Functional Studies of the MACPF Domain of Human Complement Protein C8α Reveal Sites for Simultaneous Binding of C8*β*, C8*γ*, and C9[†]

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ABSTRACT: Human C8 is one of five components of the membrane attack complex of complement (MAC). It contains three subunits (C8α, C8*β*, C8*γ*) arranged as a disulfide-linked C8α-*γ* dimer that is noncovalently associated with C8*β*. C8α, C8*β*, and complement components C6, C7, and C9 form the MAC family of proteins. All contain N- and C-terminal modules and an intervening 40-kDa segment referred to as the membrane attack complex/perforin (MACPF) domain. During MAC formation, C8α binds and mediates the self-polymerization of C9 to form a pore-like structure on target cells. The C9 binding site was previously shown to reside within a 52-kDa segment composed of the C8α N-terminal modules and MACPF domain (αMACPF). In the present study, we examined the role of the MACPF domain in binding C9. Recombinant αMACPF and a disulfide-linked αMACPF-*γ* dimer were successfully produced in *Escherichia coli* and purified. αMACPF was shown to simultaneously bind C8*β*, C8*γ*, and C9 and form a noncovalent αMACPF·C8*β*·C8*γ*·C9 complex. Similar results were obtained for the recombinant αMACPF-*γ* dimer. This dimer bound C8*β* and C9 to form a hemolytically active (αMACPF-*γ*)·C8*β*·C9 complex. These results indicate that the principal binding site for C9 lies within the MACPF domain of C8α. They also suggest this site and the binding sites for C8*β* and C8*γ* are distinct. αMACPF is the first human MACPF domain to be produced recombinantly and in a functional form. Such a result suggests that this segment of C8α and corresponding segments of the other MAC family members are independently folded domains.

Human C8 is one of five complement components (C5b, C6, C7, C8, and C9) that interact to form the membrane attack complex (MAC) (*1*, *2*). It is composed of an α (64-kDa), *β* (64-kDa), and *γ* (22-kDa) subunit, which are products of different genes (*3*, *4*). Within C8, these subunits are arranged as a disulfide-linked C8α−*γ* heterodimer that is noncovalently associated with C8*β*. C8α and C8*β* are homologous and together with C6, C7, and C9 form the MAC family of proteins (*5*, *6*). All contain tandemly arranged N- and C-terminal modules and an intervening 40-kDa segment referred to as the MACPF[1] domain. C8*γ* is unrelated to any complement protein and is a member of the lipocalin family of proteins that bind small, hydrophobic ligands (*7*, *8*). Assembly of the MAC involves highly specific protein−protein interactions; thus each component must have a binding site that recognizes the succeeding one incorporated into the MAC. Structures have not been determined for any of the MAC family proteins, and little is known about the location or properties of their binding sites.

Several C8 binding interactions are known to involve the C8α subunit. Although normally linked to C8*γ*, C8α can also bind C8*γ* noncovalently and with high affinity (*9*). Presumably, this interaction facilitates intracellular formation of the interchain disulfide bond in C8α−*γ*. C8α is also capable of independently binding C8*β*; thus C8α mediates the interaction between C8α−*γ* and C8*β* to form C8 (*10*, *11*). C8α also contains a binding site for CD59, the membrane-associated regulatory protein that inhibits formation of a functional MAC (*12*). When incorporated into the MAC, C8 binds and facilitates the self-polymerization of C9 to produce a functional pore on target cell membranes (*1*). Of the three C8 subunits, only C8α has been shown to bind C9 in solution (*13*).

Our initial efforts to localize the C9 binding site on C8α used COS cells to express C8α constructs in which the N- and/or C-terminal modules were either deleted or substituted with the corresponding modules from C8*β* (*14*, *15*). Although not expressed independently, the MACPF domain of C8α could be coexpressed with C8*γ* and secreted as a disufide-linked αMACPF-*γ* dimer. Binding was examined using density gradient centrifugation to detect the formation of complexes. Because constructs were expressed at levels insufficient for purification, potential binding interactions were studied in crude expression media. The expressed αMACPF-*γ* dimer when combined with C8*β* exhibited little affinity for C9 whereas binding could be detected with constructs containing both the N-terminal modules and MACPF portion of C8α. Although these results suggested that the N-terminal modules are involved in binding, the inability to express the αMACPF alone and the use of

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[1] Abbreviations: MACPF, membrane attack complex/perforin; αMACPF, the MACPF segment of C8α; αMACPF-*γ*, disulfide-linked dimer of αMACPF and C8*γ*; EAC1−7, sensitized sheep erythrocytes carrying human complement C1−C7.

