Function of the Factor I Modules (FIMs) of Human Complement Component C6*

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In order to elucidate the function of complement component C6, truncated C6 molecules were expressed recombinantly. These were either deleted of the factor I modules (FIMs) (C6des-748–913) or both complement control protein (CCP) modules and FIMs (C6des-611–913). C6des-748–913 exhibited approximately 60–70% of the hemolytic activity of full-length C6 when assayed for Alternative Pathway activity, but when measured for the Classical Pathway, C6des-748–914 was only 4–6% as effective as C6. The activity difference between C6 and C6des-748–913 for the two complement pathways can be explained by a greater stability of newly formed metastable C5b* when produced by the Alternative Pathway compared with that made by the Classical Pathway. The half-lives of metastable C5b* and the decay of 125I-C5b measured from cells used to activate the Alternative Pathway were found to be about 5–12-fold longer than those same parameters derived from cells that had activated the Classical Pathway. 125I-C5 binds reversibly to C6 in an ionic strength-dependent fashion, but the decay of 125I-C5 measured from cells used to activate the Alternative Pathway was found to be about 5–12-fold longer than those same parameters derived from cells that had activated the Classical Pathway. 125I-C5 binds reversibly to C6 in an ionic strength-dependent fashion, but 125I-C5 binds only weakly to C6des-FIMs and not at all to C6des-CCP/FIMs. Therefore, although the FIMs are not required absolutely for C6 activity, these modules promote interaction of C6 with C5 enabling a more efficient bimolecular coupling ultimately leading to the formation of the C5b-6 complex.

Human complement component C6 is a plasma protein of Mr = 104,000, which consists of 913 amino acids and two asparaginyl-linked oligosaccharide side chains (1, 2). The protein is a composite containing several discrete modules homologous to those found in thrombospondin, the low density lipoprotein receptor, the epidermal growth factor, factor H, and factor I. The protein also has regional homology with complement C9 and a more restricted homology with the T-lymphocyte granular protein, perforin (1, 2).

The function of C6 is to link with C5b in order to form the C5b-6 complex (3, 4). This coupling entails an interaction of C6 with a metastable intermediate of the activated fifth component of complement, designated here as C5b*. After C5 is cleaved by C5 convertase the larger fragment, C5b*, is endowed with a transient ability to form an irreversible complex with C6. If C5b* fails to bind C6 within a short period, C5b* activity decays permanently (4, 5). A further manifestation of this irreversibility is that if C5b-6 were dissociated by chaotropes, the complex cannot reform (4).

The formation of C5b-6 is the first step in the construction of the membrane attack complex (MAC),1 which is responsible for generating transmembrane channels through target cells. The MAC has the structure of a phospholipid membrane-embedded tubule having a leaflet projecting from within the interior wall. The MAC in its complete state creates a stable circular transmembrane channel of ~100 Å (for reviews on the MAC see Refs. 6–8).

How the modules and regions of C6 participate in the formation of the MAC is an ongoing topic of research. The demonstration that C6 can link with C7, C8, and C9 in forming the denaturant-resistant part of the MAC (9) suggests that the domain of C6 homologous to that of C9 is likely to be responsible for C6 integration into the cylindrical part of the MAC. The carboxyl-terminal domain has been reported to be responsible for C5b interaction (2). Binding studies using a carboxyl-terminal fragment of the C6, containing both FIMs, demonstrated that this polypeptide has a reversible affinity for C5 (10). However, investigators studying a human subtotal deficiency of C6 discovered that the molecular basis for this genetic disorder is an aberrant donor splice junction toward the 3′-end of the gene (at intron 15). This results in the translation of a protein with a truncated carboxyl terminus thereby lacking the FIMs. Because this dysmorphic C6 still possessed bactericidal activity, the role of the FIMs as linkage units for C5 was called into question (11, 12).

