An Indel within the C8α Subunit of Human Complement C8 Mediates Intracellular Binding of C8γ and Formation of C8α-γ†

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ABSTRACT: 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. It is an oligomeric protein composed of three subunits (C8α, C8β, C8γ) that are products of different genes. In C8 from serum, these are arranged as a disulfide-linked C8α-γ dimer that is noncovalently associated with C8β. In this study, the site on C8α that mediates intracellular binding of C8γ to form C8α-γ was identified. From a comparative analysis of indels (insertions/deletions) in C8α and its structural homologues C8β, C6, C7, and C9, it was determined that C8α contains a unique insertion (residues 159−175), which includes Cys^{164} that forms the disulfide bond to C8γ. Incorporation of this sequence into C8β and coexpression of the resulting construct (iC8β) with C8γ produced iC8β-γ, an atypical disulfide-linked dimer. In related experiments, C8γ was shown to bind noncovalently to mutant forms of C8α and iC8β in which Cys^{164}→Gly^{164} substitutions were made. In addition, C8γ bound specifically to an immobilized synthetic peptide containing the mutant indel sequence. Together, these results indicate (a) intracellular binding of C8γ to C8α is mediated principally by residues contained within the C8α indel, (b) binding is not strictly dependent on Cys^{164}, and (c) C8γ must contain a complementary binding site for the C8α indel.

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, each of which is produced from a different gene (*3*). In C8 purified from serum, C8α and C8γ are linked by a single disulfide bond to form a C8α-γ dimer that is noncovalently associated with C8β. Although each subunit can be expressed and secreted independently as a recombinant protein (*4*, *5*), biosynthetic studies of C8 in hepatocytes suggest that formation of C8α-γ and its association with C8β normally occurs prior to secretion (*6*).

C8α and C8β are members of the homologous MAC family of proteins, which includes C6, C7, and C9 (*2*, *7*). The N- and C-terminal portions of each family member are comprised of modules that are also found in a wide variety of functionally unrelated proteins. The central, MACPF[1] portion of each is an extended region of homology that also exhibits sequence similarity to perforin. In contrast, C8γ is unrelated and belongs to the lipocalin family of widely distributed proteins that generally bind small hydrophobic ligands such as retinol, pheromones, odorants, etc. (*8*, *9*).

C8 contains multiple binding sites that mediate interactions between the individual subunits and between the subunits and other components of the MAC. The interaction of C8α-γ with C8β involves a specific binding site located on the C8α chain, and recent studies using chimeric and truncated forms of C8α have shown that this site lies within the MACPF region of C8α (*10*, *11*). This segment also contains a recognition site for CD59, a membrane-associated regulatory protein that binds C8α and inhibits formation of a functional MAC on host cells (*12*). Evidence suggests that C8α has several other binding sites including a site that binds C8γ, a site(s) that mediates binding and incorporation of C9 into the MAC, and one or more sites that are involved in direct interaction with the surface of the target cell membrane (*13*−*15*). The location of these sites is unknown as is the location of binding sites in C8β. The C8β sites are involved in interactions with C8α, as well as other components of the MAC and the cell membrane (*15*, *16*). In contrast to C8α and C8β, C8γ has no known binding function other than interacting with C8α; however, its lipocalin-like characteristics suggest it may also bind a small but as yet unidentified ligand.

The aim of the present study was to identify the C8γ-binding site in C8α and thereby gain additional insight into structure−function relationships in C8. Previous studies have shown that C8α has the ability to interact noncovalently with C8γ after chemical cleavage of C8α-γ (*13*). This result and the fact that C8α and C8γ must associate prior to intracellular formation of C8α-γ suggests that mutual binding sites exist on each subunit. In addition, a truncated derivative consisting of only the MACPF segment of C8α was recently shown to be expressed as a heterodimer with C8γ, thus providing experimental support for a C8γ-binding site in this region (*11*). When sequences from C8α and its structural homologues C8β, C6, C7, and C9 were compared, a unique

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[1] Abbreviations: MACPF, membrane attack complex/perforin; iC8β, C8β containing the indel from C8α.

