Binding of Human Complement C8 to C9: Role of the N-Terminal Modules in the C8α Subunit†

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ABSTRACT: Human C8 is one of five components of the membrane attack complex of complement (MAC). It is composed of a disulfide-linked C8α-γ heterodimer and a noncovalently associated C8β chain. The C8α and C8β subunits contain a pair of N-terminal modules [thrombospondin type 1 (TSP1) + low-density lipoprotein receptor class A (LDLRA)] and a pair of C-terminal modules [epidermal growth factor (EGF) + TSP1]. The middle segment of each protein is referred to as the membrane attack complex/perforin domain (MACPF). During MAC formation, C8α mediates binding and self-polymerization of C9 to form a pore-like structure on the membrane of target cells. In this study, the portion of C8α involved in binding C9 was identified using recombinant C8α constructs in which the N- and/or C-terminal modules were either exchanged with those from C8β or deleted. Those constructs containing the C8α N-terminal TSP1 or LDLRA module together with the C8α MACPF domain retained the ability to bind C9 and express C8 hemolytic activity. By contrast, those containing the C8α MACPF domain alone or the C8α MACPF domain and C8α C-terminal modules lost this ability. These results indicate that both N-terminal modules in C8α have a role in forming the principal binding site for C9 and that binding may be dependent on a cooperative interaction between these modules and the C8α MACPF domain.

Human C8 is one of five components (C5b, C6, C7, C8, and C9) that interact to form the cytolytic membrane attack complex of complement (*1*, *2*). It is composed of an α (64 kDa), β (64 kDa), and γ (22 kDa) subunit, each of which are products of different genes (*3*, *4*). Within C8, the subunits are arranged as a disulfide-linked C8α-γ heterodimer that is noncovalently associated with C8β. C8α and C8β are homologous and together with C6, C7, and C9 form the MAC[1] family of proteins (*5*, *6*). Both contain a pair of tandemly arranged N-terminal modules (TSP1 + LDLRA) and a pair of C-terminal modules (EGF + TSP1). The intervening segment is referred to as the MACPF domain because of its sequence similarity to other family members and the corresponding region of the pore-forming protein perforin. The C8γ subunit is unrelated and is a member of the lipocalin family of proteins that bind small, hydrophobic ligands (*7*−*9*).

Assembly of the MAC involves highly specific and sequential interactions; therefore, each component must contain a specific binding site for the succeeding one incorporated into the complex (*1*, *6*). For C8, this laboratory has identified several binding sites that are involved in subunit interactions and the formation and function of the MAC. Binding of C8α-γ to C8β involves a site located within the MACPF domain of C8α (*10*). This domain also contains a segment that mediates intracellular binding to C8γ (*10*, *11*) and a segment recognized by CD59, the membrane-associated regulatory protein that binds C8α and inhibits formation of a functional MAC on homologous cells (*12*). In addition, C8α provides the binding site for C9 and therefore has an essential role in formation of the MAC (*13*). C8β contains at least two binding sites: one mediates the interaction with C8α-γ (*3*) and a second mediates C8 incorporation into the MAC (*14*). Binding to C8α-γ was recently shown to be dependent on a cooperative interaction between the N-terminal TSP1 module of C8β and the C8β MACPF domain (*15*).

The aim of the present study was to identify those segments of C8α which are essential for binding C9. Chimeric forms of C8α in which the modules were exchanged for those in C8β and truncated forms in which modules were deleted were prepared and characterized for their ability to bind C9 and form a functional MAC. Results indicate that both N-terminal modules in C8α have a role in binding C9, and either one together with the C8α MACPF domain is sufficient to form a binding site.

