Chimeric and Truncated Forms of Human Complement Protein C8α Reveal Binding Sites for C8β and C8γ within the Membrane Attack Complex/Perforin Region[†]

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Received March 2, 1999; Revised Manuscript Received April 27, 1999

ABSTRACT: Human C8 is one of five components of the membrane attack complex of complement. It is an oligomeric protein composed of three subunits (C8α, C8β, and C8γ) that are derived from different genes. C8α and C8β are homologous and both contain a pair of tandemly arranged N-terminal modules [thrombospondin type 1 (TSP1) + low-density lipoprotein receptor class A (LDLRA)], an extended middle segment referred to as the membrane attack complex/perforin region (MACPF), and a pair of C-terminal modules [epidermal growth factor (EGF) + TSP1]. During biosynthetic processing, C8α and C8γ associate to form a disulfide-linked dimer (C8α−γ) that binds to C8β through a site located on C8α. In this study, the location of binding sites for C8β and C8γ and the importance of the modules in these interactions were investigated by use of chimeric and truncated forms of C8α in which module pairs were either exchanged for those in C8β or deleted. Results show that exchange or deletion of one or both pairs of modules does not abrogate the ability of C8α to form a disulfide-linked dimer when coexpressed with C8γ in COS cells. Furthermore, each chimeric and truncated form of C8α−γ retains the ability to bind C8β; however, only those containing the TSP1 + LDLRA modules from C8α are hemolytically active. These results indicate that binding sites for C8β and C8γ reside within the MACPF region of C8α and that interaction with either subunit is not dependent on the modules. They also suggest that the N-terminal modules in C8α are important for C9 binding and/or expression of C8 activity.

Human C8 is one of five complement components (C5b, C6, C7, C8, and C9) that interact to form the cytolytic membrane attack complex, or MAC (*1*, *2*). It is an oligomeric protein composed of an α (M_r = 64 000), β (M_r = 64 000), and γ (M_r = 22 000) subunit, which are encoded in different genes (*3*). In C8 purified from serum, C8α and C8γ are linked by a single disulfide bond to form a dimer (C8α−γ) that is noncovalently associated with C8β. Although formation of C8α−γ and its association with C8β normally occurs prior to secretion (*4*), recent studies have shown that C8α, C8β, and C8γ can also be synthesized and secreted independently as recombinant proteins (*5*, *6*).

Assembly of the MAC involves highly specific and sequential interactions; therefore, it is likely each component contains a specific binding site(s) that recognizes the succeeding component incorporated into the complex. For C8, it is well-established that it contains multiple binding sites that are involved in C8 subunit interactions and the formation, function, and regulation of the MAC (*7*). C8α contains a site that facilitates intracellular binding of C8γ to form C8α−γ (*8*) and a site that mediates interaction between C8α−γ and C8β (*9*). It also contains a binding site(s) for C9, which functions to direct this component into the MAC (*10*), and it contains a site recognized by CD59, the membrane-associated regulatory protein that inhibits formation of a functional MAC (*11*). C8β contains at least two different sites, one that binds C8α in C8α−γ and a second that mediates C8 incorporation into the MAC by binding to the intermediate C5b−7 complex (*12*). Aside from the CD59 recognition site in C8α, the location of binding sites within each subunit is unknown.

C8α and C8β are members of the MAC family of proteins that includes C6, C7, and C9. All have highly conserved amino acid sequences and gene structures, and all contain cysteine-rich modules that are ∼40−80 residues in length and exhibit sequence similarity to modules found in several functionally unrelated proteins (*2*, *13*). Within C8α and C8β, there are a pair of tandemly arranged N-terminal modules (TSP1 + LDLRA)[1] and a pair of C-terminal modules (EGF + TSP1). Although not a module, the middle segment of

[†] Supported by NIH GM 42898.

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[1] Abbreviations: Nomenclature of protein modules follows published recommendations (*14*): TSP1, thrombospondin type I; LDLRA, low-density lipoprotein receptor class A; EGF, epidermal growth factor; MACPF, membrane attack complex/perforin. Other abbreviations: wt, wild-type; EAC1−7, sensitized sheep erythrocytes carrying human complement C1−C7.

10.1021/bi9904858 CCC: $18.00
Published on Web 06/11/1999

each protein is designated MACPF to emphasize its sequence similarity to other family members and the corresponding region of the pore-forming protein perforin. With a few exceptions, corresponding regions of the other MAC family proteins are similarly organized, with C6 and C7 having the distinction of containing an extended C-terminal region composed of additional modules. C8γ is structurally unrelated to any complement protein and is a member of the lipocalin family of widely distributed proteins that bind and transport small lipophilic ligands, e.g., retinol, pheromones, odorants, etc. (*15*, *16*).

