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Complement components C3, C4, and C5 are members of the thioester-containing α-macroglobulin protein superfamily. Within this superfamily, a unique feature of the complement proteins is a 150-residue-long C-terminal extension of their α-subunits that harbors three internal disulfide bonds. Previous reports have suggested that this is an independent structural module, homologous to modules found in other proteins, including netrins and tissue inhibitors of metalloproteinases. Because of its distribution, this putative module has been named both C345C and NTR. To assess the structures of these segments of the complement proteins, their relationships with other domains, and activities as independent structures, we expressed C345C from C3 and C5 in a bacterial strain that permits cytoplasmic disulfide bond formation. Affinity purification directly from cell lysates yielded recombinant C3- and C5-C345C with properties consistent with multiple intramolecular disulfide bonds and high β-sheet contents. rC5-, but not rC3-C345C inhibited complement hemolytic activity, and surface plasmon resonance studies revealed that rC5-C345C binds to complement components C6 and C7 with dissociation constants of 10 and 3 nM, respectively. Our results provide strong evidence that this binding corresponds to the previously described reversible binding of C5 to C6 and C7, and taken together with earlier work, indicate that the C5-C345C module interacts directly with the factor I modules in C6 and C7. The high binding affinities suggest that complexes composed of C5 bound to C6 or C7 exist in plasma before activation and may facilitate assembly of the complement membrane attack complex.


Complement components C3, C4, and C5 are evolutionarily related, ∼190,000-Da serum glycoproteins that together play key roles in the activation and regulation of complement, and in its opsonizing, chemoattractant, and lytic functions (for recent reviews, see Refs. 1 and 2). These activities require the direct interaction of one or more of the three proteins or their proteolytic fragments with ∼20 other soluble and membrane-bound blood proteins (3, 4). For C3 and C4, most binding partners are proteins that regulate complement activity or mediate its cellular interactions. C5, in contrast, is the focal point for formation of the complement membrane attack complex (MAC), binding directly to three of the four other components of the MAC (5, 6).

C3, C4, and C5 are members of a protein superfamily, which includes: 1) α-macroglobulins (7); 2) insect acute-phase thioester-containing proteins, which appear to contribute to a primitive complement-like innate immune response against bacterial infections (8, 9); and 3) a recently reported glycosylphosphatidylinositol-linked cell surface protein, CD109, which is found on hematopoietic cells, but has no known function (10). The most distinctive structural feature of this superfamily is the presence of an intrachain β-cysteinyl-γ-glutamyl thioester (7, 8, 11); C5 alone lacks this on functional group. The primary purpose of the thioester appears to be to provide a primed mechanism for creating a covalent link to neighboring proteins or carbohydrates. This process is usually triggered when a proteolysis-induced conformational change destabilizes and/or exposes the thioester, leading to an intermolecular exchange reaction that forms an amide or ester linkage between the γ-glutamyl carboxyl group of the thioester and nearby amino or hydroxyl groups (11).

Within this thioester-containing superfamily, the complement subgroup is distinguished by two features: 1) the presence of a ∼75-residue-long anaphylatoxin domain at the N terminus of the α-chains in a part of the protein in which the α-macroglobulins instead have a segment that serves as a bait for proteinases (12), and in which the insect thioester-containing proteins have a region of high sequence variability, which may serve to expand their repertoire of intermolecular interactions (8); and 2) a C-terminal extension of the α-chains of ∼150 aa residues. Ishii et al. (13) first suggested that this latter addition might be an independent protein module based on its 19% sequence identity with the C-terminal segment of the Caenorhabditis elegans UNC-6 protein, a laminin-related netrin protein (14) that guides axonal growth, and on its absence from α2-macroglobulin. Bork and Bairoch (15) subsequently named this putative module C345C, for C terminus of C3, C4, and C5. More recently, Bányai and Pathy (16) suggested that this complement/netrin domain is homologous to the N-terminal domain of tissue inhibitors of metalloproteinases (TIMPs), and that homologous representatives are also found in secreted frizzled-related proteins and type I procollagen C-proteinase enhancer (PCOLCE) proteins. They named the domain NTR, for netrin-like. More recent reports of the disulfide-bonding pattern of secreted frizzled-related protein-1 (17), and of the three-dimensional structures of the laminin-binding (Nta) domain of agrin (18) and the domain from the human PCOLCE-I (19) have supported the idea of structural homology among TIMPs and C345C/NTR.

