C3b2-IgG Complexes Retain Dimeric C3 Fragments at All Levels of Inactivation*

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C3b2-IgG complexes are formed during complement activation in serum by attachment of two C3b molecules (the proteolytically activated form of C3) to one IgG heavy chain (IgG HC) via ester bonds. Because of the presence of two C3b molecules, these complexes are very efficient activators of the alternative complement pathway. Likewise, dimeric C3b is known to enhance complement receptor 1-dependent phagocytosis, and dimeric C3d (the smallest thioester-containing fragment of C3) is linked to a protein antigen facilitates CR2-dependent B-cell proliferation. Because the efficiency of all these interactions depends on the number of C3b fragments, we investigated whether C3b2-IgG complexes retained dimeric structure upon physiological inactivation. We used two-dimensional SDS-PAGE and Western blot to study the arrangement of the C3b molecules by analyzing the fragmentation pattern after cleavage of the C3b2-IgG. Upon inactivation with factors H and I, a 185-kDa band was generated under reducing conditions. It released IgG HC and the 65-kDa fragment of C3b α’ chain after hydrolysis of the ester bonds with hydroxylamine. The two C3b molecules were not 65-kDa to 40-kDa linked, because neither ester-bonded 65 kDa HC nor 65 kDa to 40 kDa fragments were observed, nor was a 40-kDa peptide released after hydroxylamine cleavage. Factor I and CR1 cleaved the C3b2-IgG molecule to its final physiological product, C3dg-IgG, which migrated as a 133-kDa fragment in reduced form. This fragment released exclusively C3dg (the final physiological product of C3b inactivation by factor I) and IgG HC. C3dg-IgG appeared as a double band on SDS-PAGE only at low gel porosity, suggesting the presence of two conformers of the same composition. Our results suggest that upon physiological inactivation, C3b2-IgG complexes retain dimeric inactivated C3b and C3dg, which allows bivalent binding to the corresponding complement receptors.

Activation of complement by one of the three pathways, classical, alternative, or lectin, leads to the generation of nascent C3b, the proteolytically activated form of complement component C3 that can bind covalently to the cell surface or to soluble proteins in the immediate vicinity. The ability of C3b to form covalent bonds is crucial for its biological activity. This property is attributable to the presence of an internal thioester bond, which opens upon activation and forms a highly reactive intramolecular amide intermediate that is accessible to nucleophilic attack by hydroxyl groups (1, 2). The intermediate has a half-life of ~60 μs, and 90% of it is hydrolyzed by water (3, 4). Nascent C3b forms primarily ester bonds with proteins or carbohydrates (5).

Bound or free C3b can nucleate the formation of the alternative pathway C3 convertase, C3bBb, thereby amplifying the initial signal (amplification loop of complement). However, regulatory mechanisms operate in blood to prevent excess complement activation. Binding of C3b-containing immune complexes to erythrocyte complement receptor 1 (CR1, CD35) prevents C3 convertase generation and leads to its subsequent clearance (6–9). The serine protease factor I, with cofactors factor H in serum or CR1 on the surface of most blood cells, quickly inactivates free C3b (to iC3b) by proteolytic cleavage. This cleavage changes the functional properties of the molecule: (i) its ability to generate C3 convertase is abolished and (ii) its affinity for CR1 is decreased, while (iii) it becomes a ligand for other CRs. CR3 (CD11b/CD18) and CR4 (CD11c/CD18) bind iC3b, thus maintaining phagocytosis (10, 11). The final physiological product of C3b inactivation by factor I, C3dg, is a ligand for CR2 (CD21), a receptor present on B and T cells and follicular dendritic cells (12). CR2 plays an essential role in the enhancement of humoral immune response (13, 14). A key example in this respect is the 1000-to-10,000-fold enhancement of immunogenicity by the covalent attachment of two or three C3d moieties to the antigen (15). A complex containing at least two C3b molecules or fragments thereof will bind to all corresponding receptors bivalently, and therefore much more avidly, resulting in a higher efficiency of the respective process.

In covalent complex with proteins, C3b is partially protected from enzymatic inactivation. The major protein targets for nascent C3b in serum are IgG and C4b. Nascent C3b reacts with high efficiency with C4b and in the form of a C4b-C3b complex is 10-fold more stable than free C3b or C3b bound to a non-activating surface (16). On the other hand, IgG is known to have a measurable affinity for C3 and C3b (17). Covalent binding to IgG reduces the susceptibility of C3b to factor I cleavage because of impaired factor H binding (18, 19). Such complexes demonstrate an increased capacity to activate the alternative complement pathway (18, 19).