Our hypothesis is that C6 evolved its elaborate mosaic structure for functional purposes; therefore, the FIMs must impart some useful quality to the molecule. Accordingly, we designed experiments guided by this hypothesis. By using both the baculovirus and the Chinese hamster ovary (CHO) expression systems, C6 and C6-derived proteins lacking the FIMs and lacking both CCP modules and FIMs were produced by insect cells and CHO cells. These expressed proteins were isolated and studied. The new results presented here demonstrate that the association of C5 with C6 is more complex than was hitherto believed, and it involves at least two binding interactions. Although not absolutely required for C6 activity, the FIMs have been found to be C5-interactive modules that enable the optimal formation of C5b-6.

EXPERIMENTAL PROCEDURES

Materials—Sheep erythrocytes were from Colorado Serum Co. (Denver, CO). Dibutyryl phthalate and dioctyl phthalate were purchased from Aldrich. EX-Cell 400(H) was from JRH Biosciences (Lenexa, KS), and fetal bovine serum was from BioWhittaker (Walkersville, MD). Ingredients for CHO growth medium, which was comprised of Ham’s F-12

1 The abbreviations used are: MAC, membrane attack complex; CCP, complement control protein; FIM, factor I module; CVF, cobra venom factor; EAC1, sheep erythrocytes bearing antibody and complement C1; CHO, Chinese hamster ovary.

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medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum with or without geneticin (G418 sulfate), were all from Life Technologies, Inc. Nitrocellulose membranes were from Sartorius (Surrey, UK), and peroxidase-conjugated rabbit anti-murine IgG was from Bio-Rad. Complement C5–9 as well as factors B and D were isolated from a single batch of frozen recovered human plasma (13, 14). Cobra venom factor (CVF) was prepared by gel filtration followed by anion exchange chromatography (15). The plasmids, pBacPAK 9 and Bsu36I-digested BacPAK 6 were purchased from CLONTECH Laboratories (Palo Alto, CA). Guanidine HCl was from Life Technologies, Inc. Pwo DNA polymerase was obtained from Roche Molecular Biochemicals. HEPES, sodium thiosulfate, and hemolysin were from Sigma. Sodium [125I]iodide was from Amersham Pharmacia Biotech. The insect cell lines Sf9 and TN5 along with Grace’s insect cell culture medium were from Invitrogen (Carlsbad, CA), and CHO-K1 cells were purchased from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK).

C6 DNA Constructs—DNA coding for human C6 was available from the original cloning from a cDNA liver library (1). In order to subclone C6 DNA, full-length constructs were made by ligation. Then the polymerase chain reaction (Perkin-Elmer) was employed using primers that were engineered to position a Kpn1 restriction site on the 5’-end along with Not1 or XhoI restriction sites on the 3’-end. Oligonucleotides used to make full and truncated C6 proteins are as follows: C6, forward primer, 5′-GGGATCCATGCGCAGCAAGCTCTGTCCTGTGAC-3′ and reverse primer, 5′-CGGGGGGCCTGATGCGGAACACTTTCCAGGAT-3′; C6des-611–913: the forward primer was the same as that for C6 and reverse primer, 5′-GCGCGCCGCTATGGCCAGACGCTCTGACATTC-3′; and reverse primer, 5′-CCGGCTCGATCCTTCTCTCCACATTGAGA-3′. By using the engineered restriction sites DNA segments encoding full-length C6, C6des-748–913, and C6des-611–913 were subcloned into the expression vectors pBacPAK 9 (CLONTECH, Palo Alto, CA) and pCDNA3 (Invitrogen, DeSchelp, Netherlands) for the insect and mammalian systems, respectively. The inserts were sequenced by automated DNA sequencing using an ABI Prism model 377 sequencer.

