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Recombinant C345C and Factor I Modules of Complement Components C5 and C7 Inhibit C7 Incorporation into the Complement Membrane Attack Complex¹

Chuong-Thu Thai and Ronald T. Ogata²

Complement component C5 binds to components C6 and C7 in reversible reactions that are distinct from the essentially nonreversible associations that form during assembly of the complement membrane attack complex (MAC). We previously reported that the ~150-aa residue C345C domain (also known as NTR) of C5 mediates these reversible reactions, and that the corresponding recombinant module (rC5-C345C) binds directly to the tandem pair of ~75-residue factor I modules from C7 (C7-FIMs). We suggested from these and other observations that binding of the C345C module of C5 to the FIMs of C7, but not C6, is also essential for MAC assembly itself. The present report describes a novel method for assembling a complex that appears to closely resemble the MAC on the sensor chip of a surface plasmon resonance instrument using the complement-reactive lysis mechanism. This method provides the ability to monitor individually the incorporation of C7, C8, and C9 into the complex. Using this method, we found that C7 binds to surface-bound C5b,6 with a K_d of ~3 pM, and that micromolar concentrations of either rC5-C345C or rC7-FIMs inhibit this early step in MAC formation. We also found that similar concentrations of either module inhibited complement-mediated erythrocyte lysis by both the reactive lysis and classical pathway mechanisms. These results demonstrate that the interaction between the C345C domain of C5 and the FIMs of C7, which mediates reversible binding of C5 to C7 in solution, also plays an essential role in MAC formation and complement lytic activity. *The Journal of Immunology*, 2005, 174: 6227–6232.

The complement membrane attack complex (MAC)³ is a large, membrane-bound protein complex that performs the cell lysis function of complement. It is assembled from five distinct soluble plasma proteins that are incorporated in a sequential process that begins with proteolytic activation of component C5 at the target cell surface. This generates a metastable activated C5b, which forms the nucleus for addition of single molecules of C6, C7, and C8 and, finally, multiple copies of C9 to yield the lytic MAC (reviewed in Refs. 1 and 2). C5b is a heterodimer with a M_r of 180 kDa, C8 is a heterotrimer with a M_r of 151 kDa, and C6, C7, and C9 are monomers with M_r of 120, 110, and 71 kDa, respectively (1). Although the broad outlines of MAC assembly are well established, less is known about the network of protein-protein interactions that maintain this very stable complex and even less about the interacting substructures and amino acid residues within each protein. An understanding of the roles of substructures is of particular interest, because, with the exception of

C5, all the MAC proteins have almost entirely modular structures. C6 is composed of 10 modules, whereas C7, the C8 α and β subunits, and C9 all resemble truncated forms of C6 with successively fewer modules (nine, five, five, and four, respectively) (3).

In previous work (4), we expressed the ~150-residue C345C domain (also known as NTR) of C5 (C5-C345C) in a novel bacterial system and found evidence that this module mediates the reversible binding of native C5 to both C6 and C7 that was described by Müller-Eberhard et al. (5) >30 years ago. These reversible interactions take place in solution between the latent, non-activated proteins and are distinct from the relatively nonreversible reactions that lead to MAC formation. Based on studies reported >10 years ago by DiSciopio (6), we inferred that within C6 and C7, the binding sites for C5 lie within the tandem pair of ~75-residue factor I modules (FIMs; also known as FIMAC modules) that are found at the C-terminal ends of both proteins, but are absent from C8 and C9. DiSciopio reported that a tryptic fragment of C6 composed only of its two FIMs, and a larger fragment of C7, composed of its FIMs and two ~60-residue short consensus repeats (also known as CCP modules), both bind specifically to C5. To confirm that the two C7-FIMs alone mediate binding to C5, we recently expressed these modules in the bacterial system used previously for the C345C domain and found that the recombinant C7-FIMs (rC7-FIMs) does bind specifically to native C5 as well as to the isolated recombinant C5-C345C module (rC5-C345C) with affinities similar to those of intact C7 (7).