The structure of C3b2-IgG complexes has been studied extensively, yet a number of aspects remain unclear. Gadd and Reid (20) first investigated C3b2-IgG complexes that were generated in human serum from nascent C3b and preformed immuno-
to the 40-kDa fragment of the other (22). On the other hand, one depicted in Fig. 1A, the 65-kDa fragment of one C3b bound to the 40-kDa fragment of the other (22). On the other hand, C3b2-IgG complexes that were generated in human serum from immune complexes. They concluded that such complexes contained either one or two C3b molecules per IgG heavy chain (HC). Others also noted two bands on SDS-PAGE of reduced C3b2-IgG structures are shown in reduced form. For preparative purposes the arrangement and ester bond location. We have generated identical complexes both in serum and from purified C3 and IgG and have used hydroxylamine cleavage of the ester bonds to analyze the position of the C3b fragments in native and inactivated complexes. Our results clearly demonstrate that the two C3b molecules are attached to the IgG HC either separately or as a C3dg-C3dg dimer and indicate the presence of dimeric iC3b and C3dg in C3b2-IgG.

**EXPERIMENTAL PROCEDURES**

Materials—Complement proteins, factors H and I, were purchased from Advanced Research Technologies (San Diego, CA). C3 was isolated from fresh plasma as described elsewhere (24) and was purified to remove iC3 before use (25). Pooled human IgG (Redimmune) was a gift from ZLB Bioplasma AG (Berne, Switzerland). Recombinant soluble CR1 was a gift from Prof. Jürg Schifferli (University Hospital, Basle, Switzerland). The polyvinylidenefluoride membrane for blotting, Immobilon P, was a product of Millipore (Bedford, MA). Monoclonal anti-human C3d antibody A207 was from Quidel (San Diego, CA), and rabbit polyclonal anti-human C3d antibody was from DakoCytomation, Switzerland. Monoclonal antibody H206 was a gift from Prof. R. Burger, Robert Koch-Institute (Berlin, Germany). H206 was purified from culture supernatants on recombinant protein G and stored frozen in aliquots. HitTrap Protein G and Mono Q HR 5/5 columns, chromatography media, and [125I]iodide were from Amersham Biosciences.

Generation and Purification of C3b2-IgG—Complement activation in serum was performed according to Lutz et al. (19). Briefly, normal serum was diluted to a final concentration of 20% with veronal-buffered saline (4.62 mM veronal buffer, pH 7.2, 150 mM NaCl, VBS) and supplemented with 0.15 mM Ca2+, 0.5 mM Mg2+, and 1 × 106 cpm/ml of 125I-labeled C3. Heat-aggregated IgG (100 μg/ml) was added, and the samples were incubated at 37 °C for 3 min. The reaction was stopped by adding SDS-PAGE sample buffer to achieve 1% SDS, 50 mM dithiothreitol, and 10 mM EDTA. Samples were immediately heated for 3 min at 100 °C and then supplemented with an excess of N-ethylmaleimide. Aliquots of the denatured samples were either frozen at −70 °C or used immediately for SDS-PAGE.

For preparative purposes C3b2-IgG was generated in 20% factor I-deficient serum using a similar procedure (19). Factor I-deficient serum was supplemented with 1 × 106 cpm of 125I-labeled C3 or 125I-labeled IgG, 300 μg/ml of unlabeled C3, 5 mg/ml of IgG, and 40 μg/ml of factor B (Advanced Research Technologies, San Diego, CA). Complement activation was started with heat-aggregated IgG (100 μg/ml), and after 10 min at 37 °C, the sample was supplemented with 10 mM EDTA. Alternatively, unlabeled C3 and IgG were used, and C3b2-IgG was labeled after purification. Purification was performed on a Sephacryl S-300 HR column (1.6 × 95 cm) in VBS, pH 7.4, containing 1 mM NaCl and 10 mM EDTA. Fractions containing C3b2-IgG were identified by SDS-PAGE, pooled, and loaded on a QAE-Sephadex A50 column (1.5 × 5 cm) in 20 mM phosphate, pH 7.0, containing 110 mM NaCl. After washing, the bound material was eluted with a 110 to 260 mM salt gradient in the same buffer.