Expression—For the baculovirus system the engineered plasmids were used to generate recombinant viruses, which were propagated using Sf9 cells. However, for protein expression TN5 cells were employed because these cells gave better yields. For the mammalian system, CHO cells were maintained and transfected with recombinant plasmids using LipofectAMINE (Life Technologies, Inc.) as described previously (16). The expressed proteins were purified first by passage through a column (1 × 10 cm) of DEAE-Sephaloc equilibrated with 20 mM imidazole HCl, pH 7, 0.15 M NaCl. None of the recombinant C6 proteins bound to DEAE-Sephaloc using these conditions, but many contami- nant proteins were removed. The unadsorbed fraction containing re- combinant C6 proteins were immediately applied to an affinity column (1 × 3 cm) of Ni2+-Chelating Sepharose FF. After washing the column with 2 M KCl, recombinant proteins were eluted with 4 M guanidine HCl. After dialysis the proteins were concentrated in an Amicon stirred filtration unit using a PM 30 membrane (Stonehouse, UK). Western blots were performed similarly to that described (17) with the major modification being that the reactive protein bands were detected using an enhanced chemiluminescence light detection system (Amersham Pharmacia Biotech). Expressed forms of C6 were quantified by an enzyme-linked immunosorbent assay using a rabbit antibody reactive to the low density lipoprotein module within human C6 (18).

Complement Hemolytic Assays—Complement hemolytic assays and data analyses were done similarly to published methods (19–22). Briefly, for Classical Pathway assays, sensitized sheep erythrocytes bearing guinea pig C1 (EAC1) were used. The assay buffer was DGBV2+, which contained dextrose (0.14% wt), gelatin (0.1%), veronal (2.4 mM) buffer, pH 7.6, 7.0 mM NaCl, 1.0 mM MgCl2, and 0.4 mM CaCl2. Assays were performed by incubating at 37 °C for 30 min of samples expressed C6 proteins with EAC1 along with a human plasma depleted of C6. For the Alternative Pathway rabbit erythrocytes were reacted with a human plasma depleted of C6 in DGBV containing 1 mM MgCl2 and 1 mM EGTA. Reaction conditions for CH50 assays were as follows: Assays for C5b-6 were carried out using sheep erythrocytes that had previously been preincubated with 10 mM dithiothreitol for 1 h at 37 °C because this chemical treatment renders erythrocytes sensitive to reactive lysis (23). For each assay variable amounts of C5b-6 (0–0.14 μg/ml) in DGBV were preincubated with dithiothreitol-treated erythrocytes (107 cells/ml) for 1 min followed by the addition of C7 (0.15 μg), C8 (0.2 μg), and C9 (0.4 μg). After 30 min at 37 °C, 1 ml of DGBV was added, and the samples centrifuged for 5 min at 3000 rpm. Subsequently, the optical density at 413 nm was recorded. By using purified C5b-6, a standard curve relating the concentration of C5b-6 to the level of hemolysis was determined with the result that C5b-6 was calculated to contain approximately 1010 C5b-6 units/ml.

The effect of factors H and I on the Classical Pathway hemolytic activities of C6 and C6des-FIMs was performed by incubating sensitized sheep erythrocytes with isolated Classical Pathway components as follows: C2 (2 μg/ml), C3 (50 μg/ml), C4 (20 μg/ml), C5 (4 μg/ml), C7 (3 μg/ml), C8 (4 μg/ml), and C9 (5 μg/ml) with various amounts of C6 or C6des-FIMs (0–0.75 μg/ml) in presence of factors H (0–100 μg/ml) and I (0–60 μg/ml) for 30 min at 37 °C. Subsequently, the cells were centrifuged, and the absorbance at 413 nm of the supernatants was recorded.

Formation of C5b—C5b-6 was made in a fluid phase by activating C5 (0.4 μg/ml) using a preformed fluid phase C5 convertase, CVF, Bb (0.02 mg/ml) in the presence of C6 (0.2 mg/ml) for 15 at 37 °C in 20 mM Tris-HCl, pH 7.8, 7.5 mM NaCl. The complex enzyme, CVF, Bb, was prepared by combining CVF (0.13 mg/ml) with factor B (0.26 mg/ml) and factor D (0.01 mg/ml) in 20 mM imidazole HCl, pH 7, 0.075 M NaCl, 50 μM NiCl2 for 30 min at 37 °C (4). Subsequently, C5b-6 was purified by adsorption onto a DEAE-Sephaloc column (1.5 × 20 cm) followed by elution with a linear salt gradient consisting of 150 ml each of 0.075 M NaCl to 0.3 M NaCl in 20 mM Tris-HCl, pH 7.8. C5b-6 elutes after unincubated control and C6.