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expression media to study binding interactions did not allow for a rigorous assessment of their importance.

We have now extended these studies and focused specifically on the role of the C8α MACPF domain in binding C9. In this report, we describe the first production and functional characterization of a recombinant human MACPF domain. Recombinant αMACPF produced in *Escherichia coli* binds C8β and C8γ, either separately or together. When bound together, the resulting noncovalent αMACPF·C8β·C8γ complex binds C9. Similar results were obtained using a recombinant disulfide-linked αMACPF-γ dimer, which was also produced in *E. coli*. αMACPF-γ binds C8β and C9 to form a hemolytically active (αMACPF-γ)·C8β·C9 complex. Together, these results indicate that the principal C9 binding site lies within the MACPF segment of C8α. They also suggest that this segment and corresponding segments of the other MAC family members are independently folded domains.

EXPERIMENTAL PROCEDURES

Proteins. 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 strength buffer (*17*). Molar concentrations were determined using published extinction coefficients (*17*). Recombinant human C8γ containing a C-terminal 6×His tag was produced in *E. coli* and purified. The crystal structure revealed a peptide backbone identical to human C8γ produced in insect cells[2] (*8*). An experimental extinction coefficient of $\epsilon_{280}^{1\%} = 17.4$ was used to determine the concentration of recombinant C8γ. Concentrations of purified αMACPF and αMACPF-γ were determined using theoretical extinction coefficients of $\epsilon_{280}^{1\%} = 12.0$ and 11.0, respectively. Affinity-purified goat anti-human C8α, C8β, and C8γ antibodies and rabbit anti-human C8α and C8γ antibodies were prepared as described (*14*). Total chicken IgY against human C8γ was prepared by Aves Labs.

Cloning and Expression of α*MACPF*. A full-length human C8α cDNA was used as a template to create an αMACPF construct containing residues 103−462 of C8α (*14*). Overlap−extension PCR was used to create a C164 → A164 mutation that eliminates the Cys normally linked to C40 in C8γ. The second round of PCR used primers specific for incorporation of αMACPF into the ligation-independent expression vector pMCSG7 (*18*). pMCSG7 contains a 21-residue N-terminal leader sequence consisting of a 6×His tag and a TEV cleavage site. After cleavage with TEV, the αMACPF retains three vector-derived residues (SNA) at the N-terminus. To generate the ligation-independent cloning site in pMCSG7, plasmid DNA was linearized with the restriction enzyme SSPI. The vector and PCR product were treated with T4 DNA polymerase in the presence of dGTP or dCTP, respectively, to create products with complementary overhangs. The resulting plasmid was used to transform Origami B(DE3) cells (Novagen) for protein expression. Bacteria were grown at 37 °C in LB media (1% Bacto tryptone, 0.5% Bacto yeast extract, 171 mM NaCl, 50 μg/mL carbenicillin, 50 μg/mL kanamycin). Cells were induced with 1 mM IPTG, and protein was expressed at 23 °C.

[2] B. Chiswell and J. M. Sodetz, unpublished results.

Cloning and Expression of α*MACPF-*γ. αMACPF and full-length C8γ were both cloned into the *E. coli* dual expression vector pET-Duet-1 (Novagen). This vector contains cloning sites for coexpression of two target genes. For C8γ, a wild-type human C8γ cDNA containing C40 was used as a template (*14*). Overlap−extension PCR was used to introduce a silent mutation that eliminated an internal *Nco*I site. The second round of PCR was performed using primers specific for the incorporation of C8γ into the *Nco*I and *Hin*dIII sites in the pET-Duet-1 multiple cloning site 1. This introduced an alanine between the initiation methionine and the N-terminus of C8γ. Primers used in this round were also designed to incorporate a 6×His tag at the C-terminus of C8γ. The C8γ PCR product and pET-Duet-1 vector were digested with *Nco*I and *Hin*dIII, ligated, and transformed into XL1-Blue cells for plasmid purification.