The domain in TIMPs is directly involved in binding to metalloproteinases (20, 21), but its functions in other proteins are still unclear. We have previously reported (22, 23) that amino acid
residues within the C345C domain of C5 are important for proteolytic activation by the classical pathway C5 convertase, a trimeric complex composed of the activated fragments of complement proteins: C2a, C4b, and C3b (24). We infer that these residues in C5-C345C form part of a contact between C5 and the convertase, but have not yet identified the putative binding partner within the convertase. To explore further the structure and activities of this unique feature of the complement proteins, we expressed C345C from C3 and C5 in a bacterial system, and examined the structures and binding activities of the recombinant products.

Materials and Methods

Reagents

Restriction enzymes were purchased from New England Biolabs (Beverly, MA), oligonucleotides from Sigma-Genosys (The Woodlands, TX), and Phu DNA polymerase for PCR from Stratagene (Cedar Creek, TX). Plasmid pET15b, the ORIGAMI strain of Escherichia coli, and protein extraction and purification reagents including BugBuster, Benzamidine protease inhibitor cocktail, His-Bind purification kit, biotinylated thrombin, and streptavidin agarose were purchased from Novagen (Milwaukee, WI). Guanidine-HCl, 5.5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), i-cysteine hydrochloride, Tris, and Brilliant Blue R were from Sigma-Aldrich (St. Louis, MO), and isopropyl-β-D-thiogalactoside was from Fisher Biotech (Fisher Scientific, NJ). Proteoliposomes of phosphatidylcholine and all other complement components, buffers, and reagents were purchased from Advanced Research Technologies (San Diego, CA).

Construction of expression plasmids

DNA sequences encoding residues extending from Ala1452 to the C-terminal Asn1641 of human C3, and from Ala1417 to the C-terminal Cys1658 of human C5 (numbering corresponding to the pro-proteins) (25, 26) were copied from the full-length clones pSVC3 (27) and pHC5D (28), respectively, by PCR. Priming oligonucleotides were designed to include an NdeI restriction enzyme cleavage site immediately upstream of the N-terminal Ala residues and a BamHI site in the 3'-untranslated region. PCR products were digested with NdeI and BamHI, and ligated into NdeI/BamHI-digested pET15b to give pET/C3CC and pET/C5CC. Sequences of the constructs were confirmed by automated DNA sequencing with ABI model 377 Genetic Analyzer and/or ABI model 377 Automated DNA Sequencer (Perkin-Elmer, Foster City, CA).

Results

C5 was cleaved to C5a and C5b using the cobro venom factor C5 convertase, as described (30). Briefly, a solution composed of 9 μl 1 mg/ml cobro venom factor, 6 μl 1 mg/ml factor B, and 1 μl 0.1 mg/ml factor D, and 40 μl of 10 mM Tris-HCl, pH 8/50 mM NaCl/50 mM MgCl2 was incubated at 37°C for 15 min to form the convertase. This solution was then added to 250 μl C5 in PBS/5 mM MgCl2; the reaction mix was incubated at 37°C overnight and subsequently diazyed against PBS. Conversion to C5b was 80–90%, as estimated from SDS-polyacrylamide gels.

Enzymatic generation of C5b

C5b was generated by incubation of C5 with a range of concentrations of C5 convertase, as described (29). The data were fitted to the expression C5 convertase (23), which we infer to be expressed and isolated from human C5 and C5-C345C within intact C5 and C5-C345C, respectively, contain three internal disulfide bonds (34), as illustrated in Fig. 1. The reducing environment of the E. coli cytoplasm precludes disulfide bond formation. Therefore, the homologous recombinant domains from TIMPs and PCOLCE1, which also have three internal disulfide bonds, were harvested initially as insoluble inclusion bodies, and the soluble, properly folded structures subsequently were restored by dissolution under denaturing conditions, followed by dialysis to remove the denaturant and simultaneously form disulfide bonds in vitro (19, 35, 36). Because our earlier work indicated that the C345C domain from C5 harbors a site important for recognition by the C5 convertase (23), we tried first to express and isolate rC5-C345C using this strategy. Expression of pET/C5CC in the BL21(DE3) strain gave a high yield of the recombinant protein as inclusion bodies, but dissolution under denaturing conditions followed by removal of the denaturant as described for TIMPs resulted in reprecipitation of the recombinant product.

We therefore tested other methods and succeeded in obtaining soluble rC5-C345C by expressing pET/C5CC in a mutant strain of E. coli, commercially available under the trade name ORIGAMI.

Circular dichroism

Circular dichroism measurements were conducted in PBS at concentrations of 50–100 μM with a JASCO J-720 spectropolarimeter. Spectra were analyzed with the JASCO J-700 program for Windows Secondary Structure Estimation version 1.10.00, JASCO.3 to estimate secondary structure content.