To test the incorporation of recombinantly expressed C6 proteins to join with fluid phase C5b, C5 (0–0.1 mg/ml) in 20 mM Tris, pH 7.8, 0.15 M NaCl was mixed with C6, C6des-FIMs, or C6des-CCP/FIMs (0–0.05 mg/ml), along with a 1 to 20 molar ratio of CVF, Bb to C5; and the mixtures were incubated for 15 h at 37 °C. To determine the percent of C6 or C6des-FIMs incorporation into complex with C5b results of hemolytic assays were used. Levels of C5b,6 or C5b,6des-FIMs that formed were estimated by interpolation from a standard curve, described previously, relating C5b-6 hemolytic activity to the amount of C6-6.

Decay Rate of Metastable C5—Measurements of the half-lives of the activity of metastable C5b+ were determined for both major complement pathways. For the Classical Pathway, EAC1 in DGBV were combined with 1/5th volume of a human plasma depleted of C6 for 3 min at 37 °C followed by rapid washing in ice-cold DGBV/EDTA. The cells were suspended in DGBV/EDTA and incubated at 37 °C. At various times aliquots (50 μl) were withdrawn and mixed with DGBV/EDTA containing C6 (0.1 μg), C7 (0.1 μg), C8 (0.2 μg), and C9 (0.4 μg) in 50 μl. Hemolysis was measured at 413 nm after 30 min at 37 °C. For the Alternative Pathway, a similar procedure was followed except rabbit erythrocytes were used, and the preincubation time was 7 min.

Measurements on the dissociation of 125I-C5b deposited on erythrocytes as a result of the action of C5 convertases of both pathways were determined using procedures to activate the two complement pathways similar to those stated previously. Erythrocytes were preincubated for 2–7 min with a human plasma depleted of C6 along with 125I-C5, which were then reconstituted using a anti-C5-IgG-Sepharose column and washing and resuspending in DGBV/EDTA, the cells were incubated at 37 °C. At various times aliquots (55 μl) were removed, layered over 110 μl of dibutyl phthalate:diocyl phthalate (8:3, v/v), and the tubes were centrifuged at 10,000 × g for 1 min. The bottoms of the tubes, containing the cell pellets, were cut off and counted in an LKB Wallac 1282 CompuGamma counter. The data were subtracted from counts of radioactivity of 125I-C5 bound to cells, which contained decayed C5 convertase.

Deposition of C3b on Erythrocytes—The amount of C3b deposited on rabbit erythrocytes and sensitized sheep erythrocytes was determined by incubating these cells with 125I-labeled C3 (produced by using a solid phase lactoperoxidase method (24)) in DGBV, 1 mM MgCl2, 1 mM EGTA or DGBVβ2+ containing plasma depleted of C6 for 30 min at 37 °C. As controls similar mixtures were used, but the buffer was DGBV, 1 mM EDTA. Subsequently the cells were washed once in cold DGBV and resuspended. Aliquots were layered over 100 μl of oil (dibutyl phthalate:diocyl phthalate, 8:3, v/v), and the cells were centrifuged through the oil layer at 10,000 rpm for 5 min. The bottoms of the tubes were cut off, and radioactivity was measured in an LKB Wallac 1282 CompuGamma counter.
ovalbumin. After 1 h at 4 °C the wells were drained and washed once, and the radioactivity was quantified using an LKB Wallac 1282 CompuGamma counter.