The modular design and conserved structure of the MAC proteins has led to the suggestion that the modules themselves may mediate protein−protein interactions during MAC assembly (*7*, *17*, *18*). This is consistent with the view that assembly of the MAC is analogous to formation of a heteropolymer from structurally similar but distinct monomeric units. Also relevant is the fact these modules are known to mediate specific protein−protein interactions in other systems (*19*, *20*). Regarding specificity, MAC assembly requires binding interactions that are highly ordered and it is unknown if specificity is determined by differences in fine structure of the modules or sequence variations within the MACPF region of each protein. C8 is a suitable model to use for investigating the basis of this specificity because unlike the other MAC proteins, which only weakly interact until complement is activated (*10*, *21*−*23*), C8 exists in the circulation as a tightly associated complex of C8α−γ and C8β. Interactions in this complex are mediated by C8α and C8β; thus, insight into how these two proteins associate will contribute to understanding how all the MAC proteins interact.

This report describes our continuing efforts to define the location of important binding regions within C8α and thereby gain insight into the role of the modules in mediating C8 subunit interactions and MAC assembly. Chimeric forms of C8α in which modules were exchanged for those in C8β, and truncated forms in which modules were deleted, were prepared and used to narrow the location of binding sites for C8β and C8γ. Results indicate that both sites are located within the MACPF segment of C8α and that neither the N- nor C-terminal modules are required for recognition and binding of these subunits. In addition, they suggest the N-terminal modules of C8α are important for C9 interaction and/or expression of C8 activity.

EXPERIMENTAL PROCEDURES

Materials. Human C8 and C9 were purified from plasma fraction III provided by Miles Inc. (Berkeley, CA) (*24*). C8α−γ and C8β were separated by gel filtration in high ionic strength buffer (*9*). Molar concentrations were determined from published $\epsilon_{280}^{1\%}$ values (*25*). Goat antiserum against human C8 and rabbit antiserum against C8α−γ or C8β were produced by standard procedures. C8α−γ and C8β were coupled to agarose and used to isolate the respective antibodies from goat anti-human C8 antiserum. Goat anti-human C8α antibodies were prepared by passing purified C8α−γ antibodies through a C8γ−agarose column (*6*). The same resin was used to isolate anti-C8γ antibodies from rabbit anti-human C8α−γ antiserum.

Expression Constructs. Constructs containing wtC8α or wtC8γ cDNA in the COS cell expression vector pcDNA3 (Invitrogen) are described elsewhere (*6*). Chimeric constructs were generated from the above wtC8α plasmid and a wtC8β plasmid described previously (*5*). Overlap extension PCR was used to prepare chimera C8αc1. In the initial step, a cDNA fragment corresponding to residues 449−537 of C8β was generated by using an internal chimeric primer that encoded sequence flanking the C8α−C8β junction site. In a separate reaction, a C8α MACPF fragment was amplified by using a second chimeric primer containing the same flanking sequences. In the second round of PCR, these self-priming fragments were combined and used to produce a chimeric fragment containing the MACPF sequence of C8α and the C-terminal sequence of C8β. This fragment was digested with *Cla*I/*Apa*I and substituted into wtC8α pcDNA3. A chimeric fragment used to construct C8αc2 was produced by a similar strategy and was digested with *Bam*HI for substitution into wtC8α pcDNA3. To prepare C8αc3, a *Cla*I/*Apa*I fragment of C8αc1 containing MACPF sequence from C8α and the entire C-terminal region of C8β was exchanged for the same fragment in C8αc2. All constructs were sequenced to confirm fidelity of the PCR and integrity of the junction sites.

To prepare C8αt1, wtC8α cDNA in a pBluescript II plasmid was modified to remove the *Eco*RI site in the 5′ polylinker (*6*). The internal *Eco*RI site was then cleaved, blunt-ended, and ligated to a *Hin*dIII linker containing a stop codon. The truncated cDNA fragment was released with *Hin*dIII and ligated after partial fill-in to the *Xba*I site in pcDNA3. To prepare C8αt2, PCR was used to generate two overlapping C8α cDNA fragments, one that contained the leader sequence (residues −30 to −1) and one that encoded residues 105−554. These were used along with universal primers to generate the C8αt2 cDNA in a second round of PCR. This product was digested with *Hin*dIII and cloned into pcDNA3. To prepare C8αt3, the C8αt1 construct was cleaved at a *Cla*I site located in the MACPF region and at a *Stu*I site located 3′ of the insert. This fragment was isolated and exhanged with the corresponding *Cla*I/*Stu*I fragment in C8αt2.