αMACPF containing C164 was produced by PCR using wild-type human C8α cDNA as a template. Primers were designed to introduce flanking *Nde*I and *Kpn*I restriction sites for cloning into the multiple cloning site 2 of pET-Duet-1. The αMACPF PCR product and pET-Duet-1 vector containing inserted C8γ were digested with *Nde*I and *Kpn*I and ligated together. Origami B(DE3) cells were transformed with the plasmid, and the αMACPF-γ dimer was expressed as described for αMACPF.

Purification of α*MACPF and* α*MACPF-*γ. Cells containing expressed αMACPF were harvested by centrifugation and lysed using BugBuster HT protein extraction reagent (Novagen) containing 5% (v/v) Triton X-100, 0.1 mg/mL lysozyme, and the protease inhibitors AEBSF (1 mM) and E-64 (10 μM) (Calbiochem). After lysis and centrifugation, the supernatant was applied to a Ni-NTA Superflow column (Qiagen). The column was washed with 50 mM Tris, 300 mM NaCl, and 20 mM imidazole, pH 8.0. Bound αMACPF was eluted with a 20−500 mM imidazole gradient in the same buffer. Fractions containing αMACPF were collected and applied to a S-15Q anion-exchange column (Amersham) in 50 mM Tris and 50 mM NaCl, pH 8.0. The column was washed and the protein eluted with a 0.05−1.0 M NaCl gradient in the same buffer. To remove the N-terminal 6×His tag, pooled αMACPF was incubated with Ac-TEV protease (Invitrogen) in 50 mM Tris and 150 mM NaCl, pH 8.0 at 23 °C. After cleavage, the reaction mixture was passed through a Ni-NTA column to remove the tag and Ac-TEV protease. The molecular mass was determined by electrospray mass spectrometry.

Cells containing expressed αMACPF-γ were harvested and lysed as described above. Soluble protein was applied to a Ni-NTA Superflow column and eluted as described for αMACPF. Fractions containing αMACPF-γ were dialyzed into 50 mM sodium phosphate and 50 mM NaCl, pH 5.7, and applied to a S-15S cation-exchange column (Amersham). The column was washed and eluted with a 0.05−1.0 M NaCl gradient in the same buffer. Purified αMACPF-γ was dialyzed into 50 mM Tris and 150 mM NaCl, pH 8.0, for storage. The mass was determined as described above.

Binding Assays. Binding interactions were measured using sucrose density centrifugation to detect the formation of complexes. Incubations were performed in buffers typically used in C8 hemolytic activity assays (5 mM imidazole, 72 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4, 1 mg/mL BSA). Incubation mixtures were adjusted to 2.5% (w/v)

sucrose and layered onto 4 mL 5−10% (w/v) sucrose density gradients prepared in the same buffer. Gradients were centrifuged for 2 h at 4 °C in a Sorvall VTi65 rotor at 202000*g* as described (*11*). Gradients were fractionated from the top.

Binding between αMACPF, C8*β*, and C8*γ* was examined under conditions where the concentration of one was limiting (180, 280, and 100 ng/mL, respectively) and the others were added in molar excess. Binding of C9 was measured by incubating a molar excess of C9 with each individual component or with mixtures of αMACPF, C8*β*, and C8*γ*. After fractionation, the sedimentation position of each component was determined by an ELISA specific for the limiting component. To capture αMACPF, goat anti-human C8α antibody was plated onto microtiter plates in 0.1 M sodium bicarbonate, pH 8.5. Captured αMACPF was detected using rabbit anti-human C8α as the primary and HRP-conjugated goat anti-rabbit IgG as the secondary antibody. For C8*β*, goat anti-human C8*β* was plated as the capture antibody, and rabbit anti-human C8*β* was used as the primary antibody. C8*γ* was captured with plated rabbit anti-human C8*γ* antibody and detected with chicken anti-human C8*γ* as the primary and HRP-conjugated goat anti-chicken IgY as the secondary antibody. Plates were developed as described previously (*14*).

Binding between the αMACPF-*γ* dimer, C8*β*, and C9 was examined using similar assays. The concentration of αMACPF-*γ* was limiting (280 ng/mL), and the other components were added in excess. Binding of C9 was measured by adding excess C9 to αMACPF-*γ* or to a mixture of αMACPF-*γ* and C8*β*. Samples were subjected to density gradient centrifugation, and the position of complexes containing αMACPF-*γ* was determined by an ELISA.

