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The Eighth Component of Human Complement: Evidence That It Is an Oligomeric Serum Protein Assembled from Products of Three Different Genes†

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ABSTRACT: The eighth component of human complement (C8) consists of three nonidentical subunits arranged asymmetrically as a disulfide-linked α–γ dimer and a noncovalently associated β chain. Genetic studies of C8 polymorphisms established that α–γ and β are encoded at different loci. Implicit in this finding was the existence of two different genes and the likelihood that α–γ would be synthesized in single-chain precursor form. However, recent characterization of cDNA clones revealed separate mRNAs for human α and β but no evidence of a single-chain precursor for α–γ. A cDNA clone containing the entire coding region for human γ has now been characterized, and its sequence supports the existence of a separate γ mRNA. Included are a consensus translation initiation sequence, an apparent initiation methionine, and a signal peptide. By use of cDNA probes specific for human α, β, or γ, analysis of poly(A) RNA from normal baboon liver revealed separate mRNAs of 2.5, 2.6, and 1.0 kilobases (kb), respectively. Parallel analysis of poly(A) RNA from rat liver identified mRNAs of 3.4, 2.3, and 0.9 kb. These results argue against the possibility that C8 is assembled from products of two different genes (α–γ and β) and suggest it is comprised of three different gene products (α, β, and γ) that undergo both covalent and noncovalent association to yield the mature protein.

Human C8 is a glycoprotein constituent of C5b-9, the macromolecular cytolytic complex composed of complement proteins C5b, C6, C7, C8, and C9 (Müller-Eberhard, 1986). Both C8 and C9 have the exceptional ability to circulate in plasma as hydrophilic proteins but undergo hydrophilic to amphiphilic transitions leading to interaction with target membranes. This interaction is a consequence of association with other constituents and contributes directly to the cytolytic function of C5b-9. Human C8 has an atypical subunit structure consisting of α (M_r 64 000), β (M_r 64 000), and γ (M_r 22 000) subunits arranged as a disulfide-linked α–γ dimer that is noncovalently associated with β (Kolb & Müller-Eberhard, 1976; Steckel et al., 1980). Several distinct functional domains have been identified on C8. Included are those involved in interactions between subunits (Brickner & Sodetz, 1984, 1985), with other constituents of C5b-9 (Monahan & Sodetz, 1981; Stewart & Sodetz, 1985; Stewart et al., 1987), and with the target membrane bilayer (Steckel et al., 1983).

Insight into the genetic basis for the unusual subunit structure of C8 was first provided by studies of human C8 polymorphisms. Electrophoretic analysis of C8 under nonreducing conditions revealed that α–γ and β polymorphic patterns segregate independently in families, thus indicating these subunits are encoded at different genetic loci (Raum et al., 1979; Alper et al., 1983; Rittner et al., 1983). Existence of separate loci was further supported by analysis of human C8 deficiencies, where, in a given individual, α–γ or β is dysfunctional but not both (Tedesco et al., 1983a,b). On the basis of these observations, it has generally been assumed that α–γ and β are encoded in separate genes and, by analogy with other secreted proteins containing disulfide-linked chains, that α–γ is synthesized in single-chain precursor form.

To determine the C8 amino acid sequence, establish the existence of an α–γ single-chain precursor, and directly confirm that α–γ and β are products of different genes, we re-

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cently isolated and characterized cDNA clones for human C8. Characterization of a full-length α cDNA revealed structural features in the 5′ and 3′ regions that argued against the putative α–γ single-chain precursor and supported the existence of a distinct mRNA for α (Rao et al., 1987). No contiguous γ coding sequence was found at either end of the α cDNA nor was the α message size, as determined by analysis of RNA from human HepG2 cells, adequate to accommodate γ. Parallel characterization of cDNA clones for β established that it also is encoded in a separate mRNA (Howard et al., 1987). These results confirmed the existence of different loci for α–γ and β, but also suggested that α and γ are themselves products of different genes.

In this paper, we extend these studies and describe the sequence of a cDNA encoding the entire γ subunit of human C8. Using this probe and previously characterized cDNA probes, we provide unequivocal evidence that α, β, and γ are in fact encoded in separate mRNAs. This study completes the sequence of human C8 and establishes it as an oligomeric serum protein that is assembled from products of not two, but apparently three, different genes.