Expression in COS Cells. COS-7 cells were cotransfected and cultured as described previously (*6*). Control media were prepared from nontransfected cells and cells transfected with wild-type pcDNA3. After centrifugation and concentration of harvested media, samples were either precipitated with 10% (w/v) TCA or applied directly to SDS−10% polyacrylamide gels. Immunoblotting was performed with horseradish peroxidase (HRP) conjugates of either goat anti-rabbit IgG (Bio-Rad) or rabbit anti-goat IgG (Pierce) as secondary antibodies. Proteins were visualized with the Super Signal chemiluminescent substrate (Pierce).

Quantitation of Recombinant C8α−γ. Concentrations of chimeric and truncated C8α−γ were determined by quantitative immunoblotting or enzyme-linked immunosorbent assay (ELISA). Because small amounts of C8α monomer were observed in some coexpressions with C8γ, these methods were designed to specifically measure recombinant C8α−γ dimer. In the former method, SDS−PAGE and immunoblotting were performed as above using rabbit anti-human C8γ as the primary antibody and HRP-conjugated goat anti-rabbit IgG as the secondary antibody. The amount of recombinant C8α−γ was estimated by using purified human C8α−γ as the standard.

For detection of chimeric forms of C8α−γ by ELISA, goat anti-human C8α and C8β antibodies in 0.1 M sodium bicarbonate, pH 8.5, were mixed and adsorbed to microtiter plates by incubation at 25 °C for 30 min. After being washed with 20 mM imidazole, 100 mM NaCl, 5 mM $CaCl_2$, and 0.02% Tween-20, pH 7.5, wells were treated with blocking buffer [50 mM Tris, 150 mM NaCl, and 1% bovine serum albumin (BSA), pH 7.4] and washed again. Samples containing chimeric C8α−γ and controls containing wtC8α−γ or purified C8α−γ were added and incubated for 30 min at 25 °C. After washing, bound forms of C8α−γ were detected with rabbit anti-human C8γ as the primary antibody and HRP-conjugated goat anti-rabbit IgG as the secondary antibody. Color was developed with 0.25 mg/mL 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) in 0.1 M sodium citrate and 0.01% hydrogen peroxide, pH 4.0. For detection of truncated C8α−γ, only goat anti-human C8α antibodies were adsorbed to microtiter plates in the initial step.

Binding Assays. To measure binding of C8β to chimeric C8α−γ, medium was concentrated 80-fold and dialyzed into buffer of approximate physiological ionic strength (5 mM imidazole, 133.6 mM NaCl, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.4). For binding to truncated C8α−γ, medium was concentrated 40-fold and dialyzed against the same buffer prepared at a lower ionic strength (16.25 mM NaCl). Sample media and control medium containing wtC8α−γ were quantitated and diluted so that each contained the same molar concentration of recombinant C8α−γ. Purified human C8β was added at the desired molar excess and incubated for 1 h at 25 °C. Samples were applied to a 4 mL 5−10% (w/v) sucrose density gradient prepared in the same buffer containing 1 mg/mL BSA (*6*). Gradients were centrifuged for 2 h at 4 °C in a Sorvall VTi65 rotor at 202000*g*. After fractionation, recombinant forms of C8α−γ were detected by using the above ELISA.

Activity Assays. Concentrated sample media and control medium were used either directly or dialyzed into physiological ionic strength buffer. After the concentration was determined, each form of recombinant C8α−γ was incubated with 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, and 1 mg/mL BSA, pH 7.4) and assayed for hemolytic activity toward sheep EAC1−7 as described previously (*6*).