DTNB assay for free sulfhydryls

Assays were conducted, essentially as described (31). Briefly, protein solutions at 15–30 μM and parallel standard solutions of cysteine hydrochloride were incubated at room temperature in 1.5 mM DTNB/50 mM NaPO4/2 mM EDTA, pH 7.2, and the A412 was monitored for up to 3 h. Assays were also conducted under mildly denaturing conditions following the procedure of Wright and Viola (32), which is similar to the previous procedure, but substitutes 250 mM Tris-HCl, pH 7.5/4 M guanidine-HCl for the sodium phosphate buffer.

Complement inhibition

Classical pathway hemolytic inhibition assays were conducted essentially as described earlier for synthetic peptides (33). Briefly, rC3- or rC5-C345C was added to a reaction mix of Ab-sensitized sheep erythrocytes and enough human serum to give ~30% lysis of the input erythrocytes in the absence of added rC345C (~0.2% serum). Reactions were incubated for 30 min at 37°C, quenched with ice-cold isotonic veronal-buffered saline with gelatin, and centrifuged to pellet intact erythrocytes, and the supernatants were assayed for A412. Relative hemolysis is the amount of erythrocyte lysis relative to lysis in the absence of rC3- or rC5-C345C.

Surface plasmon resonance (SPR)

SPR measurements were conducted on a Biacore 3000 instrument (Biacore AB, Uppsala, Sweden) with reagents, buffers, and data analysis software from the same company. In all cases, ligands were immobilized in 10 mM sodium acetate buffer to Biacore sensor chip CMS activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and N-hydroxysuccinimide, as recommended by Biacore. All experiments were conducted at 25°C in 0.01 M HEPES, pH 7.4, 0.15 M NaCl, and 0.005% surfactant P20, and sensor chips were regenerated with 20 mM NaOH. Binding affinities and kinetics were determined by data fitting using the BIAevaluata-


which is characterized by impaired reduction of both thioredoxins and glutathione (trxB gor mutations), leading to an oxidizing cytoplasm that supports disulfide bond formation (37). The same system was used to express rC3-C345C. Typical yields of rC3-C345C were 50 mg/L of cell culture, while yields of rC5-C345C were much lower, 5 mg/L.

rC3- and rC5-C345C have substantial β-sheet structure and internal disulfide bonds

The circular dichroism spectra (not shown) of recombinant C3- and C5-C345C indicated that the recombinant products have folded structures with high β-sheet contents of 47 and 43%, respectively. This is similar to the β-sheet contents of the homologous modules from TIMPs and PCOLCE: 40 and 35% β-sheet, respectively (16, 38).

Fig. 2 shows the electrophoretic migration of rC3- and rC5-C345C on an SDS-polyacrylamide gel in the presence and absence of 2-ME. The mobilities of both modules decreased substantially in the presence of the reducing agent, consistent with the notion that the untreated modules have a more compact structure, stabilized by internal disulfide bonds. Under reducing conditions, the mobilities of both modules relative to protein standards indicated molecular masses of 20,000 Da or more, higher than expected from the sequences. However, matrix-assisted laser desorption ionization-time of flight mass spectral analyses (data not shown) of the same samples gave almost precisely the expected molecular masses of 17,852 Da and 17,197 Da for rC3-C345C and rC5-C345C, respectively. Note that removal of the His tag from rC3-C345C does not alter its electrophoretic mobility in the presence of 2-ME. rC5-C345C similarly shows a discernible, but smaller than expected mobility shift after removal of the His tag (data not shown). We assume these are artifacts of the gel system.

Although the gel mobility shift results indicated the presence of disulfide bonds, three bonds are expected in each module. To test whether all three were formed in the recombinant modules, we tested their reactivities to DTNB. Both rC3- and rC5-C345C showed no detectable reaction with DTNB after 3 h at room temperature even in the presence of 4 M guanidine hydrochloride (data not shown). Based on detection limits estimated from cysteine standards, these results indicated that less than 2% of the recombinant molecules display a free sulfhydryl group accessible to DTNB; they suggest that all cysteines in both recombinant modules are disulfide bonded.

rC5-C345, but not rC3-C345C, inhibits complement hemolytic activity

Fig. 3 shows the effects of rC3- and rC5-C345C on the hemolytic activity of dilute human serum. Under these conditions, rC5-C345C inhibited lytic activity with an IC50 of 1 × 10⁻⁶ M, whereas rC3-C345C showed no inhibitory activity in this study, and at concentrations up to 25 × 10⁻⁶ M (data not shown). Inhibition by rC5-C345C was unaffected by the His tag introduced by the expression vector. The dependence of inhibition on the concentration of rC5-C345C was consistent with simple 1:1 binding of rC5-C345C to an essential component of the hemolytic reaction; this is similar to the
effects of C5 synthetic peptides on hemolysis (33). The mechanism of inhibition is under investigation.