Hemolytic Activity Assays. Assays were performed to determine if complexes containing C9 were hemolytically active. A fixed amount of C8*β* (4 *μ*g/mL) was incubated with molar excesses of αMACPF and C8*γ* or αMACPF-*γ* before C9 was added. Incubation mixtures were serially diluted in isotonic buffer (5 mM imidazole, 72 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 EAC1−7 as described (*11*). The amount of C9 normally used in C8 hemolytic assays (100 ng/mL) was increased up to 500-fold.

RESULTS

Expression and Purification of αMACPF and αMACPF-γ. The MACPF domain of C8α was expressed in *E. coli* as a soluble fusion protein with a cleavable N-terminal 6×His tag. Cleavage with TEV protease and removal of the tag yielded a product of the predicted size (40 kDa) (Figure 1). The yield of purified αMACPF after removal of the tag was ∼2 mg/L of growth. The mass was determined to be 40857 Da, in good agreement with a theoretical mass of 40859 Da. Upon reduction with *β*-mercaptoethanol, there is a significant mobility shift on SDS−PAGE gels, indicating that one and most likely both disulfide bonds are intact.

Figure 1 also shows that a soluble disulfide-linked αMACPF-*γ* dimer can be produced when αMACPF and C8*γ* are coexpressed in *E. coli*. Recovery of purified αMACPF-*γ* was ∼2 mg/L of growth. The mass of the dimer was determined to be 61790 Da. The theoretical mass is 62099 Da. It is noted that residues penultimate to the initiation methionine on αMACPF and C8*γ* are valine and alanine, respectively. Having these as penultimate residues means it is highly likely that both initiation methionines were enzymatically removed in the *E. coli* cytosol (*19*). If so, the theroretical mass becomes 61837 Da. After reduction, proteins of the size expected for αMACPF (40 kDa) and C8*γ* (22 kDa) are observed.

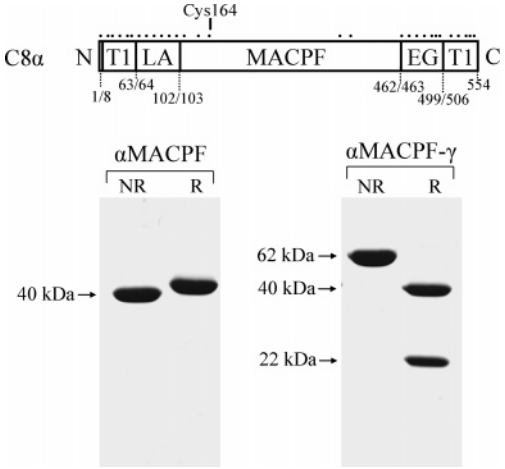

FIGURE 1: Purified αMACPF and αMACPF-*γ*. Shown at the top is a map of full-length C8α. Modules correspond to thrombospondin type 1 (T1), low-density lipoprotein receptor class A (LA), and epidermal growth factor (EG). Residue numbers identify module boundaries. Dots identify approximate locations of Cys residues. All form internal disulfide bonds except C164, which normally links to C40 in C8*γ*. The MACPF segment contains two disulfide bonds. Also shown are SDS−PAGE gels of nonreduced (NR) and reduced (R) samples of purified αMACPF and αMACPF-*γ*. Gels were stained with Coomassie Blue.

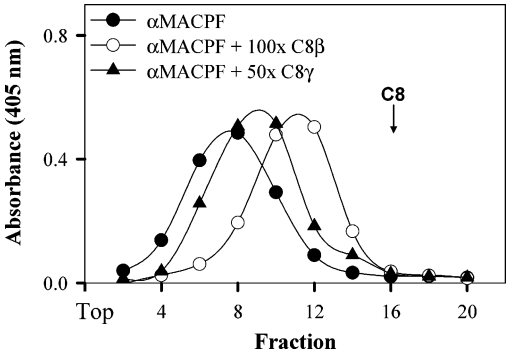

FIGURE 2: Binding of C8*β* and C8*γ* to αMACPF. Purified αMACPF was incubated with the indicated molar excess (×) of C8*β* or C8*γ* and subjected to sucrose density centrifugation. Gradients were fractionated from the top, and the sedimentation position of αMACPF was determined by an ELISA. The shift in sedimentation position of αMACPF in the presence of C8*β* or C8*γ* agrees with the predicted mass of an αMACPF·C8*β* (104 kDa) or αMACPF·C8*γ* (62 kDa) complex. Higher excesses of C8*β* or C8*γ* produced no further shifts. Control samples prepared with C8*β* or C8*γ* alone produced no signal in the ELISA. The sedimentation position of a C8 marker (151 kDa) is shown for reference.