To measure the affinity of C5 for C6, C6 was immobilized onto microtiter wells, and these were blocked and washed as described. Increasing concentrations (0–1.6 M) unlabeled C5 in 10 mM HEPES, pH 7.3, 0.15 M NaCl, or 75 mM NaCl, 10 mg/ml ovalbumin was then allowed to compete with 125I-labeled C5 for binding to C6 over 48 h at 4 °C. After draining and one brief washing bound C5 was measured in an LKB Wallac 1282 CompuGamma counter. Scatchard analysis of the data was used to estimate the affinity constants of C5 for C6 (25).

The stability of C5b-6 or C5b-C6des-FIMs was determined by incubating these complexes with various concentrations of sodium chloride or sodium thiocyanate for 1 h at 37 °C followed by dialysis and measurement of hemolytic activities.

RESULTS

An outline scheme showing a comparison of the basic features of complement C6 and the truncated expressed forms of C6 with C9 is presented in Fig. 1. As described previously C6 contains an amino-terminal domain homologous to C9, which includes a segment of ~330 amino acids related to perforin, followed by a domain consisting of two CCP modules and two FIMs. In order to elucidate the function of the carboxyl-terminal domain of this protein, C6, C6des-748–913 (C6des-FIMs), and C6des-611–913 (C6des-CCP/FIMs) were expressed using baculovirus and mammalian expression systems.

For the baculovirus system, recombinant viruses were made and propagated in Sf9 cells, but because these cells produced little heterologous protein, TN5 cells were used to synthesize all C6 types. Synthesis of the C6 proteins by the mammalian system employed CHO cells (Fig. 2). Although the yields from both systems were very low (<0.2 µg/ml), the amounts recovered were adequate to perform the necessary experiments.

Expressed forms of C6 were purified by affinity adsorption, quantified using an enzyme-linked immunosorbent assay, and assayed for complement hemolytic activities initiated by both Alternative and Classical Pathways. C6des-FIMs had between 60 and 70% of the specific activity of full-length C6 for the Alternative Pathway but only 4–6% for the Classical Pathway. C6des-CCP/FIMs had little detectable activity from either of these assays (Fig. 3). The recombinant forms of C6 gave a similar spectrum of activities independent of the mode of expression (i.e. whether synthesized by insect or CHO cells). However, since the baculovirus expression system gave better yields than the mammalian system, C6 proteins synthesized by the former method were employed for all subsequent studies.

The ability of the various forms of C6 to combine with C5b in the fluid phase to generate the C5b-6 complex was determined next. This was done by reacting a fluid phase C5 convertase, CVF,Bb with C5 and either C6, C6des-FIMs, or C6des-CCP/FIMs. The outcome of this experiment was that C6des-FIMs had only ~7% of the capacity to link with fluid phase C5b as did full-length C6, but C6des-CCP/FIMs had no activity (Fig. 4).

An explanation was required as to why C6des-FIMs had such strong Alternative Pathway activity. We noticed during assays for the Alternative Pathway that tubes containing full-length C6 exhibited lysis prior to those containing equivalents of 31813
C6des-FIMs. Therefore, we measured hemolysis as a function of time using functional equivalents of C6 and C6des-FIMs for both complement pathways. Hyperbolic relationships were observed for C6 and C6des-FIMs for the Classical Pathway, and only a small time lag for hemolysis mediated by C6des-FIMs relative to C6 was observed. In contrast for the Alternative Pathway, both C6 and C6 C6des-FIMs showed sigmoidal kinetics, and an appreciable time lag was observed for hemolysis mediated by C6des-FIMs relative to C6 (Fig. 5).