EXPERIMENTAL PROCEDURES

Materials. Human C8 and C9 were purified from plasma fraction III (Bayer Corp., Clayton, NC) as described (*16*). C8α-γ and C8β were separated by gel filtration in high ionic

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[1] Abbreviations: MAC, membrane attack complex; TSP1, thrombospondin type 1; LDLRA, low-density lipoprotein receptor class A; EGF, epidermal growth factor; MACPF, membrane attack complex/perforin; wt, wild type; EAC1−7, sensitized sheep erythrocytes carrying human complement C1−C7. Module abbreviations follow recommendations by the 1994 International Workshop on Sequence, Structure, Function and Evolution of Extracellular Protein Modules (available in ref *19* and at SWISS-PROT).

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strength buffer and the molar concentrations determined from published extinction coefficients (*17*). Goat anti-human C8α-γ, C8α, C8β, and C8γ antibodies and rabbit anti-human C8α and C8γ antibodies were affinity purified as described (*10*).

Expression Constructs. wtC8α and wtC8γ cDNA constructs, chimeras C8αc1, C8αc2, and C8αc3, and truncated constructs C8αt1, C8αt2, and C8αt3 were prepared in the COS cell expression vector pcDNA3 (Invitrogen) as described elsewhere (*10*).

Chimera C8αc4 was prepared from C8αc6. To produce C8αc6, a chimeric primer was used in PCR to generate a fragment containing residues 59−65 of C8β and 64−554 of C8α. In a separate reaction, a fragment containing the leader sequence and residues 1−65 of C8β was generated. In second round PCR, these self-annealing fragments were combined and used to generate a chimeric fragment encoding the leader sequence, residues 1−65 of C8β, and residues 64−554 of C8α. This fragment was digested with *Hin*dIII/*Cla*I and exchanged for the corresponding fragment in C8αc2 to produce C8αc6. A *Mfe*I/*Cla*I fragment from C8αc6 was then exchanged with one in C8αc3 to create C8αc4.

To prepare C8αt4, PCR was used to produce a fragment that contained a portion of the leader sequence and residues 64−554 of C8α. A second fragment encoding the 5′ UTR of C8α and the remaining portion of the leader sequence was generated. These self-annealing fragments were combined in second round PCR to generate a fragment containing the entire C8α leader sequence and residues 64−554 of C8α. This product was digested with *Hin*dIII/*Cla*I, and the released fragment was subcloned into C8αt3 that had been digested with the same enzyme.

C8αc5 and C8αt5 were prepared from C8αc7. Preparation of C8αc7 followed procedures similar to those for C8αc6. Chimeric primers were used to generate two self-annealing 5′ and 3′ fragments using wtC8α and C8αc2 as templates, respectively, with an overlap corresponding to the C8α N-terminal TSP1/C8β LDLRA junction. Fragments were combined and used with universal primers in second round PCR. The product was digested with *Hin*dIII/*Cla*I and the released fragment exchanged for the corresponding fragment in wtC8α. C8αc5 was prepared by exchanging a *Mfe*I/*Cla*I fragment in C8αc1 with one from C8αc7. C8αt5 was prepared by exchanging a *Hin*dIII/*Cla*I fragment in C8αt1 with one from C8αc7. All constructs were subjected to dideoxy sequencing to confirm PCR fidelity and junction site integrity.

Expression in COS Cells. COS-7 cells were cotransfected with each C8α construct and wtC8γ as described previously (*10*, *18*). Control medium was prepared from nontransfected cells. After centrifugation and concentration of harvested media, samples were dialyzed and applied directly to 10% SDS−PAGE gels for immunoblotting. Expression of each recombinant form of C8α-γ was confirmed by probing with the appropriate subunit-specific antibodies as described previously (*10*).

Quantitation of Recombinant Forms of C8α-γ. Concentrations of chimeric and truncated forms of C8α-γ in expression media were determined by a quantitative ELISA. In some cases, a small amount of free construct (<10%) was also secreted; therefore, antibodies specific for C8γ were used to selectively measure the concentration of the heterodimer. For detection of chimeric forms of C8α-γ, a mixture of goat anti-human C8α and C8β antibodies in 0.1 M sodium bicarbonate, pH 8.5, was plated onto microtiter plates. Media samples containing chimeric C8α-γ and controls containing wtC8α-γ or purified human C8α-γ were added and incubated for 30 min at 25 °C. Bound forms of C8α-γ were detected using rabbit anti-human C8γ as the primary antibody and HRP-conjugated goat anti-rabbit IgG as the secondary antibody. Plates were developed as described (*10*). Quantitation of truncated C8α-γ was performed similarly using goat anti-human C8α as the plated antibody.