Binding of C8β and C8γ to αMACPF. To determine if recombinant αMACPF is functional, the purified product was tested for its ability to bind C8*β* and C8*γ*. Results in Figure 2 show that incubation of αMACPF with excess C8*β* or C8*γ* produces αMACPF·C8*β* or αMACPF·C8*γ* complexes that are detectable on density gradients. Even at the low

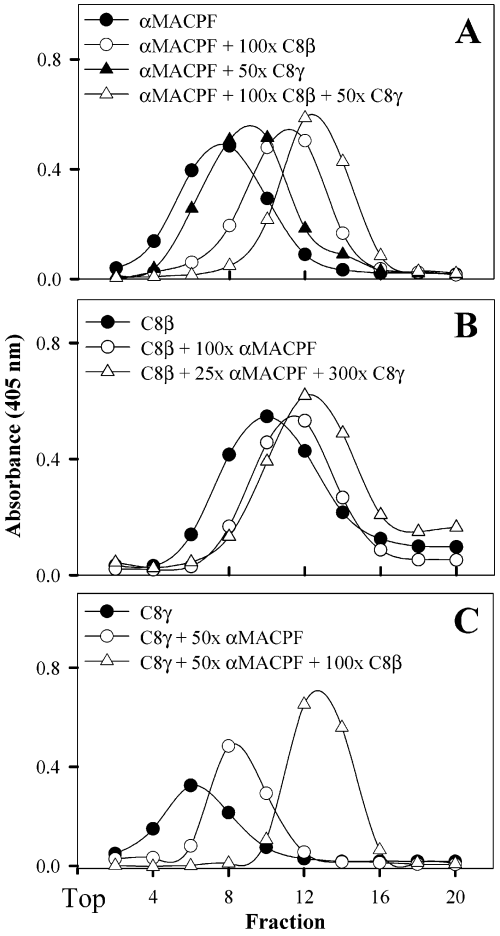

FIGURE 3: Simultaneous binding of C8β and C8γ to αMACPF. Mixtures of αMACPF, C8β, and C8γ were prepared in which the concentration of one component was limiting and one or both of the others added at the indicated molar excess (×). Samples were subjected to density gradient centrifugation, and the sedimentation position of the limiting component was determined by an ELISA. Molar excesses required for optimum formation of αMACPF·C8β·C8γ are shown. No further shift in sedimentation was observed with higher excesses. Control samples in which the limiting component was omitted produced no signal in an ELISA. The limiting component is αMACPF (A), C8β (B), and C8γ (C).

concentration of αMACPF used in the assay (~180 ng/mL), only moderate excesses of C8β or C8γ are needed for complete binding.

The ability of αMACPF to simultaneously bind C8β and C8γ was examined using a similar approach. Mixtures of αMACPF, C8β, and C8γ were prepared in which the concentration of one component was limiting and the other two were added in excess. Results from density gradient centrifugation experiments in Figure 3 show that all three components combine to form a noncovalent αMACPF·C8β·C8γ complex of the expected size (~126 kDa). The same complex is formed regardless of which component is limiting, thus confirming that all three proteins are present. It is noted that when using C8γ as the limiting component (Figure 3, panel C), the ELISA sensitivity appears to be higher for the complexes as compared to C8γ alone. This was observed in several other binding experiments described below. We attribute this to epitope changes that can occur as the monomer form of a component is incorporated into a complex.