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insertion was identified within the MACPF segment of C8α (*2*, *11*). This indel (insertion/deletion) includes the Cys residue that forms a disulfide bond with C8γ. Indels in otherwise homologous proteins have been shown to correspond to regions of functional significance (*17*−*19*); therefore, this segment of C8α was considered a potential C8γ-binding site. Accordingly, it was examined for its ability to mediate intracellular and extracellular binding of C8γ. Results indicate the C8α indel not only provides an available Cys for cross-linking to C8γ but it also contains a binding site for C8γ.

EXPERIMENTAL PROCEDURES

Materials. Human C8 was purified from plasma fraction III (Bayer Corp., Clayton, NC) (*20*). C8α-γ and C8β were separated by gel filtration in high ionic strength buffer (*21*). A mutant form of monomeric human C8γ containing a Cys^{40}→Gly^{40} substitution was produced in baculovirus-infected *Trichoplusia ni* cells (Invitrogen) and purified as described (*5*). Concentrations of purified C8 subunits were determined using published extinction coefficients (*5*, *10*). Goat antiserum against human C8 and rabbit antiserum against human C8α-γ or C8β were produced by standard procedures. Affinity resins consisting of agarose-bound C8α-γ, C8β, or mutant C8γ were used to isolate or if necessary deplete specific antibodies from the antisera. A peptide containing the C8α indel sequence was synthesized by the W. M. Keck Biotechnology Resource Center at Yale University, New Haven, CT.

Expression Constructs. cDNAs for wild-type human C8α, C8β, and C8γ, and the mutant forms of C8α and C8γ containing Cys→Gly substitutions (Cys^{164} and Cys^{40}, respectively) were previously cloned into the expression vector pcDNA3 (Invitrogen) as described (*4*, *5*). Insertion of sequence containing the C8α indel (residues 157−175) into C8β was accomplished by overlap extension PCR using the C8β pcDNA3 plasmid as a template. In the initial reaction, two internal primers each of which encoded a portion of the indel sequence and flanking C8β sequence were used in conjunction with a set of universal primers to produce self-priming 5′ and 3′ fragments. In a second reaction, these fragments were extended and amplified to produce a full-length C8β cDNA containing the C8α indel. This fragment was digested with *Xho*I/*Bsg*I and substituted into C8β pcDNA3 to generate iC8β. Positive clones were confirmed by sequencing. A mutant form of iC8β containing a Cys→Gly substitution within the C8α indel was also prepared by PCR site-directed mutagenesis as above. A full-length product containing the desired mutation was digested with HindIII/BlpI and substituted into C8β pcDNA3.

Expression in COS Cells. COS-7 cells were transfected as described previously (*5*). Control medium was prepared from identically treated but nontransfected cells. For immunoblotting, harvested medium was concentrated and subjected to SDS−PAGE. Proteins were transferred to nitrocellulose using standard methods and probed with either rabbit antisera or purified rabbit antibodies. Goat anti-rabbit IgG-horseradish peroxidase was used as the secondary antibody (Bio-Rad), and proteins were visualized using Super Signal chemiluminescent substrate (Pierce). Concentrations of expressed protein were determined using subunit-specific antibodies and quantitative immunoblotting or ELISA. Purified C8 or its subunits were used as standards.

Density Gradient Binding Studies. Binding interactions in solution were analyzed by sucrose density gradient centrifugation. Expression medium containing recombinant protein or control medium from nontransfected cells was concentrated 40−160-fold and dialyzed into gradient buffer (5 mM imidazole, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.4), which was adjusted to the desired concentration of NaCl. The concentration of expressed protein was measured and the media supplemented with 1 mg/mL BSA. Media samples were mixed with the desired C8 subunit, incubated for 1 h at 25 °C, and applied to a 5 to 10% (w/v) sucrose density gradient prepared in the same buffer containing 1 mg/mL BSA (*11*). Gradients were centrifuged for 2 h at 4 °C in a Sorvall VTi65 rotor at 202000*g*. After fractionation, the sedimentation position of the protein of interest was determined by ELISA as described below.