Details on the generation of C3b2-IgG from purified C3 and IgG have been published previously (26). Briefly, C3b2-IgG complexes were generated from 6 mg of C3 and 400 mg of IgG using trypsin to activate C3 (18, 23). Free IgG was removed by ion exchange chromatography on QAE-Sephadex A50. The sample was dialyzed and loaded on a QAE-Sephadex column (2.5 × 7 cm), equilibrated in 20 mM Tris, 140 mM NaCl, pH 8.3 (30 mM/liter, 4 °C). After washing, the bound material was eluted with a stepwise gradient using 160, 180, and 200 mM NaCl in starting buffer. C3b2-IgG eluted at 180 mM NaCl as judged by SDS-PAGE. Most of the free C3 was removed on a Sephacryl S-300 HR column (1.6 × 60 cm, fast protein liquid chromatography) and equilibrated with VBS, pH 7.4, containing 0.04% sodium azide. Fractions corresponding to C3b2-IgG complexes were dialyzed and finally purified on a MonoQ HR 5/5 (fast protein liquid chromatography, room temperature) using a stepwise NaCl gradient from 100 mM to 1 mM NaCl in 20 mM Tris (pH 7.8). Purified C3b2-IgG complexes were then precipitated with 10% trichloroacetic acid (TCA), and the pellets were washed and resuspended in 6× SDS-PAGE sample buffer. A 5-μg sample was heated for 5 min at 100 °C and subjected to 12.5% SDS-PAGE, and the gel was stained with Coomassie Blue.
Complexes were dialyzed against VBS and stored at 4 °C.

**Inactivation of C3b2-IgG and C3b with Factors H and I—** C3b2-IgG or C3b (170 μg/ml) was inactivated with 7 μg/ml of factor I and factor H at the stated concentration for 10 min at 37 °C in VBS. To stop the reaction, the sample was reduced with SDS-PAGE sample buffer containing dithiothreitol, boiled for 3 min, and alkylated.

**Inactivation of C3b2-IgG Complexes with CR1 and Factor I—** C3b2-IgG complexes (170 μg/ml) were incubated with 36 μg/ml of recombinant soluble CR1 and 37 μg/ml of factor I for 15 min at 37 °C in VBS. Inactivation was stopped by reduction and alklylation.

**SDS-PAGE and Two-dimensional SDS-PAGE with Hydroxylamine Treatment between the Dimensions—** Aliquots of intact or inactivated C3b2-IgG samples were run on SDS-PAGE at the given total acrylamide concentration. Gels were either stained with Coomassie blue and scanned with a laser densitometer (Molecular Dynamics, Sunnyvale, CA) or cut in strips for two-dimensional SDS-PAGE. The length of the strips varied according to the expected fragments. The strips were treated with 1 M hydroxylamine solution (pH 10) for 60 min at room temperature, with shaking. Every 20 min the hydroxylamine solution was replaced with a freshly prepared one. The strips were then washed with water and equilibrated in SDS-PAGE stacking gel buffer and were finally mounted on a new glass plate. After electrophoresis in the second dimension, gels were either silver-stained and scanned or blotted onto Immobilon P membrane.

For better identification of protein bands in the gel, a molecular mass marker was prepared from C3b2-IgG and C3b. The proteins, either intact or inactivated by factors H and I, were mixed at a molar ratio of 1:2, reduced, alkylated, and loaded to the second dimension gel.

**Western Blot—** Monoclonal anti-human C3d antibody A207 or rabbit polyclonal anti-human C3d as well as monoclonal antibody H206 directed against the C-terminal 40-kDa portion of the human C3 α chain was used for Western blot analysis. All antibodies were labeled with 125I as described previously (23). Blots were incubated for 3 h at room temperature in Replica buffer (20 mM Tris, 0.9% NaCl, 0.08% sodium azide, and 50 μg/ml of phenylmethyl fluoride) containing 0.5% gelatin and the corresponding antibody. Blots were then washed alternately with Replica buffer and Replica buffer completed with 0.04% Triton X-100 and 0.5% gelatin. Finally, blots were dried and exposed to PhosphorImager screen (Molecular Dynamics).