Since this result suggested that the half-life of metastable C5b* may be longer when generated by the Alternative Pathway than when made by the Classical Pathway, the half-lives of nascent C5b* after formation by both complement pathways were determined. This was accomplished by producing nascent C5b* on either rabbit erythrocytes for the Alternative Pathway or sheep EAC1 for the Classical Pathway, followed by measuring decay of hemolytic potential after incubation at 37 °C. The results shown in Fig. 6 indicate that the Classical Pathway C5b* is very labile, decaying with a half-life of 2.4 min at 37 °C, whereas that of the Alternative Pathway is considerably more stable and decays with a half-life of 30.3 min at 37 °C. Consistently, the rate of dissociation from erythrocytes of 125I-C5b deposited by the Alternative Pathway (t½ = 23.8 min) was considerably slower than that deposited by the Classical Pathway (t½ = 4.9 min) (Fig. 7). Furthermore, although similar amounts of cells, C5-, and C6-deficient plasma were used to activate both pathways, at 0 time over 10-fold more 125I-C5b was found on erythrocytes which were used to activate the Alternative Pathway than those used to activate the Classical Pathway (Fig. 7).

Since at the onset of this experiment a much higher level of 125I-C5b was found on rabbit erythrocytes than sensitized sheep erythrocytes, we hypothesized that cells, which were used to activate the Alternative Pathway, bound more C3b than those used to activate the Classical Pathway. To examine this, we measured the amount of C3b molecules bound to each cell type after incubation with plasma depleted of C6 containing 125I-labeled C3 as described under “Experimental Procedures.” The outcome was that rabbit cells bound 1.77 ± 0.05 × 10⁶ C3b/cell but sheep erythrocytes bound only 2.95 ± 0.17 × 10⁵ C3b/cell. These results along with the relative decay rates of C5b and C5b hemolytic potential suggested that bound C3b could stabilize metastable C5b*.

To test this notion further, we designed hemolytic assays using isolated Classical Pathway proteins (C2–9), and we ex-
amined the influence of the complement control proteins H and I on the hemolytic activities of both C6 and C6des-FIMs. The results, shown in Fig. 8, indicated that the specific hemolytic activities of C6 and C6des-FIMs are diminished by factors H and I but that of C6des-FIMs is more severely impacted. In the absence of the complement control proteins, the percent activity of C6des-FIMs to C6 is about 15%, but with increasing concentration of these regulatory proteins, this value declines to 1%. The combined results suggest that optimal hemolytic activity of C6des-FIMs requires more cell bound C3b than does C6, which is consistent with the notion that C3b could stabilize metastable C5b*. The binding capability of the various forms of C6 for interaction with C5 was measured next. The results show that interaction of C5 with C6 is ionic strength-dependent, and that removal of the FIMs from this protein drastically reduces the capacity of C5 to interact with C6 (Fig. 9). Relative to human serum albumin, C6des-FIMs did have a weak capacity to bind C5, but full-length C6 was clearly stronger. In order to obtain quantitative data for the interaction of C5 with C6, binding studies were performed using unlabeled C5 in competition with trace-labeled 125I-labeled C5. Scatchard analyses indicated that the affinities of C5 for C6 were 2.3 and 0.93 × 10^6 M^{-1} in 0.075 M NaCl and 0.15 M NaCl, respectively (Fig. 10).

Since it has been demonstrated that the FIMs facilitate C5 and C6 interaction, we asked whether these modules were important for stabilizing C5b-6. Both C5b-6 and C5b-6des-FIMs were produced in the fluid phase, and functional equivalents of these were assayed for activity after exposure to various concentrations of sodium chloride and sodium thiocyanate. The results were that C5b-6 and C5b-6des-FIMs exhibited nearly identical profiles of stability to concentrated solutions of sodium chloride and instability to small concentrations of sodium thiocyanate (Fig. 11).

**DISCUSSION**

The data presented here suggest that the coupling of C5 with C6 leading to the C5b-6 complex is a multi-step assembly that is more complex than previously conceived. Shortly after these proteins were first isolated from plasma, it was demonstrated that they possessed a reversible affinity for each other (26, 27). After the sequences were obtained for C6, it was demonstrated further that the carboxyl-terminal domain, containing the FIMs, was responsible for the reversible interaction with C5 (10). However, dysmorphic C6 from human subjects, who have a subtotal C6 deficiency condition, was found to be deleted of the FIMs, yet this protein still functioned for bactericidal activity and for incorporation into soluble terminal complement complexes. It was concluded that the C6 FIMs could not represent the C5b-binding site (11, 12). However, interpretation of results presented here suggests that the association of C5 with C6 entails not a single site but at least two binding sites. To evaluate the function of the CCP modules and FIMs of C6, we employed heterologous expression systems to manufacture truncated forms of this protein. Assays and binding studies were then performed using these.