To detect mutant C8γ, purified rabbit anti-C8γ antibodies in 0.1 M sodium bicarbonate, pH 8.5, were adsorbed to microtiter plates for 30 min at 25 °C. Wells were washed with 20 mM imidazole, 100 mM NaCl, 5 mM $CaCl_2$, and 0.02% Tween-20, pH 7.5, treated with blocking buffer (50 mM Tris, 150 mM NaCl, and 10 mg/mL BSA, pH 7.4) and washed again. A portion of each gradient fraction was added and incubated for 30 min at 25 °C. After washing, bound C8γ was detected using purified goat anti-C8γ antibodies, followed by HRP-conjugated rabbit anti-goat IgG. Color was developed with 0.25 mg/mL 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) in 0.1 M sodium citrate and 0.01% hydrogen peroxide, pH 4.0. Detection of iC8β was performed similarly except that purified goat anti-C8β antibodies were plated and rabbit anti-C8β antibodies were used in the second step.

Binding to Immobilized C8α Indel Peptide. A synthetic C8α indel peptide containing an N-terminal Gly and an internal Cys→Gly substitution (GELRYDSTGERLYYGDDEKY) was coupled to CNBr-activated agarose at 0.5 mg/mL in 0.1 M sodium phosphate, pH 9.0. Control resin was prepared identically but in the absence of peptide. Expression medium containing mutant human C8γ was harvested from baculovirus-infected *Trichoplusia ni* cells and dialyzed into 10 mM sodium phosphate, pH 7.0. Approximately 80 mL was applied to a column (1 × 2 cm) of indel peptide-agarose resin equilibrated in the same buffer. The resin was washed with buffer containing 10 mM NaCl and bound protein was eluted with buffer containing 0.3 M NaCl. To assess nonspecific binding, a matched column containing control resin was prepared and treated identically. Corresponding fractions from each column were pooled and the eluted material was subjected to SDS−PAGE and stained for protein.

Activity Assays. Purified C8α-γ was added to concentrated and dialyzed expression media or control medium and incubated for 30 min at 25 °C. Samples were 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 C8 hemolytic activity toward sheep EAC1−7 as described (*5*). Percent lysis was expressed relative to a water-lysed control.

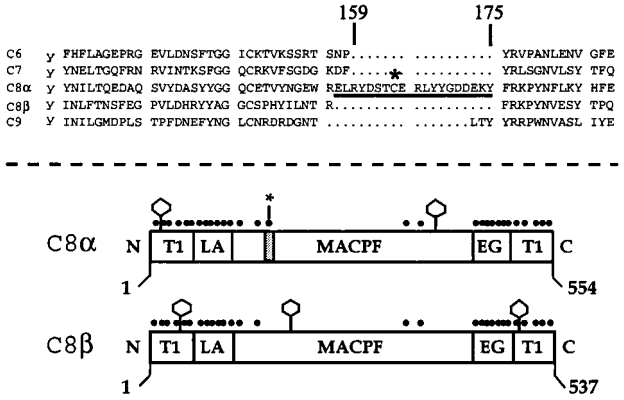

FIGURE 1: Indel sequence within C8α. (Top) Amino acid sequences corresponding to exon 5 of each member of the MAC protein family are aligned. Numbers identify the indel in C8α. Cys[164] is indicated by an asterisk. Sequence inserted into C8*β* is underlined. (Bottom) Comparison of the modular structure of C8α and C8*β*. Abbreviations correspond to the following modules: T1, thrombospondin type I; LA, low-density lipoprotein-receptor class A; EG, epidermal growth factor. Cys residues are indicated by dots; all form intrachain disulfide bonds and are conserved with the exception of Cys[164] in C8α. Hexagons identify possible N-glycosylation sites. The C8α indel is shaded.