Binding Assays. To measure binding of C9 to wtC8α-γ and chimeric or truncated C8α-γ, expression media were concentrated 40−80-fold and dialyzed into buffer of low ionic strength (5 mM imidazole, 33.6 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4). Sample media and medium containing wtC8α-γ as a control were quantitated and diluted so that each contained a similar concentration of the recombinant heterodimer. Purified human C8β was added at the desired molar excess and incubated for 15 min at 25 °C prior to adding excess C9. Samples were then applied to 5−20% or 5−30% (w/v) sucrose density gradients prepared in the same buffer containing 1 mg/mL BSA (*10*). All gradients were centrifuged for 2 h at 4 °C in a Sorvall VTi65 rotor at 202000*g*. After fractionation, the sedimentation position of each form of C8α-γ was determined using the above ELISA.

Hemolytic Activity Assays. Concentrated sample media and control medium were dialyzed into buffer of intermediate ionic strength (5 mM imidazole, 72.7 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4). Each form of C8α-γ was incubated in excess over purified human C8β for 30 min at 25 °C, serially diluted in isotonic buffer (5 mM imidazole, 72.7 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.5% glucose, 0.05% gelatin, 1 mg/mL BSA, pH 7.4), and assayed for hemolytic activity toward sheep EAC1−7 as described previously (*18*). Where indicated, the amount of C9 used in the assay was increased over that normally required for maximum C8 activity.

RESULTS AND DISCUSSION

Properties of the C8α Constructs. The chimeric and truncated C8α constructs used in this study are shown in Figure 1 along with a summary of the results from analyses of their C9 binding properties. Binding was measured directly using sucrose density gradients to detect formation of complexes or indirectly by measuring C8 hemolytic activity. The first six constructs were used previously to identify segments of C8α involved in binding C8β (*10*). When coexpressed with C8γ in COS cells, each was shown to be secreted as a heterodimer with C8γ, e.g., C8αc1-γ, C8αc2-γ, C8αc3-γ, etc. Similar results were obtained in the present study for C8αc4, C8αt4, C8αc6, and C8αc7. Immunoblot analyses using subunit-specific antibodies confirmed that each construct forms a heterodimer when coexpressed with C8γ (not shown). Constructs C8αc5 and C8αt5 were prepared for reasons described below but could not be expressed either independently or with C8γ.

Importance of the C8α N- and C-Terminal Module Pairs. The first six constructs in Figure 1 are distinguished by the absence of one or both C8α module pairs. When produced as a heterodimer with C8γ, previous experiments showed