Relative to full-length C6, C6des-FIMs exhibits very weak Classical Pathway activity and a poor capacity to integrate with fluid phase C5b (Figs. 3 and 4). However, removal of the FIMs did not have a major influence on hemolytic activity when...
Alternative Pathway assays were utilized (Fig. 3). These data are in accord with results of complement assays of a plasma derived from a subject who had a combined deficiency in C6 and C7. This plasma had negligible C6 Classical Pathway activity and C5b-6 generating capacity but surprisingly strong C6 Alternative Pathway activity (28). Since this subject’s C6 is likely to be identical with the dysmorphic C6 of the subtotal deficient subjects and would thereby lack the FIMs (11, 12, 29), a clear correlation now exists between the spectrum of activities found in C6-deficient plasmas with those of the purified recombinant proteins.

The difference exhibited by C6 and C6des-FIMs when comparing activities for the Alternative or Classical Pathways can be explained by the observation that the stability of C5b* is not the same when generated by C5 convertases of the two complement pathways. Confirming an earlier report metastable C5b* generated by the Classical Pathway has a functional half-life of only 2.4 min at 37 °C (5); however, C5b* formed by the Alternative Pathway has a considerably longer half-life of 30.3 min (Fig. 6). The decay of functional activity of C5b* correlated with the decay of 125I-C5b from cells. When the Alternative Pathway was used to generate C5 convertase, the cleavage product, 125I-C5b, dissociated from cells with a half-life of 23.8 min in contrast to a more rapid dissociation (t1/2 = 4.9 min) when 125I-C5b was generated by the Classical Pathways (Fig. 7). The longevity of metastable C5b* generated by the Alternative Pathway C5 convertase allows C6des-FIMs sufficient time for complex formation, but the half-life of C5b* formed by Classical Pathway activation is too short for efficient coupling of C6des-FIMs to C5b*.

These results suggest that membrane-bound C3b could bind and stabilize metastable C5b*. Consistent with this notion, cells that activate the Alternative Pathway were found to bind 6-fold more C3b molecules than those that were used to activate the Classical Pathway. Furthermore, when isolated components were used to activate the Classical Pathway, the hemolytic activity of C6des-FIMs showed a greater sensitivity to the influence of the complement regulatory proteins, factors H and I (Fig. 8). All the data converge on the conclusion that C6des-FIMs functions effectively when the level of C3b on target cells is high (>300,000 C3b/cell). This is accomplished readily for Alternative Pathway activators. These activators include rabbit erythrocytes, yeast, and bacterial cell walls, all of which provide a large number of nucleophilic acceptors for transacylation with nascent C3b* (30, 31). Furthermore, relative to cells that are activators of the Classical Pathway, those that engage the Alternative Pathway are better able to shield C3b from degradation by plasma control proteins (32). These observations would account for a larger number of available binding sites for C5b* on cells that were used to activate the Alternative Pathway (Fig. 7). An important consideration is that C3b deposition on activating surfaces is not random but rather occurs in clusters each containing 10–40 C3b molecules (33–35). The clustering of receptors has an effect of slowing the dissociation rate of ligand. This is demonstrated from the differing decay rates of monovalent IgE from receptors that were dissociated or clustered (36). Hence all results and published findings are consistent with the notion that bound C3b stabi-
lizes C5b*. However, C3b may not be the only protein that stabilized C5b*. The possibility exists that C7, which like C6 contains FIMs and binds C5, could also stabilize metastable C5b*.