RESULTS

With few exceptions, corresponding regions of the MAC family of proteins exhibit a high degree of sequence similarity. One of the exceptions occurs in the region corresponding to exon 5 of each protein. As shown in Figure 1, C8α contains an indel, which includes Cys[164] that forms the disulfide bond to Cys[40] in C8*γ*. To assess the importance of this indel in mediating formation of C8α-*γ*, a 19-residue segment containing this sequence was inserted into the corresponding region of C8*β* (Arg[155]−Phe[156]). The resulting iC8*β* construct was coexpressed with C8*γ* in COS-7 cells, and formation of an iC8*β*-*γ* dimer was determined by immunoblotting (Figure 2). In agreement with previous results (*5*), control experiments show that cells transfected with wild-type C8α produce a C8α monomer while cells that have been cotransfected with C8*γ* produce C8α-*γ* as the predominant product. Also as expected, corresponding controls transfected with wild-type C8*β* produce C8*β* monomer in either the absence or presence of C8*γ*. By contrast, cells transfected with iC8*β* produce a monomer in the absence of C8*γ*, but when cotransfected with C8*γ* a second product is formed which migrates at a position corresponding to an iC8*β*-*γ* dimer.

The identity of the iC8*β*-*γ* dimer was confirmed as shown in Figure 3. Immunoblots were probed separately with antibodies specific for C8*β* or C8*γ* to confirm the presence of epitopes from each subunit. Blots probed with antiserum specific for C8*β* indicate that in cells transfected with iC8*β* alone, only iC8*β* monomer is present. By comparison, samples from cells cotransfected with C8*γ* reveal both an iC8*β* monomer and iC8*β*-*γ* dimer. Upon reduction of iC8*β*-*γ*, only iC8*β* monomer is detected. A corresponding analysis with antiserum specific for C8*γ* confirms that C8*γ* is a component of iC8*β*-*γ*. Under nonreducing conditions, the iC8*β*-*γ* dimer is detected as well as some expressed C8*γ* monomer. Following reduction, only C8*γ* monomer is detected. Together, these results demonstrate that the iC8*β*-*γ* product observed in Figure 2 is in fact a disulfide-linked dimer of iC8*β* and C8*γ*.

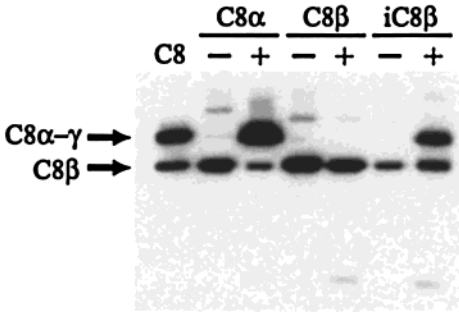

FIGURE 2: Expression of iC8*β*-*γ*. COS-7 cells were transfected with C8α, C8*β*, or iC8*β* in the absence (−) or presence (+) of wild-type C8*γ*. Media was subjected to SDS−PAGE under nonreducing conditions and immunoblotted with a mixture of rabbit anti-human C8α-*γ* and C8*β* antiserum. Arrows identify C8α-*γ* and C8*β* in the purified human C8 standard.

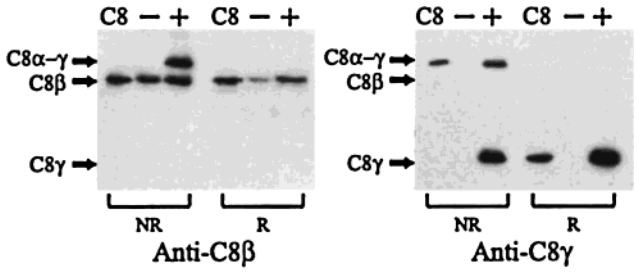

FIGURE 3: Immunoblot analysis of iC8*β*-*γ*. Medium from COS-7 cells transfected with iC8*β* in the absence (−) or presence (+) of wild-type C8*γ* was subjected to SDS−PAGE under nonreducing (NR) and reducing (R) conditions and analyzed by immunoblotting. Human C8 was used as a standard. Arrows indicate the expected mobilities of C8α-*γ*, C8*β* and C8*γ*. (Left) Immunoblot probed with rabbit antiserum specific for human C8*β*. (Right) Immunoblot probed with rabbit antiserum specific for human C8*γ*.

