, measured as described in Fig. 3. At 60 s, labeled factor B was added to C3(H₂O)-Alexa 594 (top) or C3b-Alexa 594 (bottom) in VBS-Mg²⁺. Factor H (FH) and factor I (FI) were added sequentially at 60 s (together with factor B) and at 180 s as indicated, one at each time point. The black curves show factor H added first, followed by factor I, and the grey curves show the reverse. The dotted curve shows the result for addition of buffer alone, to indicate the extent of the dilution effect.

FIGURE 5.

Regulation of AP convertases by factor I and factor H, added simultaneously. Normalized emission of C3(H₂O)-Alexa 594 (above) and C3b-Alexa 594 (below), measured as described in Fig. 3. Factor B-Alexa 546 was added at 60 s with or without unlabeled factor H and factor I (fH/I) in VBS-Mg²⁺. If not added at 60 s, fH/I was added at 180 s.

FIGURE 6.

Generation of C3a (9 kDa) by soluble AP convertases and regulation by factor I and factor H. The figure shows western blots with densitometric measurements of the C3a fragments generated from C3(H₂O)-Alexa 594 (top) or C3b-Alexa 594 (bottom) incubated with factor B-Alexa 546, unlabeled factor D, and unlabeled substrate C3 for up to 30 min at 37°C. Tests were performed without (diamonds) or with (squares) preincubation with factor H and factor I for 1 minute. The densitometric measurements were normalized to results from corresponding samples in the C3a ELISA (displayed in inserts as mean $\pm$ SD). Lane 1: MW markers as in Fig 1 (lowest band 10 kDa, followed by 15, 20, and 25 kDa); lanes 2, 3: without factor I and factor H, 0 and 30 min; lanes 4, 5: with factor I and factor H, 0 and 30 min.

FIGURE 7.

Model describing the reactions that occur when C3b/C3(H₂O) is incubated with factor B in the presence of factor D. C3b/C3(H₂O) continually associates and dissociates with uncleaved factor B (Ba + Bb, arrow 1), producing a FRET effect and increased emission. Upon addition of factor D, factor B is cleaved, and the Ba portion dissociates from the complex (arrow 2). The C3b/C3(H₂O)/Bb complex rapidly decays to C3b/C3(H₂O) alone (arrow 3). The free Ba fragments in solution, however, can reassociate with C3b/C3(H₂O) (arrows 4, 5), hindering the binding of intact factor B and thus producing a lower FRET efficiency.