**RESULTS**

**C3b2-IgG and C3b-C3b Are Generated in Serum during Complement Activation—** Generation of C3b2-IgG complexes from C3 and IgG in serum or on the surface of human red blood cells revealed two complexes, which were assumed to be C3b2-IgG and C3b-IgG according to their apparent molecular masses (19, 23). The larger complex released free IgG HC upon reduction and migrated as α2-HC with an apparent molecular mass of 263 kDa, suggesting that the two C3b molecules were bound to the same IgG HC. However, the evidence for the composition of the C3b-IgG complex was not unequivocal (19).

The complexes generated in normal and factor I-deficient serum displayed a similar banding pattern. In normal serum, with factor I operating, free C3b could be detected only in its inactivated form, which released a 65-kDa fragment and a 263-kDa complex. In normal serum, with factor I operating, free C3b could be detected only in its inactivated form, which released a 65-kDa fragment and a 263-kDa complex. In normal serum, with factor I operating, free C3b could be detected only in its inactivated form, which released a 65-kDa fragment and a 263-kDa complex. For better identification of protein bands in the gel, a molecular mass marker was prepared from C3b2-IgG and C3b. The proteins, either intact or inactivated by factors H and I, were mixed at a molar ratio of 1:2, reduced, alkylated, and loaded to the second dimension gel.

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Generation of complexes from 125I-labeled C3 or IgG in factor I-deficient serum allowed analysis of the two different complexes. We used two-dimensional SDS-PAGE accompanied by hydroxylamine cleavage of the ester bonds after the first dimension and identified C3b2-IgG and C3b-C3b with molecular masses of 263 and 208 kDa in reduced form, respectively (Fig. 2B).

Two-dimensional SDS-PAGE confirmed that C3b2-IgG was composed of α2-C3b and HC. In addition, the experiment showed clearly that the complex with a molecular mass of 208 kDa did not contain IgG and was therefore a covalent C3b dimer. Because the complexes were purified from serum, contaminants complicated the picture. The contaminants included C4b-containing, amide-bonded complexes, which remained in the diagonal because of their insensitivity to hydroxylamine cleavage (Fig. 2B), as well as α2-macroglobulin, another thioester-containing serum protein (Fig. 2C, arrow). Nevertheless, the two-dimensional pattern of complexes labeled after purification (Fig. 2C) was superimposable with the patterns obtained with either component labeled and thus confirmed the assignments (Figs. 2, B and D).

**The Two C3b Molecules Are Attached to One IgG HC via Ester Bonds—** Generation and purification of C3b2-IgG from C3 and IgG yielded complexes identical both in molecular mass and in composition to serum-derived complexes. A typical preparation contained some C3b and a small amount of covalent C3b dimers but no IgG (Fig. 3A). Therefore, the IgG HC re-
Fig. 3. A, purified C3b2-IgG complexes. C3b2-IgG complexes generated from purified C3 and IgG were boiled in sample buffer, alkylated, and run non-reduced (lane 3) or reduced (lane 6) together with the corresponding IgG standard (lanes 1 and 4). Aliquots of C3b were boiled separately under reducing or non-reducing conditions, alkylated, and mixed in equal amounts (lanes 2 and 5). A Coomassie-stained gel is shown. The five bands in lane 6 correspond to α'-HC (calculated molecular mass, 263 kDa), α'-LC (208 kDa), α' (104 kDa), β (71 kDa), and IgG HC (55 kDa). IgG LC (23 kDa) migrated at the tracking dye (TD). B, two-dimensional SDS-PAGE of C3b2-IgG complexes. Purified C3b2-IgG complexes were reduced, alkylated, and run on a 6% gel. Gel strips were cut from the top of the separating gel to the β band of C3b, treated with hydroxylamine as described under "Experimental Procedures," and loaded on an 8% gel for the second dimension. A molecular mass marker was prepared by mixing native C3b2-IgG with iC3b and run on the same gel. A picture of the silver-stained gel is shown. Calculated molecular mass are given in kDa.

released upon reduction originated from the C3b2-IgG complex. No band was detected between the α'- and α' bands where an α'-LC (129 kDa) band should have appeared, if it had formed. An α'-LC band (235 kDa) was also not observed between α'-HC and α'. Thus, complexes containing C3b bound to IgG LC were not present in our preparations.