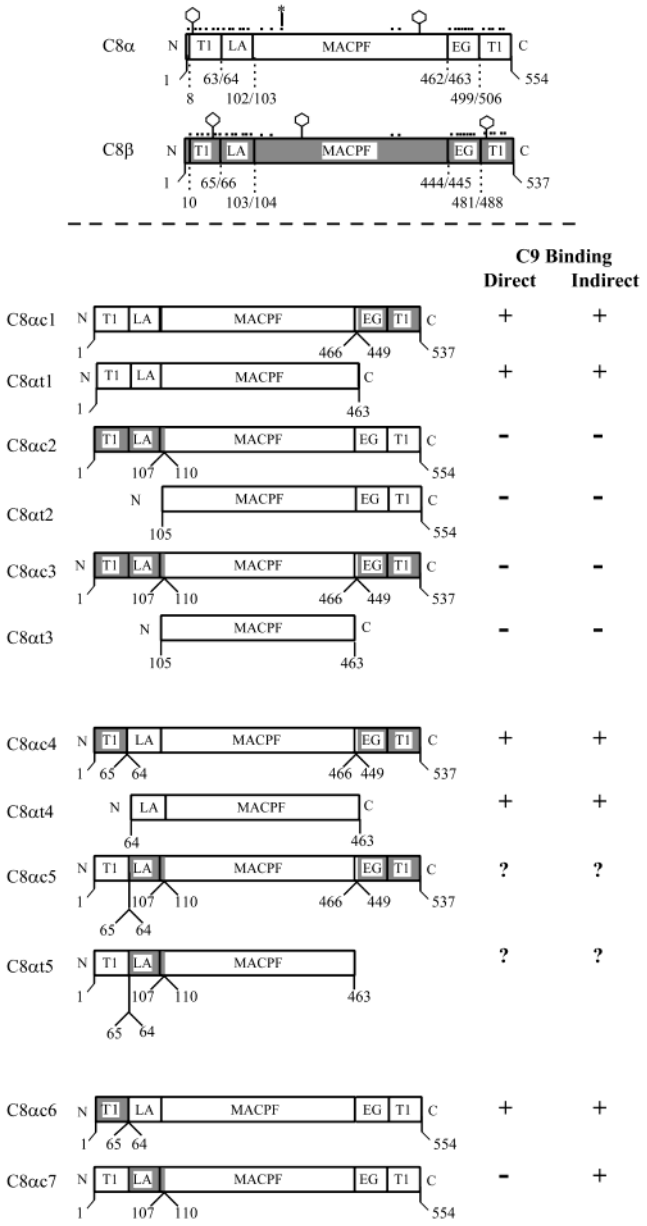

FIGURE 1: Chimeric and truncated forms of C8α. Upper panel: Full-length C8α and C8*β*. Modules are designated by the two-letter abbreviations adopted for use in figures (*19*). These correspond to TSP1 (T1), LDLRA (LA), and EGF (EG). Residue numbers identify module boundaries as defined in the SWISS-PROT Protein Sequence Data Bank. Cys residues are identified as dots above each map. All are conserved, and all form internal disulfide bonds except Cys^{164} (*), which is linked to C8*γ*. Hexagons identify potential N-glycosylation sites. Lower panel: Chimeric and truncated C8α. Junction site residues are numbered in accordance with their location in full-length C8α or C8*β*. Also shown is a summary of the C9 binding properties of each construct as measured directly by density gradient centrifugation or indirectly by hemolytic activity assays. Results for C8αc5 and C8αt5 were inconclusive for reasons described in the text.