Experimental Procedures

Purification of Proteins and Amino Acid Sequencing. Human C8 was purified from plasma fraction III that was kindly provided by Cutter Laboratories, Berkeley, CA (Steckel et al., 1980). The γ subunit was further purified after reduction and modification of α–γ with 4-vinylpyridine (Steckel et al., 1980; Rao & Sodetz, 1984). Peptides for sequencing were generated by CNBr digestion of *S*-(pyridylethyl)-γ and separated by reverse-phase high-performance liquid chromatography (HPLC) according to methodology described earlier (Rao et al., 1987). The N-terminal CNBr peptide was further digested with trypsin and fractionated by HPLC. Amino-terminal sequencing was performed by automated Edman degradation and carboxy-terminal sequencing by digestion with carboxypeptidase P (Rao et al., 1987). Carbohydrate analysis of *S*-(pyridylethyl)-γ was performed as described (Rao et al., 1987).

Isolation and Characterization of γ cDNA. Two oligonucleotide probes were used to screen an adult human liver cDNA library kindly provided by Dr. Harvey R. Colten, Washington University School of Medicine. This library contained cDNA inserted into the *Pst*I site of pKT218 plasmid by homopolymeric GC tailing (Woods et al., 1982). Approximately 40 000 recombinant clones were plated on nitrocellulose membranes and screened as described (Whitehead et al., 1983). Hybridizations were performed at 40 °C in 6 × SSC (0.9 M NaCl/90 mM sodium citrate) containing 0.05% sodium pyrophosphate. Washing was performed for 10 min in the same buffer at 45 °C for the 17-mer probe and at 52 °C for the 20-mer probe. Common positives were subjected to colony purification.

After partial digestion of plasmid DNA with *Pst*I, fragments were size-fractionated on agarose gels and recovered by electroelution. Fragments were subcloned into phage vector M13mp18 and sequenced by a modified dideoxy chain termination method (Rao et al., 1987). Restriction site overlaps were sequenced by using synthetic primers synthesized by the University of South Carolina Oligonucleotide Synthesis Facility.

RNA Isolation and Analysis. Livers from normal baboon and rat were surgically removed and immediately subjected to the guanidium thiocyanate extraction procedure for poly(A) RNA isolation (Chirgwin et al., 1979). Purified poly(A) RNA was size-fractionated on formamide–agarose gels and transferred to Hybond-N (Amersham) nylon membranes. Prehybridization, hybridization, and washing of membranes were performed as recommended by the manufacturer. The α probe consisted of a 2.0-kb fragment generated by a *Hin*dIII–*Sal*I digest of clone A1 plasmid from Rao et al. (1987). The β probe was a 2.3-kb fragment from a similar digest of plasmid from the β cDNA clone described by Howard et al. (1987). The γ probe was a 0.4-kb fragment from a *Pst*I digest of the cDNA insert described herein. Labeling of all probes was performed by nick translation with [α-^{32}P]dCTP.

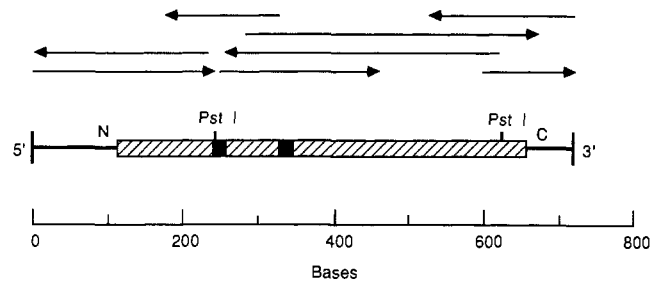

FIGURE 1: Map and sequencing strategy for γ cDNA. The cDNA insert and internal restriction sites used to generate fragments for sequencing are shown. Arrows identify segments sequenced by using synthetic primers. The hatched area corresponds to the γ coding region and includes both oligonucleotide probe sites (■).

Data Analysis. Sequence analysis was performed as described earlier (Rao et al., 1987).

Results and Discussion

Amino acid sequencing revealed that intact *S*-(pyridylethyl)-γ had a blocked N-terminal residue that could not be released with pyroglutamate aminopeptidase. Therefore, in order to design oligonucleotide probes, one CNBr peptide and two tryptic peptides were isolated and partially sequenced. The sequence Gln-Glu-Gln-Gly-His-Arg was selected to generate a mixed sequence probe

CGA/G-TGA/G/T/C-CCT/C-TGT/C-TCT/C-TG

The sequence Asp-Gly-Ile-Cys-Trp-Gln-Val yielded a second probe with the sequence

ACT/C-TGC-CAA/G-CAA/G/T-ATA/G/T/C-CCA/G-TC

These probes were highly selective when used in tandem to screen for γ cDNA clones.