Two-dimensional SDS-PAGE was used to study the composition of purified complexes. To avoid confusion arising from the unusual apparent mobility of multichain proteins, we prepared our own molecular mass marker by mixing iC3b with C3b2-IgG and ran them on the same gel. Fig. 3B shows the fragments released from the two complexes present in the purified C3b2-IgG preparation. Both C3b2-IgG and C3b2 released an α' chain at the position corresponding to that of the α' chain generated from the contaminating C3b, which remained at the diagonal. Only the larger complex, C3b2-IgG, contained IgG HC, which unequivocally confirmed that the smaller molecule was a C3b dimer and not a C3b-IgG complex. Therefore, in our subsequent experiments we used the released HC as a marker identifying the position of C3b2-IgG and its inactivation products.

Fig. 1 represents possible C3b2-IgG structures with different locations of the ester bonds. The two C3b molecules might be separately attached to the IgG HC (Fig. 1A). Alternatively, they might be attached to the HC as a dimer. Within this dimer, further theoretical possibilities exist for the orientation of the two C3b molecules. The second C3b might be linked to any of the three α'—chain fragments generated by factor I in the course of the physiological degradation (Fig. 1, B–D).

Inactivation with Factors H and I—C3b is cleaved by factors H and I at two peptide bonds in the C-terminal third of the α'—chain generating 65- and 40-kDa bands on SDS-PAGE under reducing conditions (see Fig. 2A). Cleaved and reduced C3b2-IgG and C3b2 released the 40-kDa band in the first dimension. The 65-kDa portion of the α'—chain was liberated only after cleavage of the ester bonds with hydroxylamine. It migrated in the second dimension off the diagonal under the position of the inactivated reduced C3b2-IgG and C3b2 (Fig. 4A, arrows 65-HC and 65-H). The inactivation was incomplete under the conditions used, and intact complexes appeared at their original positions. C3b2-IgG (molecular mass, 263 kDa) released α'—chain and IgG HC, whereas C3b2 (molecular mass, 208 kDa) liberated exclusively α'—chain. The only other product releasing HC was 65-HC (calculated molecular mass, 185 kDa). It migrated in the first dimension just below α', and therefore the liberated 65-kDa and HC spots were found slightly to the right from the α'—chain of C3b2. The reduced form of the inactivated C3b2, 65a (calculated molecular mass, 130 kDa), migrated closely below the α'—chain in the first dimension, and its 65-kDa fragment appeared to the right of the α'—chain spot in the diagonal. A 40-kDa spot was not released in the second dimension, suggesting that a complex with an ester bond between the 65-kDa fragment and the 40-kDa fragment of the two C3b molecules was not present.

To verify the above results, we performed Western blot analysis, making use of antibodies specific for different domains of the α'—chain. Monoclonal antibody H206 (27) recognizes the 40-kDa domain of α'-C3b, whereas monoclonal antibody A207 is specific for the C3d domain of human C3. Two identical blots were incubated with the antibodies, and a digital overlay can be expected. H206 bound strongly to the 40-kDa fragment in the diagonal and had a weak binding to the β chain. No 40-kDa spot was detected off the diagonal. Antibody A207 recognized the 65-kDa fragments released from 65-HC and 65a (Fig. 4B, arrows) as well as the 65-kDa domain of iC3b seen in the diagonal. In addition, A207 bound to the α'—chain liberated from intact C3b2. These experiments demonstrated that iC3b2-IgG complexes contained exclusively 65-kDa fragments and HC. Neither iC3b2-IgG nor iC3b2 released a 40-kDa fragment, indicating that the 65-kDa fragment was not bound to the 40-kDa fragment in any of the complexes.