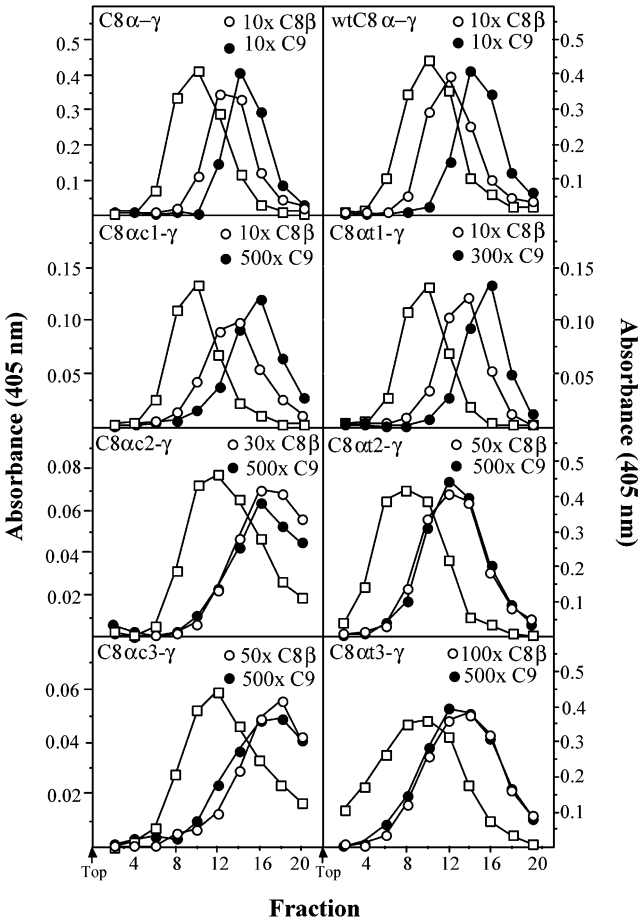

FIGURE 2: Importance of the C8α N- and C-terminal module pairs in binding C9. Purified C8α-*γ* or expression medium containing wtC8α-*γ*, chimeric C8α-*γ*, or truncated C8α-*γ* was incubated alone (□), with the indicated molar excess (×) of C8*β* (○), or with excess C8*β* and C9 (●) and subjected to sucrose density gradient centrifugation. The sedimentation position of each complex was identified by an ELISA specific for C8*γ*. Gradients were 5−20% sucrose for all constructs except C8αc2-*γ* and C8αc3-*γ*, which were 5−30%. The top of each gradient is indicated. Samples prepared with control medium from nontransfected cells produced no detectable signal when subjected to the same analysis.

that each construct has the ability to bind C8*β* (*10*). Because the C8α MACPF domain is the only segment common to all six, we concluded that this segment contains the principal binding site for C8*β*. In addition, only C8αc1-*γ* and C8αt1-*γ* exhibited C8 hemolytic activity when combined with C8*β*. This was interpreted as indirect evidence that the binding site for C9 lies within the TSP1 + LDLRA + MACPF segment of C8α, which is unique to these two constructs.

To demonstrate C9 binding more directly and explore the possibility that the other constructs may interact with C9 but are not hemolytically active, we used density gradient centrifugation to assay for the formation of complexes. As reported previously, C9 normally binds purified C8α-*γ* to form a complex that is easily detected by this method (*13*). Preliminary experiments here produced similar results with wtC8α-*γ*; however, binding of C9 to chimeric and truncated forms of C8α-*γ* could only be detected if the constructs were first combined with C8*β*. Because the apparent affinity of the constructs for C9 is comparatively low, all density gradient experiments were performed in the presence of C8*β* in order to maximize C9 binding potential.

Results from density gradient experiments using the first six constructs are shown in Figure 2. As observed previously, each has the ability to bind C8*β* as indicated by the shift in sedimentation position when C8*β* is added. The apparent affinity of each construct for C8*β* varies as indicated by the different excesses required for complete binding. When combined with C8*β*, only C8αc1-*γ* and its truncated counterpart C8αt1-*γ* bind C9, albeit with a lower apparent

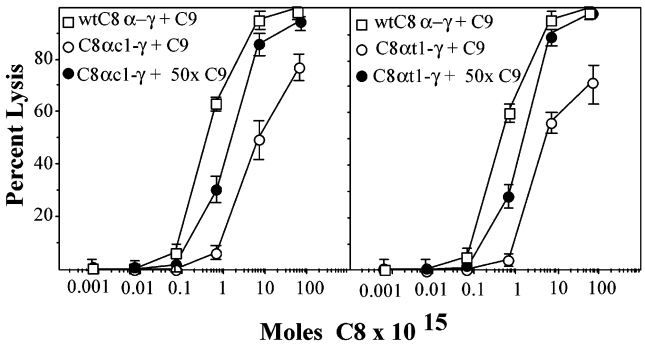

FIGURE 3: Hemolytic activity of C8αc1-γ and C8αt1-γ. Media containing wtC8α-γ, C8αc1-γ, or C8αt1-γ were incubated at a 10-fold excess over purified C8β and assayed for hemolytic activity. C9 was added at the level normally used in hemolytic assays and at a 50-fold higher level. For the wtC8α-γ control, the same activity was observed at both C9 levels. Use of higher excesses of wtC8α-γ or construct over C8β had no effect on the results. Moles of C8 are based on C8β as the limiting component. Media assayed in the absence of either C8β or C9 exhibited no measurable activity nor did controls containing only C8β and C9. Error bars show the deviation from two different assays.