To determine if the indel simply provides an available Cys for cross-linking or if it also encodes a binding site, we examined the ability of iC8*β* to bind C8*γ* independently of Cys[164]. In these experiments, mutant forms of C8α and iC8*β* that contain Cys[164]→Gly[164] substitutions were compared. Previous studies showed that mutant C8α can be expressed at relatively high levels (*5*, *11*). Mutant iC8*β* is also expressed well but at ∼50% of the level of mutant C8α. Results in Figure 4 show that when purified C8*γ* is added to expression medium containing mutant C8α, a complex is formed which sediments to the same position as a C8α-*γ* marker. Formation of a complex between mutant C8α and C8*γ* is ionic strength dependent as indicated by the need to lower the ionic strength in order to achieve complete binding (panels A−C). This is consistent with previously described results which demonstrated binding between purified C8α and C8*γ* after cleavage of C8α-*γ* (*13*). Experiments performed with medium containing wild-type C8*β* or mutant iC8*β* revealed no detectable binding of C8*γ* at higher ionic strengths (not shown); however, partial binding is observed with mutant iC8*β* at 16 mM NaCl (panel D). Complete binding could not be achieved because the amount of excess that could be added was limited by the expression level of mutant iC8*β*. Although not conclusive, these results suggest the indel alone is capable of mediating noncovalent binding of C8*γ*.

To directly demonstrate that C8*γ* recognizes the C8α indel, a synthetic peptide containing the indel sequence was coupled

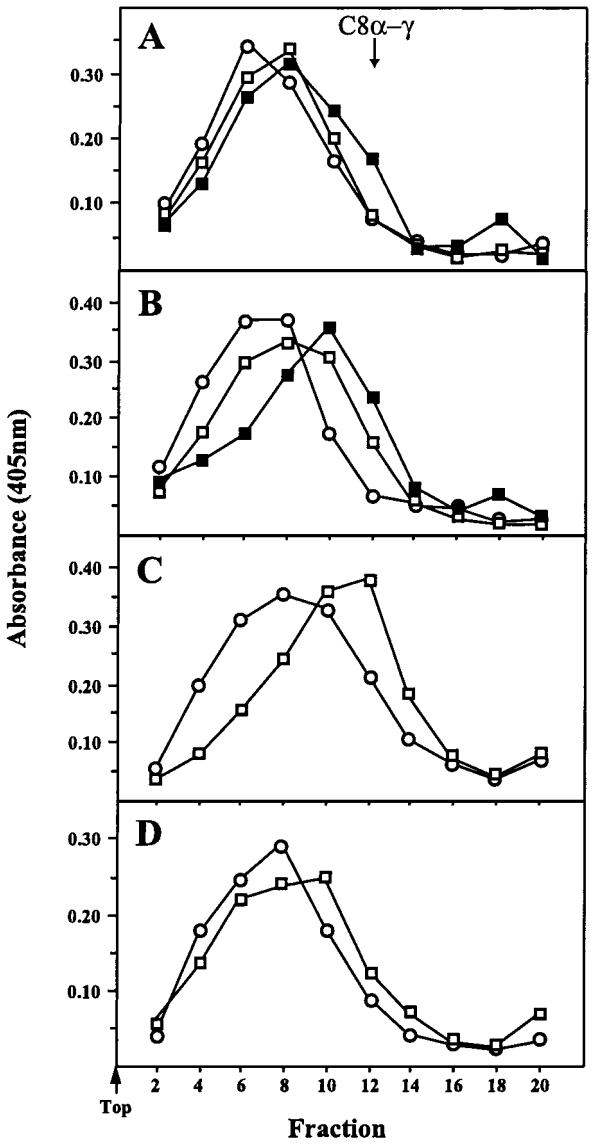

FIGURE 4: Binding of C8γ to mutant forms of C8α and iC8β. Control medium to which purified mutant C8γ was added and expression medium containing mutant forms of C8α or iC8β were dialyzed into gradient buffer supplemented with different concentrations of NaCl. Samples were mixed such that mutant C8α or mutant iC8β was in excess over C8γ and then subjected to sucrose density gradient centrifugation. After fractionation, the shift in sedimentation position of C8γ was determined by an ELISA. No signal was detected in controls that did not contain C8γ. The top of each gradient is indicated by an arrow. (A) Medium (72.7 mM NaCl) containing C8γ alone (○) or C8γ with a 100-fold (□) and 200-fold (■) molar excess of mutant C8α. The sedimentation position of a C8α-γ marker is shown in the inset. (B) Medium (33 mM NaCl) containing C8γ alone (○) or C8γ with a 100-fold (□) and 200-fold (■) excess of mutant C8α. (C) Medium (16 mM NaCl) containing C8γ alone (○) or C8γ with a 100-fold (□) excess of mutant C8α. (D) Medium (16 mM NaCl) containing C8γ alone (○) or C8γ with a 100-fold (□) excess of mutant iC8β.