The C-terminal amino acid sequence of γ was determined by digesting intact *S*-(pyridylethyl)-γ with carboxypeptidase P for various times. At 10 min, released amino acids and their mol/mol yields (in parentheses) were Arg (1.4), Val (0.4), Glu (0.3), and Asp (0.3). At 30 min, amino acids and yields were Arg (1.9), Val (1.1), Glu (0.9), and Asp (0.8). These results confirmed the C-terminal sequence Glu-Val-Arg-Arg predicted from the cDNA.

One cDNA clone with a 718 base pair (bp) insert was isolated and sequenced by using the strategy described in Figure 1. Figure 2 shows the resulting nucleotide sequence. When translated, the 5′ region yields an apparent leader sequence starting with methionine at −20. This residue is flanked by a consensus nucleotide sequence (CCACCATGY) commonly found at eukaryotic translation initiation sites (Kozak, 1981, 1984). Following the methionine, there is an extended sequence of uncharged amino acids, including a segment (−12 to −7) that is rich in hydrophobic residues. Although it is not possible to identify the N-terminal residue of mature γ, features of the leader sequence indicate glutamine at position +1 is the strongest candidate. The sequence preceding that residue conforms well to ones found near the cleavage site in signal

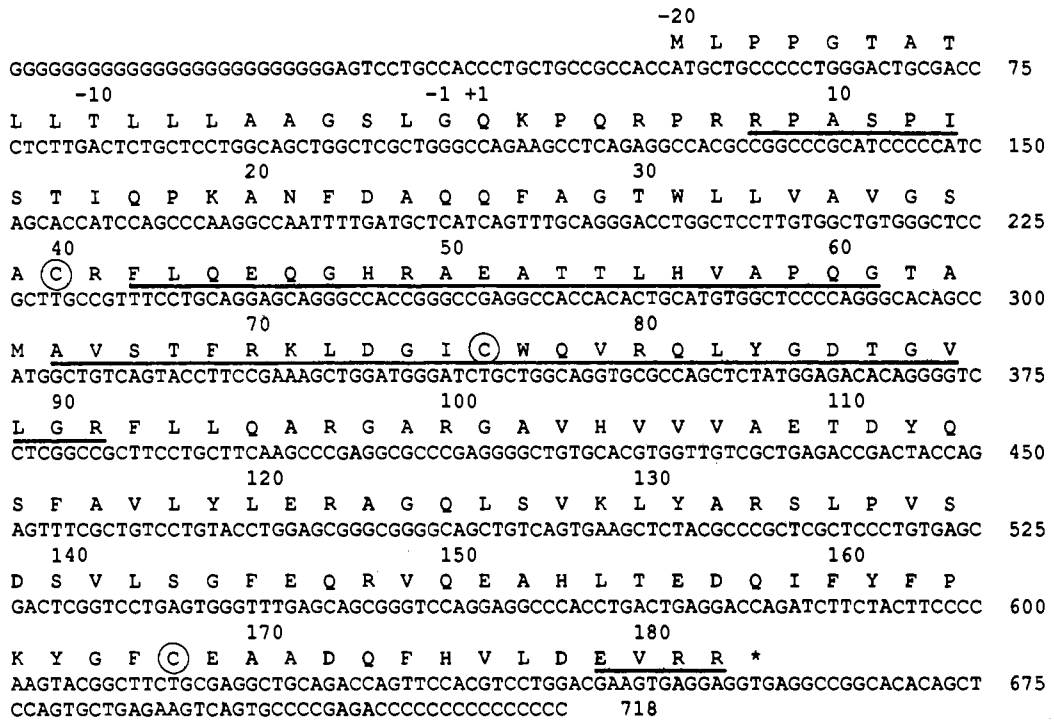

FIGURE 2: cDNA and amino acid sequence of γ. Numbering of amino acids begins with the proposed N-terminus of mature γ as +1. Cysteines are highlighted by circles, and the translation stop codon is indicated with an asterisk. Segments confirmed by amino acid sequencing are underlined.

peptides (von Heijne, 1983, 1984). This includes small neutral residues Gly and Ser at positions −1 and −3, respectively, and the absence of such a residue at −2. Location of the secondary structure breaking residue Gly at −4 is also common as is an absence of Pro between −3 and +1. Secondary structure analysis indicates a predominantly α-helical segment from −15 to −5 and a flexible region with potential for a β turn near the proposed cleavage site (−3 to +1) (Chou & Fasman, 1978). Occurrence of a β turn at this site is considered important for accessibility to the signal peptidase (Perlman & Halvorson, 1983). On the basis of these observations, we conclude that γ is synthesized with a signal peptide that is likely cleaved at position −1. Cyclization of glutamine at +1 to pyrrolidonecarboxylic acid would then yield a blocked N-terminus in the mature protein, which would be refractory to pyroglutamate aminopeptidase because of the penultimate Lys residue (Doolittle, 1972; Ferreira et al., 1970).