affinity relative to full-length C8α-γ. The amount of C9 required for complete binding to C8αc1-γ and C8αt1-γ is 500- and 300-fold, respectively, whereas purified C8α-γ and wtC8α-γ require only a 10-fold excess. Also, C8αc2-γ and C8αc3-γ form aggregates at this ionic strength, hence the need for gradients of higher density to analyze these constructs. Both constructs retain the ability to bind C8β; however, binding to C9 could not be detected. Because the effect of aggregation on C9 binding is unknown, results for these constructs are not considered reliable. Importantly, the corresponding truncated forms (C8αt2-γ and C8αt3-γ) do not form aggregates yet are also incapable of binding C9.

To detect C9 binding by density gradient centrifugation, the affinity between the constituent proteins must be relatively high. A more sensitive method is the C8 hemolytic assay which can detect interactions that lead to C9 incorporation into the MAC. These assays must be performed in isotonic buffer and at a higher ionic strength (72.7 vs 33.6 mM NaCl); however, they are highly sensitive to weak, transient interactions between C8 and C9. In earlier assays of the first six constructs, only C8αc1-γ and C8αt1-γ were found to be hemolytically active when using an amount of C9 that is typically used to assay C8 (*10*). Here, we reexamined the activity of all six constructs at higher levels of C9 in order to increase the likelihood of detecting weak interactions that may not have been detected in earlier assays. Results for C8αc1-γ and C8αt1-γ are shown in Figure 3. Consistent with earlier findings, both exhibit significant hemolytic activity when the normal amount of C9 is used in the assay. However, increasing the C9 by 50-fold increases the activity of both constructs substantially, presumably as a result of increased C9 binding. Corresponding assays performed with C8αc2-γ, C8αt2-γ, C8αc3-γ, and C8αt3-γ produced no measurable activity when C9 was increased as high as 100-fold (not shown). From these and the density gradient results, we conclude that constructs C8αc1-γ and C8αt1-γ have a substantial affinity for C9, and therefore the C8α C-terminal modules are not required for binding. Furthermore, negative results for the other four constructs suggest that the C8α MACPF domain is incapable of independently binding C9 and that the C8α N-terminal modules have an essential role.

Role of the Individual C8α N-Terminal Modules. To assess the role of each C8α N-terminal module in C9 binding, a set of constructs was prepared which lack the C8α C-terminal modules and either the C8α N-terminal TSP1 (C8αc4 and C8αt4) or LDLRA module (C8αc5 and C8αt5) (Figure 1). Of these, only the former pair of constructs could be successfully expressed as heterodimers with C8γ in COS cells. Results from density gradient experiments in Figure 4 show that both constructs form complexes with C8β and these complexes have the ability to bind C9. Furthermore, both are hemolytically active, albeit at a reduced level compared to the wtC8α-γ control. These results indicate the C8α LDLRA module and MACPF domain are sufficient for recognition and binding of C9 and for mediating formation of a functional MAC.