to agarose and used to test for specific binding of C8γ. Results in Figure 5 show that when crude insect cell expression medium containing recombinant C8γ is passed through a column of indel peptide-agarose resin, C8γ is selectively removed from the medium. Binding is specific as indicated by the relative purity of C8γ in the eluted material as compared to the starting medium. These results demonstrate that C8γ can bind directly to the C8α indel and that binding is not dependent on Cys[164].

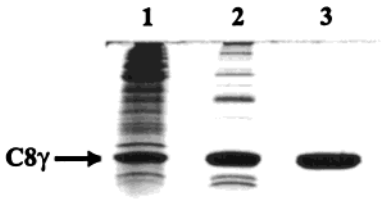

FIGURE 5: Binding of C8γ to the immobilized C8α indel peptide. Medium containing recombinant mutant C8γ was produced from baculovirus-infected insect cells and passed through a column of indel peptide-agarose resin or a matched column of control resin. After washing, bound protein was eluted, subjected to SDS−PAGE under nonreducing conditions and stained with Coomassie Blue. Lane 1: Medium applied to each column. Lane 2: Bound protein eluted from the indel peptide-agarose resin. Lane 3: Purified mutant C8γ standard. Nonspecific binding to the control resin was negligible.

The ability to express mutant iC8β and iC8β-γ indicates that insertion of the indel does not abrogate intracellular processing of C8β. To determine if the indel adversely affects C8β function, mutant iC8β was tested for its ability to form a complex with C8α-γ. Figure 6 compares the binding of purified C8β, wild-type C8β, and mutant iC8β when each is mixed with the same molar excess of C8α-γ (panels A−C). For purified and wild-type C8β, binding is complete in the presence of a 5-fold excess of C8α-γ, which is consistent with previously reported results (*4*). For mutant iC8β, only a 10-fold excess is required, which suggests its affinity for C8α-γ is comparable to that of C8β. Also noted in these experiments is a small amount of mutant iC8β that does not combine with C8α-γ. Immunoblot analysis of mutant iC8β revealed no degradation products, therefore this is likely a population of misfolded protein that is incapable of binding C8α-γ.

The activity of mutant iC8β when combined with C8α-γ is also shown in Figure 6 (panel D). Samples of purified C8β, wild-type C8β, and mutant iC8β were prepared in isotonic buffer and incubated with an excess of C8α-γ. Results indicate that purified and wild-type C8β have similar hemolytic activity, which is consistent with earlier reports (*4*). Importantly, mutant iC8β exhibits lower but significant hemolytic activity when combined with C8α-γ. When amounts needed to achieve 50% lysis are compared, it has approximately 15% of the activity of wild-type C8β.

DISCUSSION

Results in this study have established that C8α contains a distinct binding site for C8γ that is located within a segment defined by residues 157−175. This segment includes a unique indel (residues 159−175) that lies within the MACPF portion of C8α. Insertion of this indel into C8β, a close structural homologue of C8α, was sufficient to confer on this subunit the unusual ability to form a disulfide-linked dimer with C8γ. Although required for covalent cross-linking, the Cys within this indel is not essential for recognition and binding of C8γ. Mutant forms of C8α and iC8β which lack this Cys were capable of forming non-covalent complexes with C8γ at low ionic strength. Furthermore, C8γ bound specifically to an immobilized peptide that contained the mutant indel sequence. Together, these results indicate (a) intracellular binding of C8γ to C8α is mediated principally by residues contained within the C8α