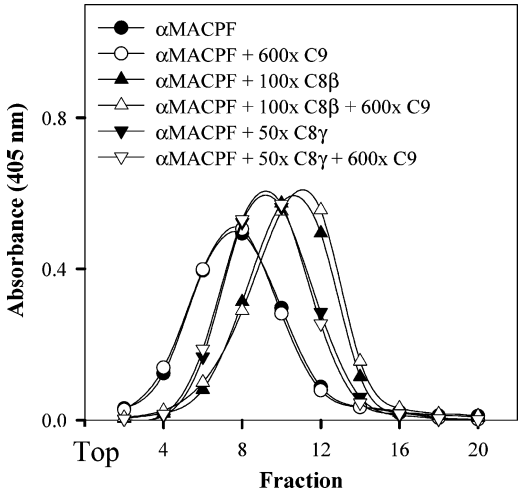

FIGURE 4: Analysis of C9 binding to αMACPF. Incubation mixtures contained αMACPF as the limiting component. C9 was added at the indicated molar excess (×) to samples containing αMACPF alone or αMACPF that had been preincubated with excess C8β or C8γ to form αMACPF·C8β and αMACPF·C8γ, respectively. Samples were subjected to density gradient centrifugation, and the sedimentation position of αMACPF was determined by an ELISA.

Binding of C9 to αMACPF. Experiments similar to those above were performed to determine if αMACPF alone can bind C9. No binding was observed with C9 excesses as high as a 600-fold (Figure 4). Binding to preformed αMACPF·C8β and αMACPF·C8γ complexes was also examined, and again no binding was observed. By contrast, results in Figure 5 show that C9 does bind to the trimeric αMACPF·C8β·C8γ complex. This complex was formed using either αMACPF, C8β, or C8γ as the limiting component. In all cases, C9 binding was complete when added at a 300-fold excess over the limiting component. The ability to detect an αMACPF·C8β·C8γ·C9 complex when following either αMACPF, C8β, or C8γ indicates that all three proteins are present in addition to C9.

Experiments to determine if the αMACPF·C8β·C8γ·C9 complex is hemolytically active toward EAC1−7 were inconclusive. In hemolytic assays that use C8 subunits, C8β must be held as the limiting component. This is because C8β alone binds to EAC1−7; thus any free C8β will compete with C8 and inhibit activity (*20*). In the present study, the excess αMACPF and C8γ needed to saturate C8β and maximize formation of αMACPF·C8β·C8γ interfered with the assay.

C9 Binding and Hemolytic Activity of αMACPF-γ. Binding between C9 and complexes containing αMACPF was also examined using recombinant αMACPF-γ. This dimer was assayed for its ability to bind C8β and C9. Results in Figure 6 show that αMACPF-γ at low concentrations (280 ng/mL) is capable of binding C8β; however, it does not bind C9. This agrees with results obtained using a mixture of αMACPF and C8γ (Figure 4). Figure 6 shows that C9 does bind to preformed (αMACPF-γ)·C8β to form (αMACPF-γ)·C8β·C9. These results agree with those obtained for C9 and a mixture of αMACPF, C8β, and C8γ (Figure 5).

Results in Figure 7 show that αMACPF-γ when combined with C8β has significant hemolytic activity in the presence of C9. C9 levels were increased over those normally used in C8 hemolytic assays because the affinity for