Because of the inability to express C8αc5 and C8αt5 we could not use this pair of constructs to evaluate the importance of the TSP1 module. To address this question, an alternative pair was prepared in which the N-terminal TSP1 or LDLRA module from C8β was inserted into full-length C8α (C8αc6 and C8αc7). Both constructs were successfully expressed as heterodimers with C8γ and their binding properties characterized. Results for C8αc6-γ (Figure 5) show that substitution of the C8α TSP1 module does not abolish C9 binding or hemolytic activity. This agrees with results in Figure 4 which indicate the C8α LDLRA module and MACPF domain alone are sufficient for binding. Corresponding results for C8αc7-γ in which the C8α LDLRA module was substituted revealed an aggregate upon density gradient analysis. C8αc7-γ retains the ability to bind C8β, but unlike the other aggregated constructs it appears to partially bind C9. This was confirmed in a corresponding hemolytic assay which shows that C8αc7-γ exhibits significant activity and therefore is capable of binding C9 and forming a functional MAC. Binding at this higher ionic strength is likely to be weak and involve the monomer form of C8αc7-γ. Indeed, density gradient experiments performed independently revealed that C8αc7-γ is a monomer at 72.7 mM NaCl (not shown). Although capable of binding C8β, formation of a complex with C9 could not be detected at this ionic strength.

In conclusion, our results indicate that binding between C8 and C9 is not dependent on the C-terminal modules in C8α. They also suggest that binding is not mediated by the C8α C-terminal modules functioning together with the C8α MACPF domain nor is it mediated by the C8α MACPF domain alone. Binding does involve the C8α N-terminal modules, and it may require cooperative interaction between these modules and the MACPF domain. Substitution or deletion of both C8α N-terminal modules abrogates binding whereas substitution of either one individually does not, provided the MACPF domain is retained. This suggests that both modules make a significant contribution to forming the C9 binding site on C8α. Whether the C8α N-terminal modules alone have the potential to bind C9 is difficult to determine experimentally. The C8α MACPF domain is involved in binding C8β, and our results indicate that formation of a complex with C8β is required to detect interaction with C9. Chimeric constructs that contain the C8α N-terminal modules but lack the C8α MACPF domain would

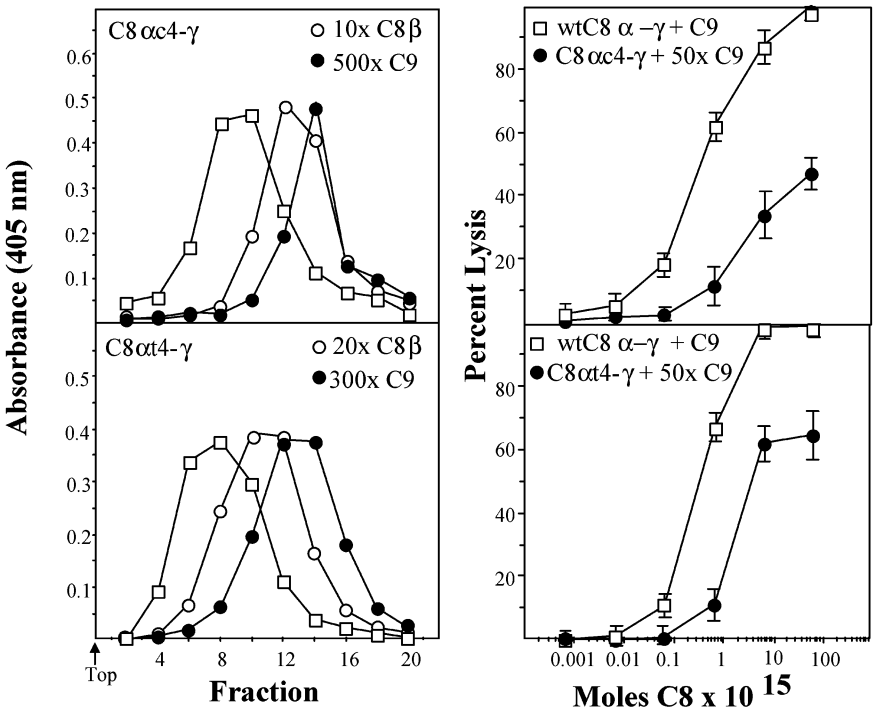

FIGURE 4: Role of the C8α LDLRA module in binding C9. Left panels: Binding of C9 to C8αc4-γ and C8αt4-γ. Constructs were incubated alone (□), with the indicated excess of C8β (○), or with excess C8β and C9 (●) and subjected to sucrose gradient centrifugation (5−20%). Complexes were detected as in Figure 2. Right panels: Hemolytic activity of C8αc4-γ and C8αt4-γ. Constructs were incubated at a 10-fold excess over C8β and assayed as in Figure 3. Assays of wtC8α-γ used the normal amount of C9 whereas assays of each construct used 50-fold higher C9. Higher excesses of construct over C8β or higher amounts of C9 produced the same results.