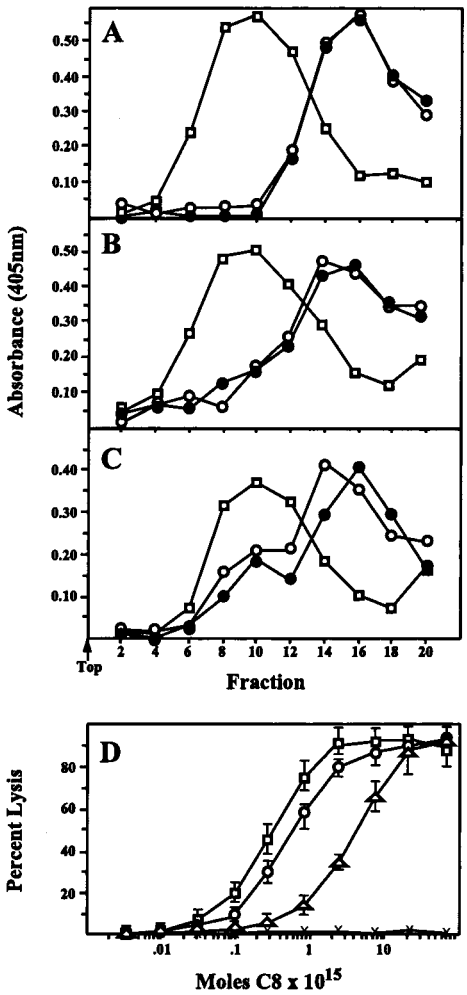

FIGURE 6: Functional properties of mutant iC8β. (Upper panels) Binding of mutant iC8β to C8α-γ. Control medium supplemented with purified C8β (panel A) or expression medium containing wild-type C8β (panel B) or mutant iC8β (panel C) were dialyzed into gradient buffer containing 72.7 mM NaCl. Each was incubated alone (□) and with a 5-fold (○) and 10-fold (●) molar excess of purified C8α-γ. After sucrose density gradient centrifugation and fractionation, sedimentation positions were determined by an ELISA specific for C8β. No signal was detected in controls that contained C8α-γ alone. (Lower panel) Hemolytic activity of mutant iC8β. The above media samples were incubated with a 10-fold molar excess of purified C8α-γ, serially diluted and assayed for C8 hemolytic activity toward EAC1−7 in the presence of C9. Moles of C8 added are based on each form of C8β as the limiting reagent. Results are shown for C8α-γ alone (×) and C8α-γ added to media containing purified C8β (□), wild-type C8β (○) and mutant iC8β (△). Media assayed in the absence of added C8α-γ exhibited <2% lysis at the highest amounts added. Error bars show the deviation observed for three different expressions.

indel, (b) binding is not strictly dependent on Cys[164], and (c) C8γ must contain a complementary binding site for the C8α indel.

Indels frequently correspond to residues located within loops on a protein surface (*22*, *23*). Accordingly, they are sometimes associated with sites of protein−protein interaction, as was recently shown for the complement C3/C4/C5 family of proteins (*17*−*19*). Analysis of the C8α indel indicates it is highly charged and therefore likely to be on the surface. This characteristic and the ionic strength dependency of binding suggests that formation of the noncovalent C8α:C8γ complex is mediated by ionic interactions. Regarding this, it is of interest that corresponding indel sequences in rabbit and pig C8α exhibit 87 and 94% similarity to human, respectively, with conservation of all charged residues (*24*, *25*). This suggests that C8α indels from different species are folded similarly and have similar binding properties. Support for this is also provided from studies showing that cells cotransfected with human C8α and rabbit C8γ or rabbit C8α and human C8γ produce the respective chimeric forms of C8α-γ (*12*).

The existence of a distinct C8γ-binding site on C8α is not unexpected in light of the fact that these two subunits must associate co- or postranslationally in order to form C8α-γ. To discover that this site is located within the C8α indel is likewise not surprising. Among the MAC family members, C8α is unique in its ability to form a disulfide-linked heterodimer, and the distinctive Cys residue involved is located within this indel. Furthermore, the indel lies within the MACPF segment of C8α. This segment alone can be expressed as a heterodimer with C8γ, thus providing independent evidence for a C8γ-binding site in this portion of C8α (*11*). The hydrophilic nature of the indel is also consistent with earlier studies which suggested a surface location for the C8γ-binding site (*13*). In those studies, an analogue of C8 prepared by combining C8α with C8β (C8α:C8β) was shown to be capable of forming a noncovalent complex with C8γ in solution. This analogue when incorporated into the MAC retains its ability to bind C8γ. This could only occur if the site involved is located on the surface of C8α and remains so after incorporation of C8 into the MAC.