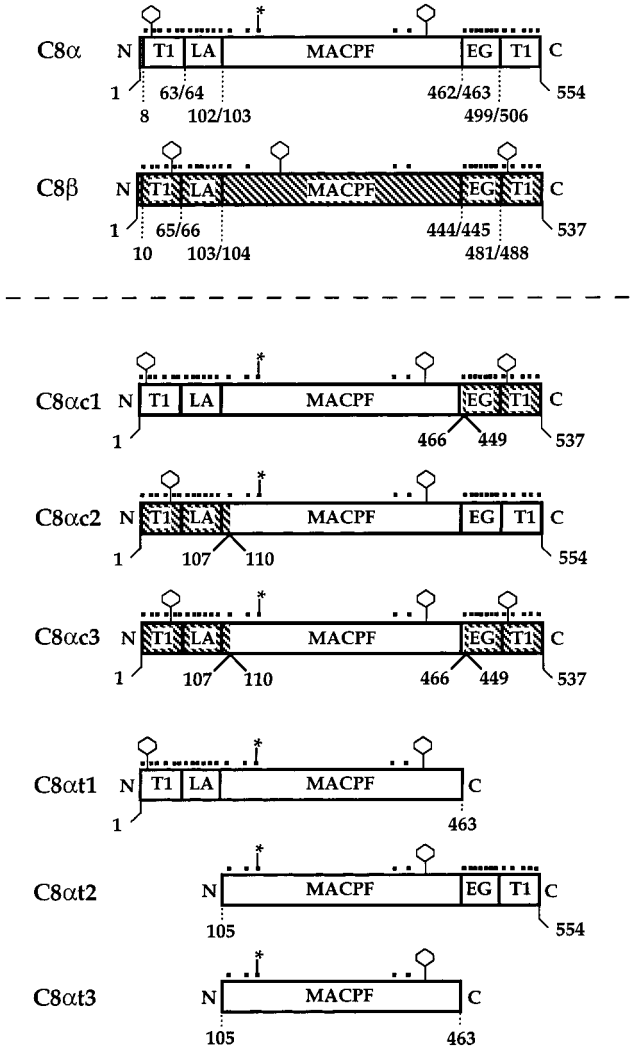

FIGURE 1: Maps of chimeric and truncated forms of C8α. Upper panel: Full-length C8α and C8β. Abbreviations correspond to the TSP1 (T1), LDLRA (LA), and EGF (EG) modules and the MACPF region. Residue numbers identify module boundaries as defined in the SWISS−PROT Protein Sequence Data Bank (*2*). Approximate location of Cys residues is indicated by dots above each map. Aside from Cys^{164} (∗), which is linked to C8γ, all are conserved and all form intrachain disulfide bonds. Hexagonal symbols identify potential N-glycosylation sites. Lower panel: Chimeric (C8αc1, C8αc2, C8αc3) and truncated (C8αt1, C8αt2, C8αt3) forms of C8α. Numbers correspond to residues in full-length C8α or C8β. Junction sites occur at conserved Cys residues and therefore do not correspond precisely with module boundaries.

RESULTS

To investigate the role of the C8α modules in intracellular recognition and binding of C8γ, chimeras were prepared in which one or both pairs of modules were exchanged for the corresponding ones in C8β (Figure 1). Results in Figure 2 show that, when coexpressed with C8γ in COS-7 cells, each chimera retains the ability to associate intracellularly with C8γ to produce a dimer that is similar in size to human C8α−γ. Each form of C8α−γ reacts with antibodies specific for C8α, C8β, or C8γ, thus confirming the presence of epitopes from all three subunits. Differences in signal intensity among the samples probed with anti-C8α or anti-C8β antibodies reflect differences in the epitopes represented in each construct. Also noted are small amounts of chimeric C8α monomer in samples of C8αc2−γ and C8αc3−γ. This is not unexpected since previous experiments have shown that C8α can be secreted independently of C8γ (*6*). Results from analysis of reduced samples confirmed that each C8α chimera is disulfide-linked to C8γ (not shown).

Because it is common to all three C8α−γ chimeras, these results suggested the MACPF segment of C8α contains the site that mediates intracellular interaction with C8γ. To demonstrate this more directly, a second set of constructs was prepared in which the N- and/or C-terminal modules were deleted (Figure 1). Results in Figure 3 show that, when coexpressed with C8γ, all three truncated forms of C8α have the ability to form a disulfide-linked dimer with C8γ. Each secreted dimer reacts to antibodies against C8α−γ or C8γ and each yields the expected products upon reduction. Regarding the size of the truncated products, it is noted that C8αt1−γ and C8αt2−γ have significantly different mobili-