We suggest that the FIMs of C6 exert their influence for Classical Pathway activity by providing a reversible binding site for C5 or C5b. This interaction evidently facilitates the coupling of C5b* with C6. Studies of reversible binding directly implicated the FIMs as binding modules of C6 because only full-length C6 exhibited appreciable interaction for C5 (Fig. 8). Relative to human serum albumin, C6des-FIMs do have weak interactive capacity for C5, but for C6des-CCP/FIMs this binding ability is essentially negligible (Fig. 9). Hence the observation made over 25 years ago of a reversible interaction capacity between C5 and C6 (26, 27) was a consequence of C5 binding to the FIMs of C6. This binding is reversible and ionic strength-dependent and is characterized by affinity constants of 0.93 and $2.3 \times 10^8 \text{ M}^{-1}$ in 0.075 and 0.15 M NaCl, respectively.

Although the FIMs are specific C5 binding modules, they are not required absolutely for the activity of C6 nor are they required for the stability of C5b-6 complex. Nearly identical profiles for stability to high concentrations of sodium chloride or sodium thiocyanate were found for C5b-6 and C5b-6des-FIMs (Fig. 11). These results indicate that another potential binding interaction between C5b and C6 exists apart from that mediated by the FIMs. The fact that chaotropic anions ($\text{SCN}^-$) but not lyotropic ones ($\text{Cl}^-$) can dissociate C5b-6 (Fig. 11) suggests that this second binding site is mediated by hydrophobic interaction. Also the hydrophobic nature of this second binding site is implied by the observation that if C5b were formed in the fluid phase at concentrations above 100 $\mu$g/ml in the absence of C6, large aggregates of C5b are generated. These C5b aggregates are not dissociated by high concentrations of sodium chloride, indicative of hydrophobic complexionation (4).

Although the specific regions of C5b and C6 that take part in this hydrophobic interaction have not yet been identified, potential importance of the third thrombospondin module of C6 for C5b interaction has been suggested because an epitope within this module is lost upon complex formation (37). Also the CCP modules could participate because deleting these regions of C5b could also stabilize metastable C5b*.

The covalent structure of the FIMs within C6 has been determined, and the disulfide bridges are arranged as follows: 1–3, 2–9, 4–7, 5–10, and 6–8 (44). Since these 5–S bonds overlap, the resultant polypeptide chain is expected to create an ellipsoid that should contain several tight turns and loops linking twisted and irregular $\beta$-strands. This model is consistent with the circular dichroism spectroscopy of a fragment of C6 containing the two FIMs (1). Such a structure would be an excellent scaffold for fixing interactive side chains. Indeed it is now generally recognized that modules are fundamentally and autonomously folding units of large proteins, and one reason for the evolution of modules is to endow proteins with ligand binding capacity (45–47).

The observation that the FIMs are facilitating devices that promote interaction of C5 and C6, which is important for antibody-mediated complement activation, conveys implications as to how the terminal pathway of complement evolved. We suggest that the most likely scenario is that the membranolytic pathway of complement evolved in retrograde. In this scheme we propose that the original molecule was a perforin-like granular membranolytic protein. The gene for this original granular protein duplicated and rearranged to generate a gene coding for a plasma-soluble C9-like molecule, which was coupled functionally to the innate immune system. Subsequently the C9 gene then duplicated serially creating those for C8a, C8b, and then C6/7. We propose that the initial C6/7 molecule lacked the FIMs but contained the CCP modules, and it was this form of the protein that coupled the membranolytic pathway of complement with the Alternative Pathway through interaction with C5. Thereafter, the gene for C6/7 evolved to include the FIMs, which enabled C6/7 to forge a link with the evolving Classical Pathway. The culmination of this process was the duplication of the C6/7 gene to produce C6 and C7. The concept of retrograde evolution of the membranolytic pathway of complement conflicts with the suggested forward evolution of this pathway (48). These differing ideas can be critically tested using comparative biochemical studies of the complement components of modern lower chordates especially those in the subphylum Agnatha and class Chondrichthyese.

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