The significance of reversible binding reactions among MAC proteins is uncertain. Müller-Eberhard et al. (5, 8) reasoned, from the limited number of such interactions, that they must result from topological relationships that reflect the arrangement of individual components within the stable, fully assembled MAC. The C345C/FIMs-mediated reversible interaction between C5 and C6 appears to be nonessential, however, because a truncated C6 variant responsible for clinical subtotal deficiency has no FIMs, but retains

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³ Abbreviations used in this paper: MAC, membrane attack complex of complement; C5b,6, the stable complex composed of complement components C5b and C6; C5b-7, the partially assembled MAC intermediate composed of C5b, C6, and C7; C5b-8, the partially assembled MAC intermediate composed of C5b, C6, C7, and C8; C345C, the C345C module, also known as NTR; FIM, factor I module, also known as FIMAC; GVB⁰, veronal-buffered saline with 0.1% gelatin; HBS-EP, 0.01 M HEPES (pH 7.4), 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant P20; RU, response unit in the SPR instrument; SPR, surface plasmon resonance.

bactericidal activity (9); a recombinant C6 engineered without the FIMs is also active, although somewhat disabled (10); and the common A form of mouse C6 also lacks FIMs, but is active (11). Reversible binding of C5 to C7 is stronger than that to C6, however, and C7 efficiently displaces C6 already bound to C5 (4, 5). Based on this and other indirect evidence, we suggested that in contrast with C6, the interaction of the C7-FIMs with C5-C345C is essential for MAC assembly and probably provides one of two or more essential contacts between C7 and C5b,6 (7).

To test this idea, we developed a cell-free method for assembling a complex that closely resembles the genuine MAC on the surface of the sensor chip of a surface plasmon resonance (SPR) instrument. With this method, we tested the ability of rC7-FIMs and rC5-C345C to block binding of C7 to surface-bound C5b,6. We found that both recombinant modules inhibit C7 uptake by C5b,6, and that they also block complement-mediated erythrocyte lysis by the classical pathway and reactive lysis (12, 13) mechanisms.

Materials and Methods

Reagents

Sheep erythrocytes and all human complement components and buffers were purchased from Advanced Research Technologies, including C5b,6, which, according to the supplier, was made from purified components by activating C5 in the presence of C6 with the cobra venom factor C3/C5 convertase, C5b,6, and isolated by gel filtration chromatography. The human C6-specific mouse mAb (catalogue no. A219) and the rabbit anti-mouse Ig polyclonal Ab (catalogue no. BR-1005-14) were purchased from Quidel and Biacore, respectively. Recombinant C3-C345C, C5-C345C, and C7-FIMs were expressed in bacteria and purified as previously described (4, 7).

Surface plasmon resonance

All measurements were conducted on a Biacore 3000 instrument, with reagents, buffers, and data analysis software from the same company. All experiments were performed at 25°C in 0.01 M HEPES (pH 7.4), 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant P20 (HBS-EP).

For MAC assembly, ~13,000 response units in the SPR instrument (RU) of the rabbit anti-mouse Ig Ab at 50 µg/ml was immobilized by amine coupling onto Biacore sensor chip CM5 as recommended by the supplier. Approximately 800 RU of the human C6-specific mAb was then captured by pulsing 50 µl of a 4 µg/ml solution of mAb in HBS-EP over the surface at 10 µl/min. Finally, ~500 RU of C5b,6 was captured with a 50-µl pulse of a 40 µg/ml solution at the same flow rate. Sensor chips were regenerated to remove the mAb from the amine-coupled rabbit Ab with 10 mM glycine-HCl, pH 1.7, again as recommended by the supplier. In all binding and competition experiments, analyte solutions were pulsed sequentially, first through a control flow cell bearing only the amine-coupled anti-mouse Ig Ab and then through the flow cell bearing mAb-captured C5b,6; the response from the control flow cell was subtracted from the binding sensorgram.

All binding and competition experiments used a flow rate of 40 µl/min. Inhibition of C7 binding was assessed by measuring the amount of C7 bound (in RU) in the presence of module, relative to the amount bound in the absence of module: relative binding = $(RU_m - RU_b)/(RU_0 - RU_b)$, where RU_m , RU_0 , and RU_b are the RU values displayed, respectively, by solutions containing C7 and a recombinant module, by solutions of C7 alone, and by buffer only pulsed over the sensor chip. All RU measurements were made at time = $t_b + 40$ s, where t_b is the time at which the pulse of solution containing C7 and the modules ends, and the flow of buffer begins. This time was selected to avoid the interfering signal caused by the recombinant domains themselves binding to immobilized C5b,6. The domains associate and dissociate very quickly, within a few seconds, whereas C7 dissociates very slowly ($t_{1/2}$, ~30 h; see *Results*). Therefore, at $t_b + 40$ s, the SPR response is essentially entirely due to bound C7. The C5b,6 on the sensor chip is removed along with the C6-specific mAb after each binding cycle; hence, the amount of C5b,6 captured varies for each cycle. This variation was usually 2–3% and was not >5% within each concentration series in binding/inhibition experiments. All data are the results of duplicate independent measurements.