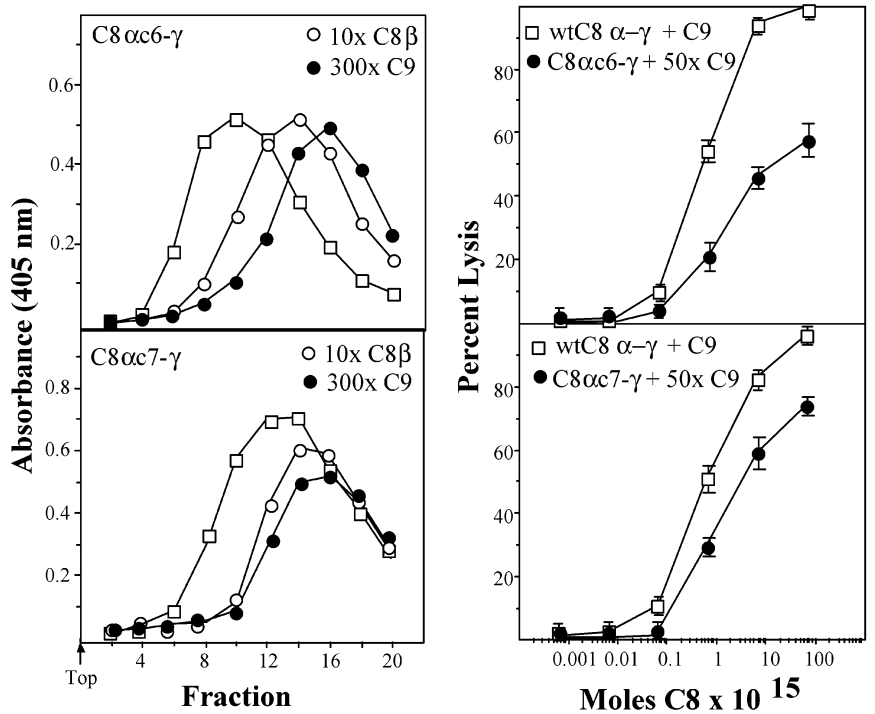

FIGURE 5: Role of the C8α N-terminal TSP1 module in binding C9. Left panels: Binding of C9 to C8αc6-γ and C8αc7-γ. Constructs were incubated alone (□), with the indicated excess of C8β (○), or with excess C8β and C9 (●) and analyzed on 5−20% (C8αc6-γ) or 5−30% (C8αc7-γ) sucrose density gradients. Complexes were detected as in Figure 2. Right panels: Hemolytic activity of C8αc6-γ and C8αc7-γ. Constructs were incubated at a 10-fold excess over C8β and assayed as in Figure 3. Assays of wtC8α-γ used the normal amount of C9 whereas assays of the constructs used 50-fold higher C9. Higher excesses of construct over C8β or higher amounts of C9 produced the same results.

not bind C8β and therefore would not be informative with respect to C9 binding. Interestingly, the MACPF domain of C8α is capable of independently binding C8β, which suggests that the binding site for C8β lies exclusively within this segment (*10*). This differs from C9 binding which is dependent on the N-terminal modules, and it is in contrast to recently reported results for C8β and its interaction with C8α-γ. In C8β, the N-terminal TSP1 module appears to act

cooperatively with the C8β MACPF domain to form a binding site for C8α (*15*). Considering that C8α and C8β are capable of multiple, simultaneous binding interactions, it is not surprising to find that for some of these interactions different segments of each protein are involved.

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