**rC5-C345C, but not rC3-C345C, binds to C6 and C7**

Fig. 4 is an SPR sensogram showing the response of a sensor chip bearing ~100 response units (RU) of immobilized rC5-C345C to solutions of C6, C7, and C8, each at 100 μg/ml. These data clearly show binding to C6 and C7, but not to C8. A parallel experiment, with ~260 RU of rC3-C345C immobilized on the sensor chip, showed no response above baseline to any of these three proteins. In the same assay, neither rC3- nor rC5-C345C showed any affinity for C3, C3b, C4, C4b, C5, C5b, or factor I (data not shown).

**Immobile rC5-C345C binds more C7 than C6**

As shown in Fig. 4, the SPR signal for binding of C7 to rC5-C345C was ~5 times greater than for C6. The saturating amounts of C6 and C7 bound to immobilized rC5-C345C ($R_{\text{max}}$) determined by curve fitting (see below) were similarly ~5 times greater for C7. The SPR signal is proportional to the mass of the protein binding to the immobilized target. Therefore, because C6 and C7 are approximately the same size, at 120,000 and 110,000 Da, respectively, this result indicates that on a molar basis, about 5 times more C7 than C6 binds to immobilized rC5-C345C.

The amounts of C7 and C6 bound at saturation are both lower than the maximum expected binding capacity of the immobilized rC5-C345C. In the SPR method, the amount of soluble analyte protein expected to bind to a fully active immobilized ligand protein at saturation, $R_{\text{max}}$, is calculated using the expression, $R_{\text{max}} = (R_0)(\text{MW}_A)/(\text{MW}_L)$, where $R_0$ is, in instrument RU, the amount of ligand (rC5-C345C in this case) immobilized on the sensor chip, and $\text{MW}_L$ and $\text{MW}_A$ are, respectively, the molecular masses of the ligand (rC5-C345C) and analyte (C6 or C7). In these studies, the experimental saturation level, determined from curve fitting of the kinetic sensogram responses illustrated in Figs. 5 and 6, was $\sim$25% of the expected $R_{\text{max}}$ for C7 and $\sim$5% for C6. This indicates that while more binding sites are available to C7, most of the immobilized rC5-C345C is not available for binding to either C7 or C6.

Figs. 5 and 6 show normalized SPR kinetic responses (solid lines) and global curve fits (dashed lines) for immobilized rC5-C345C pulsed with varying concentrations of C6 and C7, respectively. Curve fitting of these and similar replicate data from several sensor chips and with varying levels of immobilized rC5-C345C was used to extract the equilibrium and kinetic constants listed in Table I.

**C6 and C7 bind to distinct, but neighboring sites on rC5-C345C**

Fig. 7 shows the SPR signal observed when C6, C7, and C8 are pulsed in sequence over a surface-bearing immobilized rC5-C345C. In this experiment, C6, C7, and C8 were first pulsed in sequence over the surface, the surface was then regenerated (at R in the figure) with 20 mM NaOH to remove all bound proteins, and finally C6 and C7 were pulsed again in reverse order, as indicated in Table I.

### Table I. Equilibrium and kinetic constants from SPR for binding of rC5-C345C and C5 to C6 and C7

<table>
<thead>
<tr>
<th>Ligand Combination</th>
<th>$K_D$ (nM)</th>
<th>$k_{\text{on}}$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_{\text{off}}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rC5-C345C + C6</td>
<td>10</td>
<td>$2 \times 10^4$</td>
<td>$20 \times 10^{-5}$</td>
</tr>
<tr>
<td>rC5-C345C + C7</td>
<td>3</td>
<td>$3 \times 10^4$</td>
<td>$9 \times 10^{-5}$</td>
</tr>
<tr>
<td>C5 + C6</td>
<td>0.4</td>
<td>$5 \times 10^4$</td>
<td>$2 \times 10^{-5}$</td>
</tr>
<tr>
<td>C5 + C7</td>
<td>0.1</td>
<td>$4 \times 10^4$</td>
<td>$0.4 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

*In 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.005% surfactant P20, at 25°C.*
The capacity of immobilized C5 (RU\textsubscript{max}) was similar to that of immobilized C345C. Times of exposure to C6, C7, and C8 are shown by dashed lines so labeled over the sensorgram plot. R notes the times the surface is exposed to 20 mM NaOH.