Complement inhibition

Inhibition of the complement classical pathway was measured as previously described (4). Briefly, the recombinant module was added to a reaction mix of Ab-sensitized sheep erythrocytes and human serum at a series of concentrations as noted. Reactions were incubated for 30 min at 37°C, quenched with ice-cold veronal-buffered saline with 0.1% gelatin (GVB⁰), 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 the recombinant module. Hemolysis in the absence of module was ~20, 65, and 95% of the input sensitized erythrocytes in 0.3, 0.6, and 1% serum, respectively. All data are the results of duplicate independent measurements.

Inhibition of reactive lysis-mediated hemolysis (12, 13) was determined in duplicate independent measurements by incubating (for 20 min at 37°C) 10 µl of recombinant module in 10 mM Tris-HCl (pH 7.5)/150 mM NaCl (or buffer alone) with 35 µl of 10 µg/ml C5b,6 (final concentration, 4.4 µg/ml), 30 µl of 5×10^8 /ml sheep erythrocytes, and 5 µl of a solution containing equal amounts (by weight) of C7, C8, and C9, all in GVB⁰/10 mM EDTA. Reactions were quenched with 1 ml of ice-cold GVB⁰/10 mM EDTA and centrifuged to pellet intact erythrocytes, and the supernatants were assayed for A₄₁₂. Relative hemolysis was measured as described for classical pathway hemolysis. Approximately 20, 40, or 50% of the input erythrocytes was lysed when the C7/C8/C9 solution contained each of the components at 2, 4, or 6 µg/ml (final concentration, 0.125, 0.25, or 0.375 µg/ml), respectively.

Results

Formation of the MAC on an SPR sensor chip

Complex formation was based on the reactive lysis mechanism, which requires only the MAC proteins (12, 13). Reactive lysis is initiated by binding of C5b,6 to C7 in solution, followed by deposition of the heterotrimer onto a target surface. Subsequent addition of C8 and C9 yields the complete MAC. To reproduce this pathway, we immobilized C5b,6 on the SPR surface by first linking a mouse Ig-specific rabbit Ab to sensor chip CM5 by amine coupling, then using this to capture in sequence a human C6-specific mAb and C5b,6. A commercially available (Quidel) human C6-specific mAb was used for convenience, but any mAb that recognizes C5b or C6 within the membrane-bound MAC should work as well, for example, the human C5-specific mAb 558 (14).

The SPR traces in Fig. 1 show the effects of pulsing in order C9, C8, C7, C9, C8, and C9 over immobilized C5b,6. The two traces illustrate the concentration dependence of the reactions; C7, C8, and C9 are all at ~50 µg/ml in plasma (1). Note that only C7 binds to C5b,6, whereas C8 binds only to C5b-7, and C9 binds primarily to C5b-8. The SPR response is directly proportional to the mass of protein bound to the sensor chip, and adjusting for the molecular

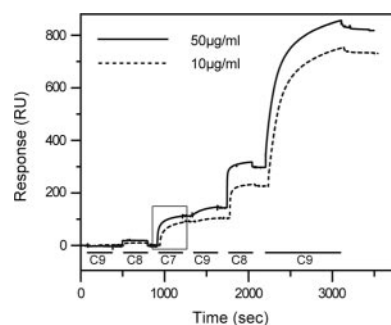


FIGURE 1. Formation of the MAC-like complex on an SPR sensor chip. This SPR sensorgram shows the effects of pulsing solutions of C7, C8, or C9 at 50 µg/ml (solid line) and 10 µg/ml (dashed line), at the times indicated under the sensorgram trace, over C5b,6 immobilized on the surface of the sensor chip. The binding stoichiometry was 1.6:1:1.1:9 at 10 µg/ml and 1.5:1:1.1:8 at 50 µg/ml; 50 µg/ml is the approximate concentration of C7, C8, and C9 in plasma. The boxed region traces the binding of C7 to C5b,6, which is the focus of this report.