Inactivation with CR1 and Factor I—C3b inactivation is different on the cell surface because it involves CR1 as a cofactor for factor I. In addition to the two peptide bonds mentioned above, a third bond is cleaved, and a 23-kDa portion from the N terminus of the α'—chain is cut. As a result, two fragments are formed: C3dg remains bound to the cell surface (or to a protein), whereas C3e is released. We therefore investigated the fragmentation pattern after incubation of C3b2-IgG and C3b2 with CR1 and factor I (Fig. 5A). C3dg has a molecular mass of 39 kDa, and thus C3dg-HC, with a calculated molecular mass of
133 kDa, migrated close to α’-C3b. Correspondingly, C3dg₂ overlapped with the β-chain or migrated slightly below it. Upon hydroxylamine cleavage, C3dg₂-HC released a 39-kDa C3dg and IgG HC below the diagonal, whereas under C3dg₂, only a 39-kDa C3dg spot was observed (arrows). The silver staining revealed no trace of smaller fragments, e.g. a 23-kDa fragment which could be expected if one assumed that the two C3b molecules were bound 65- to 23-kDa. The inactivated complexes contained exclusively the C3dg portion of C3b, as predicted by the 65–65-kDa model (Fig. 1C). However, two complexes with different mobility around 133 kDa in the first dimension released identical HC and 39-kDa fragments. No other fragments that could account for this difference were visible anywhere in the gel. A Western blot, performed with antibody H206, revealed only the residual α’-chain and no other spots that might have been undetectable with the silver staining (not shown).

The heterogeneity of the C3dg₂-HC fragment was unexpected because equal composition would suggest equal size, particularly when samples had been reduced beforehand. Still, structural or conformational variability may result in different sizes, leading to different apparent mobility in SDS-PAGE. Incubation with a rabbit polyclonal anti-human C3d antibody confirmed the existence of 39-kDa fragments in the form of a doublet, despite the unexpected binding to the β chain. Interestingly, the two C3dg₂-HC spots were differently resolved at varying gel porosities: the spots were better separated in the 8% gel (Fig. 5A) than in the 6% gel (Fig. 5B). We therefore studied the migration of the two bands on SDS-PAGE at various acrylamide concentrations (Fig. 6A). The scanned gel images were electronically aligned using the CR1 band and the β-chain band as references. The α’-chain bands on all gels aligned to the same position, confirming that the relative mobility of single-chain molecular species remained unchanged (see the 104-kDa dashed line, bands not shown). The two C3dg₂-HC bands, on the other hand, migrated differently, depending on the porosity of the gel. They were best separated at 8% total acrylamide concentration, meaning that some conformational difference existed. Thus, the bands represent two alternative conformational states of the same complex rather than two complexes of different compositions. Fig. 6B shows schematically the main variations that could account for the different apparent sizes in SDS-PAGE. One possibility is that the two bands correspond to two structural variants of C3dg₂-HC, in which the C3dg portions are attached either individually or sequentially to IgG HC (Fig. 6B, part I). Alternatively, two sequentially attached C3dg moieties may be oriented in two ways with respect to each other and/or to IgG HC, resulting in different conformers of the same structure (Fig. 6B, part II).

**DISCUSSION**

C3b₂-IgG complexes are the major C3b-containing high molecular mass species formed in serum upon complement activation (19, 23). In the present study, we demonstrate that these complexes contain two C3b molecules linked to one IgG HC in
their native form and that they retain dimeric iC3b or C3dg during inactivation. The same is true for the second most abundant complex, a C3b dimer. Such a structure facilitates alternative pathway C3 convertase generation. In fact, C3b₂-IgG complexes are partially protected from inactivation with CR1 and factor I as in Fig. 5 and separated at different acrylamide concentrations. Gels were stained with Coomassie, scanned, and digitally aligned using the CR1 and the β-C3b bands as reference. The α' band of C3b on all gels aligned on the same level (104 kDa, dashed line). The strips containing the doublet are shown. B, schematic presentation of possible C3dg₂-HC structures and conformers. The complexes are shown in their fully inactivated and reduced forms.

FIG. 6. Separation of the C3dg₂-HC doublet and possible arrangement of the C3dg moieties. A, SDS-PAGE of C3dg₂-IgG. C3b₂-
IgG complexes were inactivated with CR1 and factor I as in Fig. 5 and separated at different acrylamide concentrations. Gels were stained with Coomassie, scanned, and digitally aligned using the CR1 and the β-C3b bands as reference. The α' band of C3b on all gels aligned on the same level (104 kDa, dashed line). The strips containing the doublet are shown. B, schematic presentation of possible C3dg₂-HC structures and conformers. The complexes are shown in their fully inactivated and reduced forms.

CR2 as well. Because CR2 links specific ligand binding to signal transduction events mediated by the CD21/CD19/ TAPA-1 complex, bridging of CR2 to Fc receptors facilitates immune response (32, 33). Thus, the existence of two C3b in C3b₂-IgG has major implications for their biological function, because it is a structural prerequisite for bivalent binding.