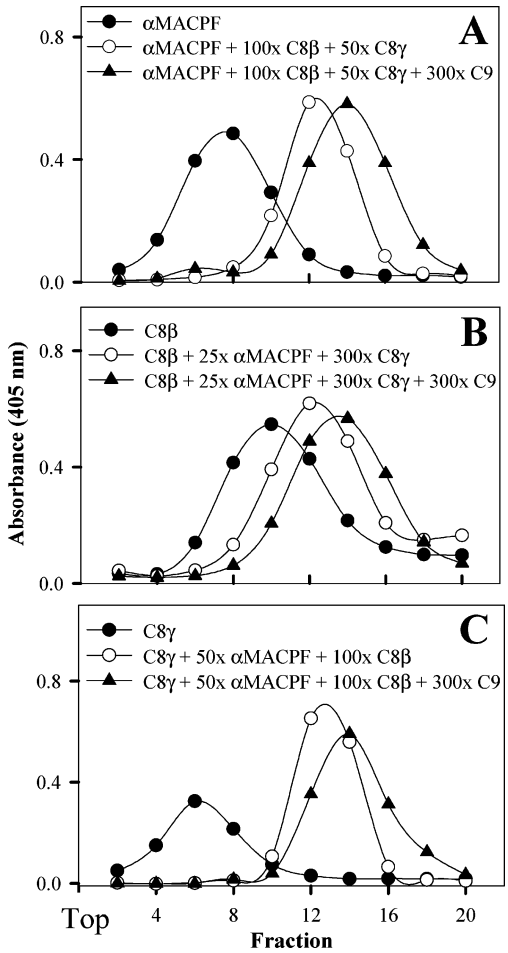

FIGURE 5: Binding of C9 to αMACPF·C8β·C8γ. Incubation mixtures of αMACPF, C8β, and C8γ were prepared in which one component was limiting and the others were added at a molar excess (×). C9 was then added in excess over the limiting component, and the sedimentation position of that component was followed by an ELISA. In all cases, higher excesses of C9 produced no further shift in sedimentation. Controls showed no C9 binding to C8β or C8γ alone. The limiting component is αMACPF (A), C8β (B), and C8γ (C).

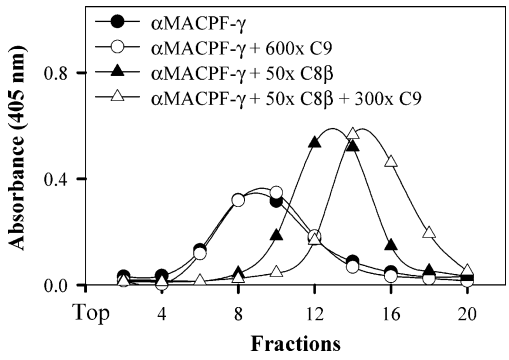

FIGURE 6: Binding of C8β and C9 to αMACPF-γ. αMACPF-γ was incubated with the indicated excesses (×) of C8β and C9. Samples were subjected to density gradient centrifugation, and the sedimentation position of αMACPF-γ was followed by an ELISA.

(αMACPF-γ)·C8β as compared to C8 was expected to be lower. Lower affinity is apparent from the results. For each amount of C9 used in the assay, the activity reaches a plateau when increasing amounts of (αMACPF-γ)·C8β are added. This indicates saturation of EAC1−7 with (αMACPF-γ)·C8β. The level of activity at saturation varies in accordance with the amount of C9 added, which is consistent with increased C9 binding to (αMACPF-γ)·C8β.

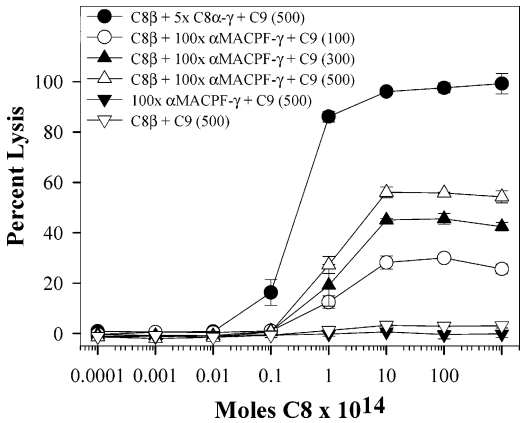

FIGURE 7: Hemolytic activity of αMACPF-γ. A limiting amount of C8β was preincubated with a molar excess (×) of αMACPF-γ, serially diluted, and assayed for hemolytic activity in the presence of C9. The amount of C9 was increased (100-, 300-, and 500-fold) over that normally used in assays of C8. A mixture of C8β + 5× C8α-γ was used as a C8 reference. Theoretical moles of C8 formed are based on C8β as the limiting component. Results are the average of two different assays.

DISCUSSION

Results from this study represent a significant advance toward understanding details of the molecular interactions that occur during MAC formation. We have shown for the first time that a human MACPF domain can be produced in *E. coli* and on a scale sufficient for purification and characterization. This is in contrast to our earlier efforts to produce the αMACPF and related C8α constructs in a COS cell expression system (*14*, *15*). In that system, the αMACPF was not expressed independently but could be coexpressed with C8γ as a disufide-linked αMACPF-γ dimer. Intracellular formation and secretion of this dimer was interpreted as evidence that αMACPF has a binding site for C8γ. This was later confirmed when the binding site was localized to residues 157−175 within C8α (*21*). The αMACPF-γ produced by COS cells also bound C8β; thus it was proposed that the MACPF segment of C8α contains a second binding site for C8β. The present study supports these conclusions and shows that when expressed independently and purified, αMACPF can bind C8β and C8γ. The ability to bind both subunits simultaneously indicates that the respective binding sites are distinct. Binding of both subunits also suggests that the conformation of recombinant αMACPF is similar to that of the MACPF domain in full-length C8α.