weight of each protein, the molar ratios of C5b,6:C7:C8:C9 in the final complex were approximately 1.5:1:1.1:8, respectively, consistent with the composition of the MAC (1, 2). The ratio of C5b,6 to C7 varied from 1.3 to 1.6 in five experiments; we infer that it is >1 at least in part because the C5b,6 is not fully active (the specific activity of C5b,6 is very sensitive to freeze-thaw cycles according to the supplier). The same ratios were found when only C7, C8, and C9 were pulsed in order over immobilized C5b,6.

Fig. 1 shows a small amount of C9 binding to C5b-7 that is apparently specific to the heterotrimer, because it is not seen with C5b,6. This is probably not due to contamination, because the C9 preparation contained no detectable C8 according to the supplier. It is a relatively small effect that amounts to only a fraction of the level of C9 that binds to C5b-8: $\sim 2\%$ at $10 \mu\text{g/ml}$ and 5% at $50 \mu\text{g/ml}$. The amount of C8 that binds to C5b-7 is the same when C8 is added directly to the heterotrimer or after pulsing with C9. Therefore, the low level of C9 binding to C5b-7 does not affect binding of C8 and is probably an artifact that is not important for complement function or MAC assembly.

C7 reinforces a labile association with the sensor chip surface

As shown in Fig. 2, the SPR signal from a surface bearing immobilized C5b,6 decreases slowly at $\sim 1 \text{ RU/min}$ as buffer is passed over the sensor chip. This implies that protein is slowly released from the chip surface. C6 alone bound to the mAb showed the same slow loss of RU (Fig. 2), but there was no loss of signal with only the C6-specific mAb bound to the mouse Ig-specific Ab (not shown). Therefore, the decrease in signal seen with immobilized C5b,6 is due to dissociation of the entire heterodimer from the chip, presumably because of a relatively weak bond between C6 and the C6-specific mAb.

Fig. 2 also shows that in contrast to C5b,6, the SPR response from the heterotrimer C5b-7 is stable, indicating that it is more firmly attached to the sensor chip. Any dissociation of the heterotrimer from the chip would result in an even greater loss of RU, because the response is proportional to the mass of the bound protein, and C5b-7 is $\sim 30\%$ larger than C5b,6. This increased stability could be caused by a conformational change induced by incorporation of C7 that results in a more stable bond between C6 and the mAb. Another possibility is that C7 is itself binding to the chip surface. During MAC formation on a biological membrane, binding to C5b,6 induces a conformational change in C7 that generates a membrane binding site and gives the nascent MAC its first firm hold on the target surface (1); a similar process may be oc-

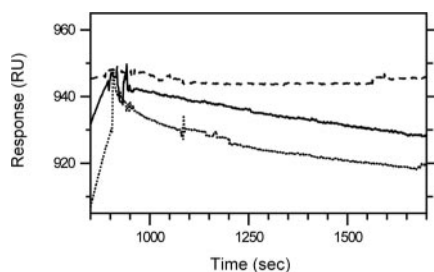


FIGURE 2. C6 dissociates slowly from the SPR chip surface; C5b-7 is bound stably. These sensorgrams show the signal from a sensor chip bearing the C6-specific mAb bound to C6 (solid line), C5b,6 (dotted line), and C5b-7 (dashed line) as HBS-EP is passed over the chip surface at $10 \mu\text{l/min}$. The absolute time and response are arbitrary; sensorgrams were shifted to align the points marking termination of the pulses of C6, C5b,6, and C7 that yielded immobilized C6, C5b,6, and C5b-7. Levels of 212, 400, and 550 RU of C6, C5b,6, and C5b-7, respectively, were immobilized initially on the sensor surface.

curing on the chip surface. C5b-7 binds to agarose (12) and may bind similarly to the dextran matrix on the SPR chip.