In the figure. These data show again that C6 and C7 bind to rC5-C345C, that C8 does not bind to rC5-C345C even when C7 is already bound, and that the mass of C7 binding to the immobilized module is several-fold higher than C6. In addition, the further increase in the SPR signal displayed by pulsing with C7 after C6 was already bound suggests that additional C7 molecules are binding to rC5-C345C at a site distinct from the C6 binding site. As shown in the second part of the experiment, however, the inverse was not true: there was no further increase in signal on adding C6 when C7 was already bound to the surface. This implies that C6 cannot bind to rC5-C345C with C7 already in place. Note in both parts of the experiment that decay of the SPR signal following the end of the C7 pulse closely resembles the decay due to C7 dissociating from immobilized rC5-C345C in buffer alone shown in Fig. 6. Hence, C7 dissociation is not substantially affected by the presence of C8 or C6, and C6 does not appear to bind to newly vacated C7 binding sites.

These results suggest that the C6 and C7 binding sites overlap, or that C7 obstructs the C6 binding site. In addition, because several-fold more C7 binds than C6, apparently only a subset of C7 binding sites is associated with C6 binding sites. A simple interpretation of these observations is that chemical coupling of rC5-C345C to the SPR chip surface produced a heterogeneous collection of molecules linked to the surface at different amino acid residues. Within this collection, most of the molecules cannot bind to either C6 or C7, the binding site for C7 is available in ~25% of the molecules, and the binding site for C6 is accessible at only 20% of the C7 binding sites. It is also possible that multiple C7 molecules bind to each rC5-C345C molecule, but the binding sites on the module would need to be largely independent and equivalent to fit the data.

**Immobilized C5 binds to C6 and C7**

Because previous studies have reported that intact, native C5 binds to C6 and C7 (5, 39, 40), and to C8 (6, 41), we tested for binding of C5 to C6, C7, and C8 using the SPR method. Fig. 8 is an SPR sensogram showing the response of a sensor chip bearing ~1000 RU of immobilized C5 to solutions of C6, C7, and C8, each at 50 \(\mu\)g/ml. At 1000 RU, the molar quantity of immobilized C5 is equivalent to ~100 RU of immobilized rC5-C345C, because C5 is ~10-fold larger than the recombinant module. These data clearly show strong responses to C6 and C7, but not to C8. The binding capacity of immobilized C5 (RU\textsubscript{max}) was similar to that of immobilized C5-C345C: 10 and 20% for C6 and C7, respectively. This was surprising, because we expected that the larger protein would allow coupling at a higher proportion of sites distant from the C6 and C7 binding sites, and hence a higher RU\textsubscript{max}. We infer that this is an artifact of the chemical immobilization method and/or the SPR method. Equilibrium and kinetic constants for binding of C5 to C6 and C7 were derived from binding data collected at different concentrations of C6 and C7, with different amounts of immobilized C5, as illustrated for rC5-C345C in Figs. 5 and 6; these are also listed in Table I.

Note in Figs. 4 and 8 that binding to intact C5 and to rC5-C345C is similar in that at saturation more C7 than C6 binds to immobilized C5. As mentioned above, the saturating amounts of C6 and C7 were ~10 and 20%, respectively, of the levels expected from the amount of C5 immobilized on the sensor chip (the calculated RU\textsubscript{max}). Although the difference is less with intact C5, 2-fold instead of 5-fold, the greater binding of C7 is still somewhat surprising, because we would not expect that accessibility of binding sites would differ much in an immobilized target as large as C5. Therefore, the greater mass of C7 binding seems to be due to a difference in the binding stoichiometries or an unknown artifact of the SPR method. C7 molecules do form dimers and higher multimers, but only after treatment with deoxycholate (42); and there is no evidence of multiple C7 molecules binding to C5. As discussed below, most studies have focused on the MAC itself, and association of C5 with multiple C7 molecules may occur in the reversible binding reaction of C5 to C7.

**C6 and C7 also bind to neighboring sites on C5**

Fig. 9 shows the results of pulsing C6, C7, and C8 in sequence over C5 immobilized on an SPR sensor chip. The results in this study are essentially identical with those in Fig. 7, in which rC5-C345C was immobilized on the surface: C8 does not bind at all even after C6 and C7 are bound, the amount of C7 bound at saturation is greater than C6, and C7 precludes binding of C6.

rC5-C345C inhibits binding of C6 and C7 to immobilized C5

Figs. 10 and 11 show the results of pulsing 0.25 \(\mu\)M solutions of C6 and C7, respectively, containing rC5-C345C at concentrations equal to 200 RU of immobilized C5. Hence, C6 cannot bind to rC5-C345C with C7 already in place. Note in both parts of the experiment that decay of the SPR signal following the end of the C7 pulse closely resembles the decay due to C7 dissociating from immobilized rC5-C345C in buffer alone shown in Fig. 6. Hence, C7 dissociation is not substantially affected by the presence of C8 or C6, and C6 does not appear to bind to newly vacated C7 binding sites.