The existence of a covalent C3b dimer as found in C3b₂ and possibly in C3b₂-IgG is not surprising, because C3b has long been known to form covalent dimers on the cell surface (34). In particular, C3b₂ complexes are formed very efficiently at the sites of amplification, where C3b is deposited in dense clusters (35). In our hands, complexes containing only one C3b per IgG were never detected, nor were bands corresponding to α'-LC.

All C3dg-containing inactivation fragments of the two C3b molecules remained bound to IgG HC and were released by hydroxylamine cleavage (Figs. 4 and 5). Inactivation with factors I and H resulted in the release of 65-kDa fragments from both complexes after reduction and failed to yield a 40-kDa peptide, suggesting that the C3b molecules were not 65–40-kDa bound. Accordingly, a 23-kDa peptide was not detected in the complexes after inactivation with factors I and CR1, which ruled out the 39–23-kDa binding. The remaining possibilities are (i) attachment of individual C3b moieties to IgG HC or (ii) linkage of a 39–39-kDa C3b dimer to a single amino acid residue in HC. Our results do not allow discrimination between these structures. Attempts to prove the sequential C3b arrangement via partial inactivation yielding e.g. a 65-α'-HC product were unsuccessful. On the other hand, physiological concentrations of both factors I and H were required to achieve complete inactivation of C3b₂-IgG (not shown), supporting the known partial resistance of protein-bound C3b to inactivation (18, 19). Evidently, the two C3b molecules within C3b₂-IgG were equally protected from cleavage by factor I.

The C3dg₂-HC fragments migrated as a double band. Given that the degree of their separation was dependent on the porosity of the gel, the observed electrophoretic mobility difference is likely attributable to conformational variants rather than to different structure. Because the released fragments were of equal size, issues such as differential HC glycosylation or variations in the IgG subclass were ruled out. Most importantly, this molecular variation was revealed only after digestion with CR1, whereas the 65-α'-HC product gave a single band. Thus, the difference either became apparent in the smaller fragments or occurred only after removing the rest of the molecule. Both possibilities would allow structural variants e.g. sequentially or individually attached C3b as well as conformational alternatives (Fig. 6B).

There are several arguments in favor of the sequential binding model: (i) The formation of covalent C3b dimers on cell surfaces and in serum has been well documented (34, 35). A C3b dimer may be a precursor for the formation of a C3b₂-IgG complex. (ii) Multiple attachment sites on IgG HC have been reported, but simultaneous usage of two amino acid residues on the same IgG HC has never been observed. The exact location of the attachment site for C3b on IgG HC is another disputable point of attachment lies within the Fab fragments, extending from the end of the variable region through the first 20 residues of CH2 (36, 37), Thr-144 in the CH1 domain being one binding site (38). Attachment to the CH3 domain and formation of C3b-IgG complexes after deletion of the CH1 domain were, however, reported as well (39, 40). Although the binding site on IgG HC is not within the scope of the present work, we believe that multiple attachment points on IgG are likely to exist. Their utilization needs to be compatible with the functions of
both C3 and IgG molecules: binding to antigen and receptors, complement activation, and others. From this point of view, the CH1-domain region appears to be the best candidate. (iii) The proposed two-domain structure of C3b, based on X-ray- and neutron-scattering solution analysis, depicts the molecule as a flat ellipsoid 18 nm long × 2 nm thick × 8–10 nm wide (41). The overall length of an IgG molecule has been estimated to be ~15 nm. Therefore, it is difficult to imagine two bulky C3b molecules accommodated separately on one HC. (iv) Comparison of the crystal structure of C3d and C4d (42) has revealed the two protein domains to be essentially superimposable α-α barrels. On the convex thioester surface, C4d has a loop including Ser-1217, which is known to be the acceptor site for the thioester. On the opposite, concave barrel, two CR2-binding amino acid clusters have been identified (44). In the C3b-IgG complex, the two thioester surfaces of the C3dg fragments would be brought together, leaving the CR2 binding site on the opposite side of the barrel open for interaction.

We have shown that C3b2-IgG complexes contain two C3b, iC3b or C3dg, in all forms of their physiological existence. Regardless of the actual attachment mode, sequential or independent, the two C3b molecules are close enough to represent a dimeric structure to which complement proteins and CRs can bind bivalently.

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