Results from the expression and characterization of mutant iC8β have significance with regard to the location of binding sites in C8β. C8β has a high affinity for C8α-γ, and binding is believed to be mediated by a specific site on C8β. The fact that mutant iC8β exhibits near-normal affinity for C8α-γ suggests this site is not located in the immediate vicinity of the insertion. Furthermore, mutant iC8β exhibits significant hemolytic activity when combined with C8α-γ. This suggests the insertion does not completely disrupt the ability of C8β to interact with other components of the MAC. Although the activity of mutant iC8β appears to be lower than that of C8β, this could be an anomaly caused by a population of misfolded, nonfunctional mutant iC8β. Alternatively, the insertion may in fact perturb interactions that are necessary to form a fully functional MAC.

Identification of the C8α indel as the binding site for C8γ suggests that C8γ has a complementary binding site for the indel. Regarding this, it is of interest that C8γ belongs to the lipocalin family and therefore is likely to have a tertiary structure typical of many family members. This structure is referred to as the "lipocalin fold", and it consists of an eight-stranded, antiparallel β-barrel which defines a calyx- or cup-shaped structure that encloses a binding site for small and primarily hydrophobic ligands (*26*). The potential for C8γ to bind small molecules has led to speculation about possible ligands (reviewed in ref *27*). One study reported that C8γ binds retinol (*28*); however, a later study concluded that this binding was nonspecific (*5*). It has also been suggested that the putative ligand binding site may recognize and bind to a hydrophobic region on C8α (*27*). Regarding this, homology modeling of C8γ against the lipocalin β-lactoglobulin predicts a ligand-binding pocket that is too narrow to accommodate a loop structure and too hydrophobic and deep

to bind an amino acid side chain (28). In view of this and the hydrophilic nature of the C8α indel, it seems unlikely the indel itself is the "natural" ligand for C8γ, and it suggests the complementary site on C8γ may be located outside the cavity of the β-barrel.

Most lipocalins occur as monomers and in some cases higher order multimers; however, several including C8γ are distinct in that they occur as heterodimers with other macromolecules. For example, neutrophil gelatinase associated lipocalin (NGAL) from neutrophil granules forms a disulfide-linked dimer with gelatinase B (matrix metalloproteinase 9) (*29*). A large percentage of plasma apolipoprotein D circulates as a disulfide-linked complex with apoA-II and apoB-100, and a substantial portion of serum α_1-microglobulin occurs as a complex with IgA (*30*, *31*). Several other lipocalins form noncovalent complexes such as the one between the holoform of serum retinol binding protein and transthyretin (*32*). These binding interactions are thought to be mediated by one or more loops near the open end of the β-barrel (*9*). Variation in loop composition and length is believed to be the principal means by which different lipocalins selectively bind certain macromolecules. Regarding this, it is of interest to note that modeling of C8γ predicts that Cys^{40} resides in a large loop near the opening of the ligand binding pocket. If this loop also serves as the indel binding site then it may place Cys^{164} from C8α in close proximity to Cys^{40} and thereby facilitate disulfide bond formation.

In conclusion, the C8γ-binding site is now the third functionally distinct site to be mapped to the MACPF portion of C8α. The C8β-binding site also resides in this segment as does the principal recognition site for CD59, which has been mapped to within a region defined by residues 320−415 (*11*, *12*). Thus far, only one other member of the MAC family has been shown to have a functional site associated with an indel. Human C9 contains a unique insertion that corresponds to the putative "hinge" region, which also contains the only thrombin-cleavage site in C9 (*33*). Overall, the MAC family proteins have only a few indels of sizable length. In view of the observations reported here and those regarding C9, it is likely that some of these other indels have functional significance as well.

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