C7 binds to C5b,6 immobilized on the sensor chip with a K_d of $\sim 3 \text{ pM}$

Fig. 3 shows SPR sensorgrams tracing the binding of C7 at different concentrations to immobilized C5b,6. In these experiments, $\sim 100 \text{ RU}$ of C7 binds at saturation to the immobilized C5b,6 (see boxed area in Fig. 1). Note again the steady loss of response with buffer alone flowing over the sensor chip. Curve fitting of these data yielded for this reaction, $K_d = 3 \text{ pM}$, $k_{\text{on}} = 2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, and $k_{\text{off}} = 5 \times 10^{-6} \text{ s}^{-1}$. These values agree quite well with the previously reported (15) $K_d = 0.2\text{--}2 \text{ pM}$ at 37°C and $k_{\text{on}} = 8 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (our extrapolation to 25°C from their data) for this reaction in solution. This affinity applies to the essentially nonreversible interaction between C5b,6 and C7 that takes place during MAC formation. It is ~ 30 times greater than the reversible affinity of C5 for C7 previously described (5, 7). The accuracy of our measurement is limited by the very slow off-rate measured in this study, which at a half-life of 39 h makes model fitting problematic, and by dissociation of C5b,6 from the chip during the measurement, as shown in Fig. 2. The inhibition experiments described below do not depend on having an accurate measurement of the affinity of C7 for immobilized C5b,6, however, only that this interaction on the SPR chip be representative of the same interaction within a genuine MAC forming on a target surface.

Recombinant C5-C345C and rC7-FIMs bind to C5b,6 with K_d of $\sim 3 \mu\text{M}$

We expect from our previous work that rC5-C345C and rC7-FIMs should also bind to immobilized C5b,6 by interacting reversibly with the FIMs in C6 and the C345C domain in C5b, respectively. This is confirmed by the results presented in Fig. 4, which show SPR sensorgrams tracing the binding of rC5-C345C and rC7-FIMs to immobilized C5b,6. Note the very low total SPR responses of only 5–10 RU, and the very fast on- and off-rates, indicated by the rapid increases and decreases in signal at the beginning and end of the pulses. The SPR response is directly proportional to the molecular mass of the binding analyte, and therefore under our conditions, where 100 RU of C7 bind at saturation, a maximum of only 15 RU of either module is expected to bind to the chip. The low signals and affinities make quantitation difficult, but assuming that binding is at equilibrium at each module concentration, we estimate from simple equilibrium binding (Scatchard) plots of these and replicate data (not shown) that the dissociation constants for these binding reactions are $\sim 3 \mu\text{M}$ for both modules. As with

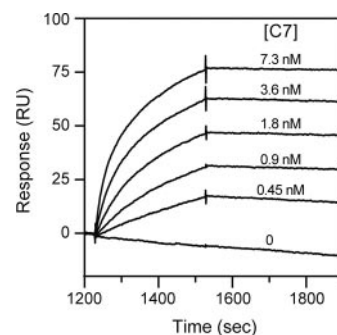


FIGURE 3. Binding of C7 to immobilized C5b,6. These representative sensorgrams show the effects of pulsing C7 at the concentrations shown from 1250 to 1550 s over a surface bearing $\sim 500 \text{ RU}$ of immobilized C5b,6. The theoretical global fit of these responses gave $K_d = 3 \text{ pM}$, $k_{\text{on}} = 2 \times 10^6$, and $k_{\text{off}} = 5 \times 10^{-6}$, with $\text{RU}_{\text{max}} = 75$ and $\chi^2 = 4.3$.

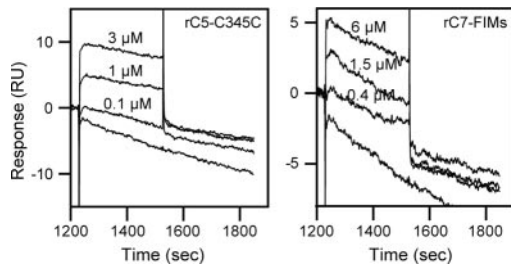


FIGURE 4. Recombinant C5-C345C and rC7-FIMs bind to immobilized C5b,6. These representative sensorgrams show the effects of pulsing rC5-C345C (*left panel*) and rC7-FIMs (*right panel*) from 1250 to 1550 s over a sensor chip bearing ~ 500 RU of immobilized C5b,6 (which binds ~ 100 RU of C7). Note that the difference in scale causes the loss of signal to appear faster with rC7-FIMs, and that the absolute responses for both modules are quite small because of the low mass of the recombinant modules. For equimolar binding, and accounting for the $\sim 65\%$ efficiency of C7 binding, a maximum of ~ 15 RU of either module would be expected to bind to 500 RU of C5b,6.