It is noted that an arginine-rich tetrapeptide begins at position +5. Although typical of proteolytic processing sites in propeptides, our inability to sequence either intact γ or an N-terminal CNBr peptide argues against processing at this site. The sequence beginning at +8 was obtained for a tryptic peptide from γ.

Translation of the 3′ extension yields an in-frame stop codon corresponding to the C-terminus of γ. No polyadenylation signal nor poly(A) sequence was found. Assuming glutamine at +1 is the N-terminus, γ would contain 182 amino acids for an M_r of 20329. This agrees well with the earlier M_r of 22000 and 194 amino acids estimated from amino acid analysis (Steckel et al., 1980). No carbohydrate was found associated with γ.

The amino acid sequence of γ exhibits no significant homology to other proteins. Hydropathy analysis revealed no extended regions of hydrophobicity (Kyte & Doolittle, 1982). Likewise, no segments with potential for interacting with membranes were found when predictive methods to identify such domains were used (Eisenberg et al., 1984). The predicted inability of γ to interact with membranes contrasts with results from a similar analysis of α and β (Rao et al., 1987; Howard et al., 1987). On the basis of its sequence, α was predicted to contain a potential transmembrane domain located in a large cysteine-free region of the protein. This was considered functionally significant because intrinsic conformational flexibility in this region could modulate exposure of this domain to target membranes. Both α and β also contain segments with potential for interacting with membrane surfaces.

Experimental evidence in support of these predictions does exist. Photolabeling studies using membrane-restricted probes showed that, within C5b-9, α interacts directly with the target membrane bilayer (Steckel et al., 1983). The extent of interaction suggested a major role in membrane perturbation, a conclusion now supported by the prediction of a transmembrane domain in α. The β subunit also interacts but to a lesser extent, as might be expected if it contains strictly membrane surface seeking domains. Importantly, these same photolabeling studies yielded no evidence that γ interacts directly with the target membrane. A similar conclusion was reached from studies of an analogue of C8 prepared by combining equimolar amounts of purified α and β (Brickner & Sodetz, 1984). Although lacking γ, this analogue is similar to native C8 in its ability to lyse membranes after incorporation into C5b-9. Thus, structural predictions and functional evidence indicate γ has no direct role in membrane perturbation.

The absence of distinctive features in the sequence and the lack of homologies with other proteins give us little insight into the function of γ. Because it is not essential for C8 activity, its exact role in C5b-9 assembly and function has been and continues to be unclear. This contrasts with α, which has at least one identifiable role, that of providing a binding site for C9 and directing its incorporation into the nascent C5b-9 complex (Stewart & Sodetz, 1985). Likewise, β has a binding site for C5b that mediates C8 interaction with the precursor C5b-7 complex (Monahan & Sodetz, 1981; Stewart et al., 1987). In addition, both subunits have the intrinsic ability to interact with target membranes, which undoubtedly relates

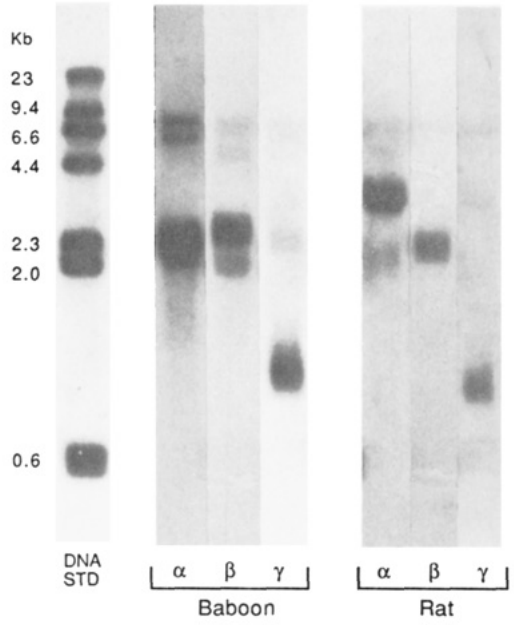

FIGURE 3: RNA blot analysis of α, β, and γ mRNAs. Approximately 20 μg of purified poly(A) RNA from baboon or rat liver was size-fractionated by electrophoresis on agarose and blotted. The same blot was probed sequentially with radiolabeled fragments of α, β, and γ cDNAs. Molecular weight standards consist of a *Hin*dIII digest of ^{32}P-labeled λ phage DNA. The figure shows the autoradiograph obtained with each probe.