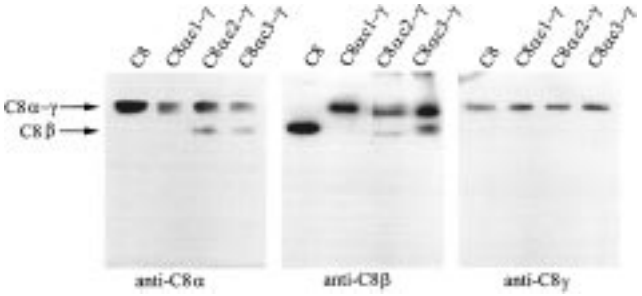

FIGURE 2: Expression of chimeric forms of C8α−γ. COS-7 cells were cotransfected with chimeric C8α and wtC8γ. Expression media were harvested and analyzed by SDS−PAGE under nonreducing conditions. Immunoblotting was performed with the indicated subunit-specific antibodies. Arrows indicate the location of C8α−γ and C8β in the human C8 standard. Small amounts of C8αc2 and C8αc3 monomer are also visible on blots probed with anti-C8α or anti-C8β (lanes 3 and 4). Control medium from nontransfected cells or cells transfected with wild-type pcDNA3 produced no signal.

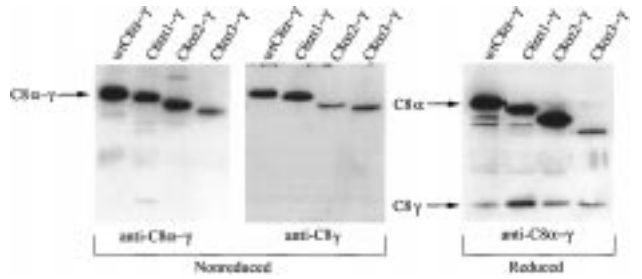

FIGURE 3: Expression of truncated forms of C8α−γ. COS-7 cells were cotransfected with wtC8α or truncated C8α and wtC8γ. Expression media were analyzed by SDS−PAGE under nonreducing and reducing conditions. Immunoblotting was performed with the indicated antibodies. Arrows identify the location of C8α−γ, C8α, and C8γ in the wtC8α−γ control. Trace amounts of degraded wtC8α or C8αt1 are visible in some of the reduced samples (lanes 1 and 2).

ties despite the fact their sequences predict a molecular weight difference of only ~1900. This is likely the result of differences in glycosylation. Human C8α has two potential N-glycosylation sites ($Asn^{13,407}$) but contains only one N-linked chain that is predicted to be at Asn^{13} (*26*). Truncate C8αt1 contains both sites; however, deletion of the C-terminal modules may render Asn^{407} accessible to N-glycosylation along with Asn^{13}. In contrast, C8αt2 lacks Asn^{13} and it has an intact C-terminus, thus it is not likely to contain any N-linked carbohydrate. The net difference of two N-linked chains would account for the apparent difference in molecular weights between C8αt1-γ and C8αt2-γ.

Binding of human C8α−γ to C8β is known to be mediated through a site located on C8α. To determine if this site lies within the modules, the above chimeric forms of C8α−γ were tested for their ability to bind C8β at physiological ionic strength (Figure 4). In these experiments, shifts in sedimentation position on density gradients were detected by using a capture ELISA to determine the location of recombinant forms of C8α−γ. Results for wtC8α−γ show that addition of either a 10- or 50-fold excess of C8β produces a high molecular weight complex. The position of this complex corresponds to that of C8, as was shown previously with purified human C8α−γ or wtC8α−γ and radiolabeled C8β (*6*). Although binding to C8α−γ is normally complete at a 2-fold excess of C8β, higher levels were used in these

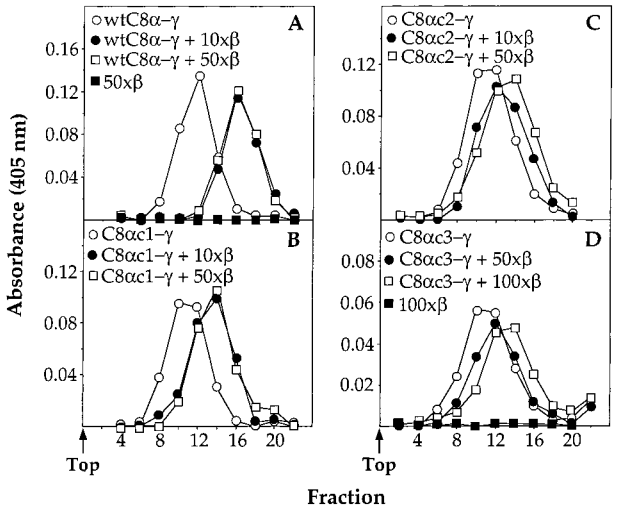

FIGURE 4: Binding of C8β to chimeric forms of C8α−γ. The indicated molar excesses (*x*-fold) of C8β were incubated with each form of C8α−γ and subjected to sucrose density gradient centrifugation. Gradients were fractionated and analyzed by an ELISA that selectively detects C8α−γ dimers. The top of each gradient is identified by an arrow. Panel A: Binding to wtC8α−γ. Controls prepared with media from nontransfected cells or cells transfected with wild-type pcDNA3 produced no detectable signal. Human C8 typically sediments at fraction 15−16 under these conditions. Panels B−D: Binding to chimeric forms of C8α−γ.