C7 binding described above, the inhibition studies described below do not depend on having accurate measurements of the affinities of the recombinant modules for immobilized C5b,6.

Although these results are consistent with the idea that rC5-C345C binds to the C6 FIMs within immobilized C5b,6, the mAb used to immobilize C5b,6 to the sensor chip recognizes a site that lies somewhere within the last 65-aa residues of the C-terminal-most FIM in C6 (9). This indicates that the two FIMs in C6 can accommodate both the mAb and rC5-C345C. Both recombinant modules bind more weakly to C5b,6 than to the isolated proteins themselves; the affinity of rC5-C345C for C6 and that of rC7-FIMs for C5 are 40- to 200-fold higher than those for binding to immobilized C5b,6 (4, 7). We assume that the weaker binding of the recombinant modules to immobilized C5b,6 is due primarily to competition from the high local concentrations of the C345C module in C5b and the C6-FIMs, respectively, within the heterodimer.

Recombinant C5-C345C and rC7-FIMs inhibit uptake of C7 by immobilized C5b,6

The sensorgrams in Fig. 5 show the effects of rC5-C345C and rC7-FIMs on binding of C7 to immobilized C5b,6. Note that sensorgrams monitoring binding in the presence of the recombinant

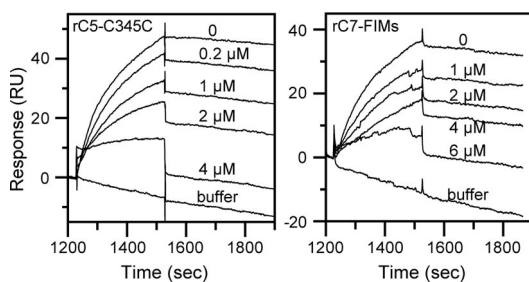


FIGURE 5. Recombinant C5-C345C and rC7-FIMs inhibit binding of C7 to immobilized C5b,6. These sensorgrams show the effects of rC5-C345C (*left panel*) and rC7-FIMs (*right panel*) on C7 binding to immobilized C5b,6. Solutions containing C7 at 3 nM ($0.3 \mu\text{g/ml}$) and the recombinant modules at the concentrations listed adjacent to each sensorgram trace were pulsed over immobilized C5b,6 from 1225 to 1525 s. Relative amounts of C7 binding were assessed at about 1560 s to avoid interference from the modules themselves binding to C5b,6, as described in *Materials and Methods*. The SPR responses continue to decrease even after the pulse, because at 3 nM, C7 associates with slightly $<50\%$ of the available immobilized C5b,6.

modules show two components. The first resembles the sensorgrams in Fig. 3 and is due to C7 binding to C5b,6. The second resembles the sensorgrams in Fig. 4 and is due to reversible binding of the recombinant modules to the immobilized heterodimer. Note also that the second component increases with increasing module concentrations, whereas the first component decreases. The SPR response due to C7 binding is lower in this study than the maximum of 100 RU mentioned above, because the total C7 concentration in this experiment is much lower, at $0.3 \mu\text{g/ml}$, than the $50 \mu\text{g/ml}$ used in Fig. 1. These results indicate that both recombinant modules inhibit binding of C7 to C5b,6 and are effective at similar concentrations, as might be expected. We assume that rC5-C345C and rC7-FIMs block binding of C7 to immobilized C5b,6 by binding to C7 in solution and to C5b in immobilized C5b,6, respectively. Therefore, the binding of rC7-FIMs to immobilized C5b,6 is directly responsible for blocking C7 uptake, whereas binding of rC5-C345C to immobilized C5b,6 at the C6-FIMs only reflects the nonessential association with C6.

Inhibition is specific to rC5-C345C and rC7-FIMs

In a parallel experiment (not shown), the homologous recombinant C345C domain from C3 (rC3-C345C), which does not bind C6 or C7 (4), showed no evidence of binding to immobilized C5b,6 (none of the second component); at a concentration of $3 \mu\text{M}$, it decreased C7 binding only slightly, by $<10\%$, at a total C7 concentration of 1.5 nM, the lowest and most sensitive level. In contrast, the homologous domain from C5 inhibited C7 binding by $\sim 90\%$ under the same conditions. Fig. 6 summarizes the inhibitory effects of rC5-C345C and rC7-FIMs at total C7 concentrations of 1.5, 3, and 5 nM, which correspond to the C7 concentrations in 0.3, 0.6, and 1% serum, respectively (16). These concentrations were chosen to correspond to the serum levels used in the inhibition experiments described below.