FIGURE 7. SPR sensogram showing the effects of pulsing C6, C7, and C8, all at 100 \(\mu\)g/ml, in sequence followed by surface regeneration with 20 mM NaOH to remove all bound proteins and subsequent pulsing with the same solutions of C7 and C6 over a surface bearing immobilized rC5-C345C. Times of exposure to C6, C7, and C8 are shown by dashed lines so labeled over the sensorgram plot. R notes the times the surface is exposed to 20 mM NaOH.

FIGURE 8. SPR sensograms showing the effects of pulsing C6 (solid line), C7 (dotted line), and C8 (dashed line), all at 50 \(\mu\)g/ml, over a surface bearing 1000 RU of immobilized C5. The pulse extends from time = 150 to 450 s, after which buffer is passed over the sensor chip and the complex dissociates.
ranging from 0 to 2.4 μM, over a sensor surface bearing immobilized C5; they show clearly that the module prevents both C6 and C7 from binding to C5 in a concentration-dependent manner. In a parallel experiment (data not shown), we found that rC3-C345C at 0.95 and 2.4 μM in 0.25 μM solutions of C6 and C7 had no discernible effect on binding of either protein to immobilized C5. Hence, inhibition is specific to the rC5 module. These results indicate that rC5-C345C and C5 compete for the same binding sites on C6 and C7.

Discussion

The C345C modules of C3, C4, and C5 are ~150-residue-long C-terminal segments that are unique to these members of the α-macroglobulin protein superfamily. To explore further the structures and activities of these domains, we expressed C345C from C3 and C5 in a bacterial system that supports cytoplasmic disulfide bond formation. With this expression system, rC3- and rC5-C345C were isolated as soluble proteins containing intramolecular disulfide bonds. Expression of C345C of C5 was particularly efficient, yielding up to 50 mg of highly purified protein per liter of cultured cells. The circular dichroism spectra of both recombinant products were consistent with the high β-sheet content that is predicted from homology with the modules from TIMPs and PCOLCE (16). We infer from the presence of disulfide bonding and the high β-sheet content that both recombinant modules have a native folded structure representative of the structure of the module in the intact parent C3 and C5 proteins.

To assess the binding capabilities of these recombinant modules, we tested for the ability to inhibit complement hemolytic activity and for direct binding to purified complement components using the SPR method. Protein-protein interactions can often be obstructed by a protein segment or peptide that corresponds to all or part of one of the protein-binding interfaces (43). Reasoning that interference with complement-mediated hemolysis could provide a broad screen of potential binding capacities, we tested rC3- and rC5-C345C for the ability to inhibit hemolysis by the classical complement pathway. As shown in Fig. 3, rC3-C345C showed no inhibitory activity. SPR assays likewise did not detect binding of rC3-C345C to any of the complement components tested: C3, C3b, C4, C4b, C5, C5b, C6, C7, C8, and factor I. It is possible that rC3-C345C showed no activity because the recombinant product is improperly folded; however, the same criteria were used to infer proper folding of the modules from both C3 and C5. Confirmation of the structures of these recombinant modules will come in part from ongoing high resolution nuclear magnetic resonance studies, but identity with the native structures cannot be certain in the absence of three-dimensional structures of the intact proteins themselves or of the modules excised from the native proteins.

Our failure to detect any inhibitory or binding activities with rC3-C345C is perhaps not surprising, because all of the known binding sites on C3 (many reviewed in Ref. 4) are quite distant from C345C in the amino acid sequence. These include binding sites for: 1) receptors C3aR, CR1 (44), CR2 (45, 46), and CR3 (47); 2) regulatory proteins and cofactors H (44), membrane cofactor protein (48), and properdin (4); and 3) associated subunits of the classical and alternative pathway convertases C4 (49) and factor B (47, 48). In addition, the proteolytic cleavage sites that activate and inactivate C3 (25) all lie away from the C345C module. We previously found that some mutations in the C5-C345C region do substantially affect sp. act., but the same mutations also affect expression of the protein in a mammalian cell culture system (50). Hence, C3-C345C may not be involved in direct binding interactions with other proteins of the complement pathways.

In contrast with the C3 module, rC5-C345C displayed inhibitory and binding activities that indicate an important function for this segment of C5. It inhibited erythrocyte lysis with an IC50 of 1 μM, and SPR assays showed strong, specific binding to C6 and C7 at distinct binding sites. We previously reported that a 13-aa-residue-long peptide corresponding to a segment of C5-C345C (dashed underlined in Fig. 1) inhibits complement hemolytic activity, apparently by obstructing binding of C5 to the C5 convertase, as it prevented proteolytic activation of C5 (22, 23). This peptide exhibits an IC50 of 10 μM (originally reported incorrectly as 100 μM (22)), so rC5-C345C is a much more potent inhibitor. We did not find evidence of rC5-C345C binding to the noncatalytic subunits of the C5 convertase, C3b or C4b, but interactions between the isolated subunits and the module may be too weak to be detected by SPR. The earlier peptide and the current SPR results suggest that C5-C345C harbors three distinct binding sites: for the convertase, C6, and C7. Therefore, the high inhibitory activity of rC5-C345C may be due to an ability to interfere with three different binding reactions. Identifying the individual binding sites in C5-C345C will help to resolve this issue.