controls to confirm that the stoichiometry remains 1:1 at greater excesses. Corresponding results for chimeric forms of C8α−γ revealed that all are likewise capable of forming a 1:1 complex with C8β. Binding to C8αc1−γ is complete at a 10-fold excess of C8β, whereas C8αc2−γ and C8αc3−γ require higher excesses to achieve complete binding (50- and 100-fold, respectively). Binding could be observed at lower excesses as the ionic strength was reduced; however, self-aggregation of the chimeras made interpretation difficult.

These results suggested that the MACPF region of C8α is involved in binding C8β. To confirm this, similar binding studies were performed with the truncated forms of C8α−γ. Low ionic strength buffers were used in these experiments because the truncates did not self-aggregate under these conditions. Moreover, the sizable deletions in these constructs made it unlikely their affinity for C8β at physiological ionic strength would be high enough to allow detection of complexes on density gradients. Results in Figure 5 show that at low ionic strength each truncate has the ability to form a 1:1 complex with C8β. Only a 10-fold excess of C8β is required to achieve complete binding as indicated by lack of a further shift in sedimentation position at higher excesses (not shown). When considered together with results for the C8α−γ chimeras at physiological ionic strength, these observations support the conclusion that recognition and binding of C8β is mediated through the MACPF region of C8α.

The functional potential of each chimeric and truncated form of C8α−γ when combined with C8β was assessed in hemolytic assays. Each was incubated in excess over C8β and added to EAC1−7 cells in the presence of C9. Use of C8α−γ as the excess component was necessary because free C8β binds to EAC1−7, competes with C8 binding, and thereby inhibits hemolytic activity (*12*). By adding excess C8α−γ, the amount of free C8β is restricted and its inhibitory potential is minimized. Results in Figure 6 show

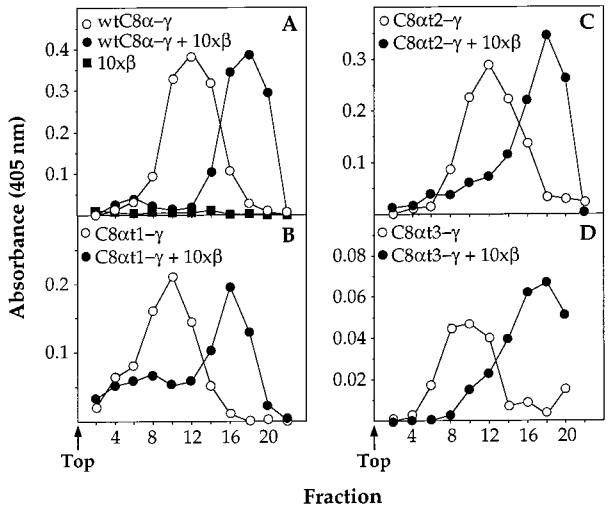

FIGURE 5: Binding of C8β to truncated forms of C8$\alpha-\gamma$. A 10-fold molar excess of C8β was incubated with each form of C8$\alpha-\gamma$ and subjected to centrifugation on sucrose density gradients as in Figure 4. Fractions were analyzed by ELISA to detect recombinant C8$\alpha-\gamma$ dimers. Panel A: Binding to wtC8$\alpha-\gamma$. Panels B−D: Binding to truncated forms of C8$\alpha-\gamma$.