Recombinant C5-C345C and rC7-FIMs inhibit classical pathway- and reactive lysis-mediated hemolysis

Figs. 7 and 8 illustrate the inhibitory effects of rC5-C345C and rC7-FIMs on hemolysis mediated by the complement classical pathway and reactive lysis mechanisms. Assay conditions were chosen to give approximately the same range of C7 concentrations as those used in the binding inhibition experiments described above. Classical pathway inhibition was measured in 0.3, 0.6, and 1% serum, whereas inhibition of the reactive lysis mechanism was measured with C7 at 0.125, 0.25, and $0.375 \mu\text{g/ml}$, which correspond to the C7 concentrations in 0.2, 0.4, and 0.7% serum.

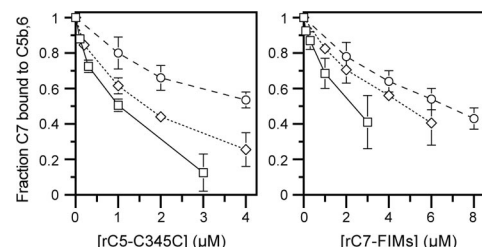


FIGURE 6. Summary of inhibition of C7 binding to immobilized C5b,6 by recombinant modules. These graphs show the effects of rC5-C345C (*left panel*) and rC7-FIMs (*right panel*) on binding of C7 to immobilized C5b,6 at total C7 concentrations of 1.5 nM (solid lines), 3 nM (dotted lines), and 5 nM (dashed lines). Relative binding is the amount of C7 bound (in RU) in the presence of recombinant module relative to the amount bound in the absence of module.

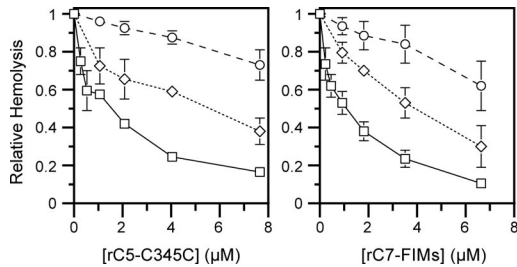


FIGURE 7. Inhibition of classical pathway hemolytic activity. These graphs show the effects of rC5-C345C (*left panel*) and rC7-FIMs (*right panel*) on classical pathway-mediated erythrocyte lysis at serum concentrations of 0.3% (solid lines), 0.6% (dotted lines), and 1% (dashed lines). Relative hemolysis is the amount of erythrocyte lysis relative to lysis in the absence of the recombinant module.

Discussion

The aim of the present study was to test the idea that the interaction between the C345C domain in C5b and the FIM domains in C7 is essential for assembly of the complement lytic complex. For this, we tested the abilities of the corresponding recombinant modules from C5 and C7 to specifically inhibit the interaction between C5b,6 and C7 during MAC formation, and their abilities to inhibit complement-mediated erythrocyte lysis; these assays tested for the ability to inhibit that precise step in MAC assembly and the authentic lytic process. We reasoned that if the interaction between C345C and the FIMs in C5 and C7, respectively, is essential for MAC assembly and complement lysis, then rC5-C345C and rC7-FIMs should competitively inhibit C7 uptake by C5b,6 and complement-mediated cell lysis by binding to the FIMs in native C7 and the C345C domain in C5b,6, respectively.