Although previous studies suggested a role for the N-terminal modules and MACPF domain of C8α in binding C9, the use of COS cell expression media rather than purified constructs limited the sensitivity of the binding assays. As a result, the relative importance of the modules as compared to the MACPF domain could not be determined. In the present study, availability of a purified C8α MACPF domain enabled us to focus more specifically on its role in binding C9. Results indicate that αMACPF alone has little affinity for C9 even when the latter is present in large excess (Figure 4). Similar results were obtained with the noncovalent αMACPF·C8β and αMACPF·C8γ complexes. By contrast,

C9 readily binds to αMACPF·C8β·C8γ. Binding requires only a moderate excess of C9 and is not dependent on the order in which the trimeric complex is assembled (Figure 5). The affinity for C9 must be relatively high since components are incubated at extremely low concentrations, yet complexes can be detected after centrifugation in the presence of serum albumin.

Corresponding results were obtained with αMACPF-γ. Production of this dimer in *E. coli* is in itself significant. The ability to associate with C8γ in the cytoplasm and spontaneously form an interchain disulfide bond is compelling evidence that bacterially expressed αMACPF is properly folded. When assayed, αMACPF-γ alone did not bind C9. However, C9 did bind to (αMACPF-γ)·C8β, which agrees with the results obtained for the noncovalent αMACPF·C8β·C8γ complex. Importantly, (αMACPF-γ)·C8β exhibits hemolytic activity in the presence of C9, thus confirming that binding interactions observed in solution are functionally significant. Hemolytic activity of (αMACPF-γ)·C8β means that this complex is recognized by C5b-7, an intermediate in the MAC assembly pathway. C5b-7 normally binds C8 to form C5b-8, the immediate precursor of the MAC. The fact that (αMACPF-γ)·C8β binds to C5b-7 and then mediates incorporation of C9 to form a functional MAC suggests that the respective binding sites in this complex are structurally similar to the corresponding ones in intact C8.

C9 binding was only observed with trimeric complexes containing αMACPF, C8β, and C8γ. Binding was not detected with αMACPF alone or with complexes of αMACPF and C8β or C8γ. Apparently, the affinity for C9 increases as αMACPF combines with C8β and C8γ to form a complex whose structure more closely resembles that of C8. A similar trend occurs with full-length C8α; the affinity for C9 increases in the order C8α → C8α−γ → C8 (*13*). Because C9 binds to C8α, but not C8β or C8γ, one must conclude that interaction between C9 and αMACPF·C8β·C8γ or (αMACPF-γ)·C8β is mediated by a binding site located in αMACPF. These results indicate that the principal binding site for C9 is located within the MACPF domain of C8α, and while they may stabilize binding interactions, the C8α N-terminal modules are not essential.

In summary, the C8α MACPF domain has now been shown to contain binding sites for four different proteins. Sites for C8β, C8γ, and C9 appear to be distinct, whereas sites for C9 and CD59 are likely to be overlapping. The latter is suggested by the fact that CD59 is known to bind at a site located within the MACPF domain of C8α and inhibit incorporation of C9 into the MAC (*12*). We have shown that C9 also binds to the C8α MACPF domain; thus it is reasonable to suggest that the respective binding sites are mutually exclusive and either overlapping or in close proximity. Two different binding sites have also recently been identified in the MACPF domain of C8β.[3] This segment of C8β contains binding sites for C8α−γ and C5b-7. In light of these results, one can speculate that MACPF domains in C6, C7, and C9 also have specific binding functions. If so, binding interactions during MAC formation are not restricted to these domains. In at least one case, the modules themselves were found to have a key role in recognition and binding (*22*, *23*). Factor I modules in C7 were shown to mediate reversible binding to C5b within C5b-6, the initial complex formed in the MAC assembly pathway. It remains to be determined whether the modules or the MACPF domains are primarily involved in interactions between C6 and C7 or between these components and C8 and C9.

[3] C. L. Brannen and J. M. Sodetz, manuscript submitted for publication.

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