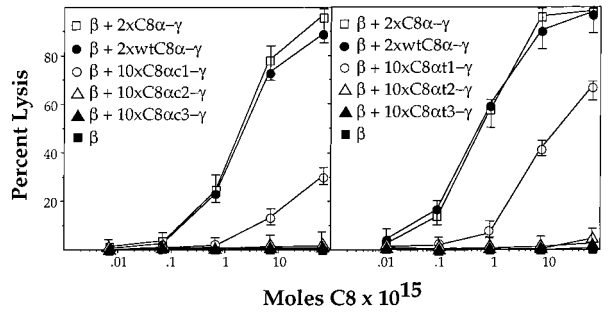

FIGURE 6: Activity of chimeric and truncated C8$\alpha-\gamma$. Hemolysis of EAC1−7 was used to measure the activity of chimeric (left) and truncated (right) forms of C8$\alpha-\gamma$. Controls of human C8$\alpha-\gamma$ or wtC8$\alpha-\gamma$ and samples of chimeric or truncated C8$\alpha-\gamma$ were incubated at the indicated molar excess (*x*-fold) over C8β and assayed for hemolytic activity in the presence of C9. Moles of C8 added are based on C8β as the limiting reagent. Samples assayed in the absence of C8β or C9 exhibited no measurable activity. Shown are results from a representative experiment. Error bars indicate the deviation observed for samples from three different expressions.

that chimera C8αc1$-\gamma$ and its truncated analogue C8αt1$-\gamma$ are hemolytically active when incubated at a 10-fold molar excess over C8β. As expected, activities of human C8$\alpha-\gamma$ and wtC8$\alpha-\gamma$ controls are identical under these conditions (*6*). When the amounts of C8$\alpha-\gamma$ required to achieve 30% lysis are compared, the respective activities of C8αc1$-\gamma$ and C8αt1$-\gamma$ are ~1% and 10% of the controls. Addition of higher excesses of each chimera or truncate (20- and 40-fold over C8β) produced the same results, suggesting that low activities are not due to incomplete combination with C8β (not shown). Although low, the activities of C8αc1$-\gamma$ and C8αt1$-\gamma$ are significant when compared to the other chimeric and truncated forms of C8$\alpha-\gamma$, which all have <0.01% the activity of the control.

DISCUSSION

Previous studies have shown that human C8α, C8β, and C8γ can be expressed independently as recombinant proteins in mammalian and insect cell lines (*5*, *6*). Furthermore, coexpression of C8α and C8γ in the same cell produces a recombinant form of C8$\alpha-\gamma$ that is capable of combining with human C8β and expressing C8 lytic activity. Results in the present study extend these observations by demonstrating for the first time that chimeric and truncated forms of recombinant C8$\alpha-\gamma$ can also be produced when the respective mutant forms of C8α are coexpressed with C8γ.

The highly conserved and modular structure of C8α and C8β makes them well-suited for designing chimeric analogues that can provide insight into the location of functionally important sites in C8. In this study, pairs rather than individual modules were exchanged in order to achieve proper cross-linking of intrachain disulfide bonds. Although disulfide cross-links within the N-terminal TSP1 and LDLR modules of C8α and C8β are predicted to be internal to each module, the C-terminal EGF and TSP1 modules contain an odd number of Cys and thus are presumed to share one disulfide bond (*2*, *27*). By exchanging pairs, the likelihood for proper cross-linking is increased. For similar reasons as well as a desire to gain information on the role of the modules in mediating C8 subunit interactions, pairs rather than individual modules were deleted to produce truncated C8α.

Our results indicate the N- and C-terminal modules in C8α are of little importance to the interaction with C8γ. Evidence that this interaction involves a specific binding site on C8α was initially obtained in studies using subunits that were isolated after selective cleavage of the interchain disulfide bond in C8$\alpha-\gamma$ (*8*). It was shown that purified C8α and C8γ retain the capacity to interact noncovalently and form a 1:1 complex in solution. The significance of this interaction relates to the fact that C8α and C8γ are products of different genes. Because they are translated independently, they must associate intracellularly prior to disulfide bond formation and secretion of C8$\alpha-\gamma$. Results in the present study provide evidence that this interaction involves the MACPF segment of C8α. Chimeras containing this segment of C8α and modules from C8β were capable of intracellular association with C8γ to produce a disulfide-linked dimer that was secreted by transfected cells. Truncated forms of C8α lacking one or both pairs of modules were likewise secreted as disulfide-linked dimers with C8γ. Particularly significant is the observation that the MACPF fragment alone can form a dimer with C8γ. This is compelling evidence that the site for intracellular binding of C8γ is contained entirely within this segment of C8α. Furthermore, it indicates that neither the N-terminal TSP1 + LDLRA nor the C-terminal EGF + TSP1 modules contibute directly to the formation of this site.