To observe C7 binding to C5b,6 directly, we developed a novel cell-free method for assembling a complex that appears to be very similar to the genuine MAC. The specific order and relative stoichiometries of C7, C8, and C9 binding to C5b,6 immobilized on an SPR sensor chip provide strong evidence that essentially all the protein-protein interactions and conformational changes necessary for MAC assembly on a target cell surface are duplicated on the chip. This implies that a structure very similar to the genuine MAC is formed on the chip. The SPR chip surface is not a cell membrane, however, and hence, the complex on the chip cannot represent the exact MAC structure; indeed, it may more closely resemble the soluble form of the terminal complement complex, SC5b-9. However, SC5b-9 contains one or two molecules of S protein and clusterin and only two to four molecules of C9 (1, 2). In contrast, the complex on the SPR chip does not contain S protein or clusterin, because it was assembled from purified proteins, and it has 8 or 9 copies of C9, suggesting the possibility of some degree of C9 polymerization (1). These features indicate that the complex on the SPR chip more closely resembles the MAC than SC5b-9; this is certainly the case with respect to specific protein-protein interactions. Apparently, immobilization on the chip surface allows a more MAC-like structure with a higher C9 content to form by physically preventing the aggregation of intermediates in the assembly process and of the MAC itself that ordinarily occurs in the fluid phase (15, 17, 18). The inhibition results presented in Figs. 5 and 6 are valid in either case, because they focus only on the binding of C7 to C5b,6, which takes place before a distinction is made between the structures of the soluble and membrane-bound complexes.

The present results provide strong evidence that binding of the FIMs in C7 to the C345C domain in C5 is essential for incorporation of C7 into C5b,6 and hence for successful MAC assembly.

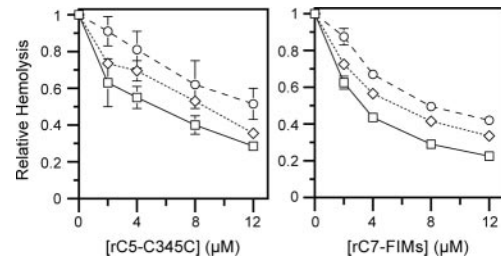


FIGURE 8. Inhibition of reactive lysis. These graphs show the effects of rC5-C345C (*left panel*) and rC7-FIMs (*right panel*) on hemolysis by the reactive lysis mechanism with C5b,6 at a concentration of 4.4 μg/ml and C7 at concentrations of 0.125 μg/ml (solid lines), 0.25 μg/ml (dotted lines), and 0.375 μg/ml (dashed lines). Relative hemolysis is defined in Fig. 7.

We found that micromolar concentrations of both rC5-C345C and rC7-FIMs inhibited binding of C7 to surface-bound C5b,6. Similar concentrations of the recombinant domains inhibited complement hemolysis by two distinct assays as well, with one involving MAC components alone. Therefore, recombinant domains harboring complementary binding sites showed almost the same inhibitory activity and dependence on C7 concentration in three different protocols, including two that involve authentic complement-mediated cell lysis.

These results may also provide an explanation for the highly asymmetric affinities we previously measured for the reversible binding reaction between C5 and C7 (7) and perhaps some insights into the conformational changes that take place in C6 and C7 during MAC formation. Using SPR, we previously found that the affinity of C5 for C7 measured with C5 immobilized is several hundred times higher than that in the reverse orientation, with C7 immobilized on the sensor chip (7). The results in Fig. 2 suggest that when C7 binds to immobilized C5b,6, it may also bind to the sensor chip itself. If binding to immobilized C5 alone facilitates a similar interaction with the chip, then this explains the much higher affinity we observed in this orientation. Although this is an attractive idea, C6 also has a very high affinity for immobilized C5, only several-fold lower than C7 (4). Therefore, the similar high affinities for immobilized C5 imply that both C6 and C7 bind to the sensor chip. Unfortunately, C6 immobilized by either amine coupling or Ab capture does not bind C5, so we have not been able to determine the affinity in the reverse orientation. C6 is not known to bind to membranes, but the work of Whitlow and colleagues (19, 20) has emphasized the importance of ionic interactions between C5b,6 and anions on target membrane surfaces. Therefore, C6 may bind through ionic interactions to the negatively charged carboxymethyl dextran matrix on the chip. It may be that binding to C5 or C5b facilitates similar conformational changes in C6 and C7 that expose anion and membrane binding sites, respectively.

In summary, the results obtained in this study support the idea that although the FIMs in both C6 and C7 mediate reversible binding to C5, bound C6 is easily displaced by C7, and only the reaction with the C7 FIMs is important for MAC assembly. They also affirm the early suggestion that the reversible binding reactions among the five MAC components can provide insights into the protein-protein interactions that take place within the MAC itself (5, 8).

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Disclosures

The authors have no financial conflict of interest.

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