The binding of C5 and rC5-C345C to C6 and C7 that we observed by SPR closely parallels results reported 30 years ago by Arroyave and Müller-Eberhard (39), who used sucrose density-gradient ultracentrifugation to demonstrate that: 1) C5 binds to
both C6 and C7 in isolation; 2) C5 binds preferentially to C7; and 
3) C6 appears to enhance the association of C5 and C7. More 
recently, DiScipio (5) used a solid-phase assay involving C5 co-
valently linked to yeast cell walls and similarly found that C6 and 
C7, but not C8, bind to the immobilized C5. The strong similarities 
among the ultracentrifugation, solid-phase, and SPR results sug-
gest that all three methods are detecting the same reversible bind-
ing of C5 to C6 and C7. Furthermore, the observations that rC5-
C345C, but not rC3-C345C, inhibits binding of C5 to C6 and C7, 
and that C5 and rC5-C345C bind to C6 and C7 with very similar 
features (Figs. 4 and 8, and 7 and 9) together provide strong evi-
dence that within C5, the C345C domain binds directly to C6 and 
C7 and mediates the reversible interactions.

Within C6, the region responsible for reversible binding to C5 
has been localized to the two ~70-residue-long factor I modules 
(FIMs (51); also known as FIMAC modules (52), for factor I mem-
brane attack complex) at the C-terminal end of the protein. Using 
the solid-phase method described earlier, DiScipio (5) showed that 
immobilized C5 binds equally well to intact C6, and a C-terminal 
peptide of C6 containing only the two FIMs. He further showed that 
truncated rC6 molecules lacking the FIMs retained almost none of 
the reversible C5-binding activity displayed by intact C6 (40). Be-
cause reversible binding between C5 and C6 is mediated by the 
C345C module in C5, and by the FIMs in C6, we conclude that this 
interaction involves direct contact between the FIM and C345C 
domains. We infer that this is probably the case with C7 as well, 
because it has a domain structure very similar to that of C6 (51). 
This suggests that C345C domains might in general bind to FIMs 
and therefore that C3-C345C might bind to the FIMs in factor I. 
However, our SPR studies failed to detect binding of either rC3- 
or rC5-C345C to factor I. Therefore, either the interaction between 
C3 and factor I is not mediated by an interaction between the 
C345C and FIM domains in the two proteins, or binding of factor 
I to the isolated rC3-C345C domain immobilized on a sensor chip 
is too weak to be detected by SPR.

The reversible interaction discussed in this work between C5 
and C6 is distinct from the interaction that occurs between Cs5b 
and C6 within the MAC. The latter interaction is very stable, irre-
versible, and takes place only between C6 and a metastable form of 
newly activated C5, referred to as Cs5b* (30, 53). In addition, the 
FIMs in C6 are essential for the reversible interaction with C5, but 
not for C6 hemolytic or bactericidal activity (54), and they are not 
important for the stability of the irreversible Cs5b,6 complex (40). 
Therefore, different regions of C6 mediate the reversible reaction 
with C5 and the nonreversible reaction with Cs5b* that leads to 
MAC formation. The similarities shared by C6 and C7 again make 
it likely that the interactions between Cs5b,6 and C7 that occur in 
the MAC are also distinct from those responsible for reversible 
binding of C5 to C7.

Reversible binding of C5 to C8 has been observed using sucrose 
gradient ultracentrifugation (6, 41), but the binding mechanism is 
probably distinct from the interactions with C6 and C7, because 
neither we nor DiScipio et al. (30) detected binding of C5 to C8 
using solid-phase methods. This view is consistent with the notion 
that reversible binding to C6 and C7 is mediated by C5-C345C and 
that this domain binds directly to FIMs because C8 does not carry 
this module (51). C8 also appears to use the same site on its β-sub-
unit both for reversible binding to C5 and nonreversible binding to 
Cs5b within the nascent MAC (6), whereas these sites are distinct 
in C6, as discussed above.