The ability of the MACPF fragment alone to form a dimer with C8γ suggests the binding site in C8α may be associated with a specific sequence rather than a conformationally defined feature. Regarding this, it is of interest to note that when conserved sequences of all five MAC family members are aligned, C8α is unique in that it contains an insertion of 17 residues at position 159−175 (*2*). This includes Cys^{164} that is normally linked to C8γ. Insertions in otherwise homologous proteins are frequently associated with sites of protein−protein interaction because they often contain residues located in loops at the protein surface (*28*, *29*). In the case of the C3/C4/C5 family, insertion/deletions (indels) have been correlated with distinct binding functions performed by each family member (*30*, *31*). While the above sequence may confer on C8α the distinctive ability to form

a dimer with C8γ, it remains to be determined if it constitutes the actual binding site or if it simply provides an available, surface-exposed Cys residue.

Our results indicate the MACPF region of C8α also mediates the interaction between C8α−γ and C8β. At physiological ionic strength, all three C8α−γ chimeras were capable of forming a 1:1 complex with C8β. Formation of complexes at the low protein concentrations (0.2−0.4 μg/mL) used in these experiments is a qualitative indication that the affinity for C8β must be high. When results are compared, C8αc1−γ and wtC8α−γ have similar affinities for C8β as evidenced by the requirement of only a 10-fold molar excess to achieve complete combination. In contrast, C8αc2−γ and C8αc3−γ required higher excesses, which suggests a lower but nonetheless significant affinity for C8β. Corresponding experiments with truncated forms of C8α−γ yielded similar conclusions about the role of the MACPF. All three truncates were capable of forming a 1:1 complex with C8β, including C8αt3−γ, which contains only the MACPF segment of C8α.

The observation that C8αc1−γ and the corresponding truncate C8αt1−γ are hemolytically active in the presence of C8β is evidence that the binding observed on density gradients is functionally significant. Incorporation of C8 into the MAC is mediated by C8β, whereas incorporation of C9 is mediated by C8α; therefore expression of hemolytic activity can only be expected if there is appropriate physical interaction between C8α−γ and C8β either prior to or during formation of the MAC (*10*, *12*). Also noteworthy is the ionic strength of the isotonic buffer used in the activity assays, which is higher than that used in binding experiments with truncated forms of C8α−γ (72.7 vs 16.25 mM NaCl). Activity of C8αt1−γ in hemolytic assays is evidence that C8β physically interacts with this truncate even at this higher ionic strength. We cannot unequivocally conclude the same for the other two truncates of C8α−γ because neither is hemolytically active and binding studies using these constructs were only performed at the lower ionic strength. However, the fact that no activity was detected at several different excesses over C8β suggests the lack of hemolytic function is not due to failure to combine with C8β at this ionic strength.

Expression of hemolytic activity by C8αc1−γ and C8αt1−γ is evidence that both retain the ability to bind C9 and form a functional MAC. C8α has the capacity to bind at least one C9 and our results indicate that a site(s) contained within residues 1−463 (TSP1 + LDLRA + MACPF) of C8α is involved in this interaction. The lack of activity observed for those constructs in which the N-terminal modules have been exchanged or deleted suggests the TSP1 + LDLRA segment is particularly important for C9 binding and/or expression of C8 activity. It should be emphasized that our data do not exclude the possibility of a direct role for the C-terminal modules in either C9 binding or C8 function. Indeed, the higher hemolytic activity of wtC8α−γ suggests the C-terminal modules enhance C9 binding and/or the lytic function of the MAC, perhaps by providing additional contact sites for C9. Experiments to measure direct binding of C9 to each recombinant form of C8α−γ are currently in progress and should clarify the role of the modules in this interaction.

In conclusion, our results provide evidence that structural features located within the MACPF region of C8α confer on this subunit the ability to specifically interact with C8β and C8γ. Moreover, neither the N- nor C-terminal modules in C8α are absolutely required for binding to either subunit. For C8γ, this is not surprising since the Cys to which it is linked is located within the MACPF of C8α. What is surprising is the ability of the MACPF fragment alone to form a dimer when coexpressed with C8γ. This suggests the MACPF may be an independently folded segment of C8α that does not require an intact N- or C-terminus for biosynthetic processing. With respect to C8β, binding of this subunit also involves a site located within the MACPF of C8α. However, the modules appear to contribute to this binding as evidenced by the higher affinity of C8β for wt C8α−γ as compared to chimeric C8α−γ at physiological ionic strength. Whether the MACPF region of C8β has a corresponding function in binding to C8α is currently under investigation. It may be that the unique interaction between C8α and C8β within C8 involves the MACPF region of each subunit, whereas sequential binding of components during MAC formation is dependent on specific interactions between modules.

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BI9904858