Although the reversible binding of C5 to C6 and C7 appears to 
volve interactions that are distinct from those that form the MAC 
itself, it is consistent with the notion that the proteins of the MAC 
exist, in their native form, stereochemical affinity for each other 
(41). It has been speculated that the reversible interaction between 
C5 and C6 facilitates formation of the Cs5b,6 complex (40) and 
thereby enhances complement function. How this happens is un-
clear, however.

One possibility is that the reversible interactions promote for-
mation of Cs5b,6 and Cs5b,6,7 simply by increasing the local con-
centrations of C6 and C7 near C5. This assumes that preactivation 
complexes between C5 and C6 or C7 exist in plasma, however, 
and there is little evidence that this is the case. The nanomolar 
dissociation constants measured in this study for these reactions do 
suggest that most of the C5 in plasma should be complexed with 
C6 or C7, but the strengths of these interactions in plasma are 
doubtless lower than those measured in this study with a solid-
phase assay and purified components. Moreover, DiScipio et al. 
(40) measured a much higher dissociation constant of 0.11 
μM for the reaction between C5 and C6 using a solid-phase assay with C5 
immobilized on polystyrene (the published value of 1.1 μM for the 
binding reaction has been revised because it was miscalculated (R. 
DiScipio, personal communication)). Nevertheless, even with this 
much higher dissociation constant, almost 90% of the C5 in plasma 
would be complexed with C6 or C7. Kolb et al. have observed 
complexes of C5, C6, C7, C8, and C9 in whole human, rabbit, and 
guinea pig sera, by sucrose density-gradient ultracentrifugation 
(41). Although those complexes were observed only at a subphysi-
ological ionic strength of 0.05 and at 4°C, we estimate that the 
dissociation constants in this experiment must have been 25 nM or 
less, because the initial concentrations of individual components 
were ~0.5 μM and all detectable C5 activity migrated as higher 
molecular mass complexes even after enduring the dissociative 
effects of sample dilution and differential sedimentation of free and 
bound C5. Therefore, these centrifugation results are consistent 
with the idea that C5 is associated with C6 or C7 in plasma, but 
with affinities too low for the complexes to be detected under phys-
iological conditions. They would be consistent, for example, with 
a dissociation constant in plasma of 250 nM, which is probably too 
low to detect the complexes by sucrose density-gradient ultracen-
trifugation, but high enough that almost 70% of the C5 would be 
associated with C6 or C7. Based on the available information, we 
believe it is likely that most of the C5 in plasma is associated with 
either C6 or C7 before activation.

Do preactivation complexes have a biological function? Revers-
ible interactions between C5 and C6 or C7 would increase the local 
centrations of C6 and C7 near newly activated Cs5b*, and may 
thereby lower the threshold for MAC formation in plasma by in-
creasing the proportion of Cs5b* that goes on to assemble the
MAC. Such a mechanism would be more important in situations in which the density of C5 convertases on the target surface is low or formation of C5b,6 or C5b,6,7 complexes is relatively inefficient. How much this added level of efficiency contributes to human health is unclear, because individuals with a subtotal C6 deficiency possess a truncated C6 that lacks FIMs and is present at only 1–2% of the normal C6 level, yet they do not appear to have increased susceptibility to infections (54). In addition, the consequences of deficiencies of MAC components in humans are in general relatively mild. In contrast, the substantial nonhepatic synthesis of C7 by monocytes and macrophages at sites of inflammation suggests that efficient MAC formation is important to the host, because C7 is often the limiting factor in this process (55). Indeed, that the C7 is often limiting may explain the stronger reversible binding of C5 to C7 than C6 that we observed. Although the value of MAC formation in host defense may be uncertain, it is clearly a major factor in complement-induced pathology (56). Preactivation complexes may exacerbate these conditions by facilitating MAC formation. If this is the case, deliberate disruption of these complexes in plasma (by introduction of small molecule inhibitors that mimic binding sites on C5-C345C, for example) might attenuate and allow fine tuning of MAC formation in situations in which it causes injury. A better understanding of the reversible binding of C5 to C6 and C7 may provide a novel route to controlling complement activity.

In summary, the present work presents evidence that the C5-C345C module of C5 binds to C6 and C7 at what appear to be independent, but neighboring or overlapping binding sites. Evidence is also presented that rC5-C345C and intact C5 compete for binding to C6 and C7 and that the features of these interactions are very similar. We conclude from these results that the C5-C345C module contains the binding sites for the previously reported reversible interactions of C5 with C6 and C7, and based on previous work by DiScipio et al. (40), that these interactions involve direct contact between C5-C345C and the FIMs in C6 and C7. In earlier work, we identified a recognition site for the classical pathway C5 convertase in the C5-C345C module (23). Therefore, this module appears to display binding sites for at least three different proteins, and to provide a focal point for protein-protein interactions before and in the initial phase of MAC formation.

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