to their striking homology and similarity in structural organization. One can only speculate on possible roles for γ. It may serve to impart a functional difference in two otherwise structurally similar subunits, i.e., α and β. If so, this difference must be subtle because α alone can still associate with β and function in cytolysis. Alternatively, it may be essential for biosynthetic processing of α–γ or stability of C8 in the circulation. Most intriguing is its possible role in regulating C8 membranolytic activity and thereby protecting host cells from lysis. Evidence suggests that interaction between γ and what could be a ubiquitous cell-surface protein (homologous restriction factor) may be an essential step in the mechanism by which homologous cells protect themselves from C5b-9-mediated lysis (Hänsch et al., 1986; Schönermark et al., 1986; Zalman et al., 1986). Such a possibility would be consistent with a passive role for γ in C8 function.

Results in Figure 3 confirm that γ is encoded in a separate mRNA. Sequential probing of baboon poly(A) RNA revealed predominant messages of 2.5, 2.6, and 1.0 kb for α, β, and γ, respectively. Corresponding analysis of rat poly(A) RNA identified messages of 3.4, 2.3, and 0.9 kb. Absence of a common band indicates the α and γ messages are not alternatively processed forms of a larger mRNA coding for α–γ. Lengths of baboon α and β mRNAs agree with the corresponding human messages (2.5 kb each) found in total RNA from HepG2 cells (Rao et al., 1987; Howard et al., 1987). The length of the γ mRNA produced by this cell line was not established earlier because γ cDNA clones had not been isolated. However, on the basis of our results for a closely related primate, one can predict the γ message to be approximately 1.0 kb in humans.

Existence of separate mRNAs for α, β, and γ suggests these chains are encoded in different genes. The absence of nucleotide sequence homology between cDNAs for α, β, and γ supports this conclusion and indicates their respective messages are not likely to arise from alternative processing of a common primary transcript. Although unexpected, our results do not conflict with evidence that α–γ and β are encoded at different genetic loci. Indeed, they confirm this conclusion but further indicate that separate loci exist for α and γ. The latter can be reconciled with polymorphism studies if one realizes those studies relied strictly on electrophoretic behavior of α–γ and β under nonreducing conditions. Consequently, polymorphisms in α or γ could not be distinguished nor followed independently. In one study, where α–γ was analyzed under reducing conditions, only α exhibited polymorphism (Rogde et al., 1985). On the basis of these observations and our results, one must conclude that α, β, and γ are encoded at different loci but only α and β exhibit polymorphisms that are readily detectable by electrophoretic analysis.

Two predictions about the synthesis and assembly of C8 can be made on the basis of our results. First, α–γ synthesis must involve independent translation of α and γ mRNA, followed by specific recognition and noncovalent association of products and co- or posttranslational disulfide bond formation. Such a mechanism is not unique and would be analogous to immunoglobulin G synthesis where an intrinsic affinity between heavy and light chains facilitates their noncovalent interaction while the nascent heavy chain is still on the polyribosome (Schubert, 1968). This same mechanism could be operative in the synthesis of α–γ because α and γ do have a high affinity for each other (Brickner & Sodetz, 1985). This affinity persists even after partial reduction and alkylation, indicating that an interaction could occur early in the synthesis process, i.e., before covalent linkage. Second, the affinity between α–γ and β must be high to ensure efficient and complete post-synthetic association. Their affinity is in fact remarkably high, as evidenced by the in vitro association of purified α–γ and β when mixed 1:1 at dilute concentrations (50 ng/mL) and in the presence of high concentrations of serum albumin carrier (5 mg/mL) (Steckel et al., 1980). Support for these predictions also comes from studies of C8 synthesis by rat hepatocytes.[1] Pulse–chase experiments showed that intracellular disulfide bonding between α and γ occurs rapidly, with no detectable accumulation of free subunit. These same studies also showed that α–γ and β associate prior to secretion, thus confirming a high intracellular affinity.

Human C8 is considered unusual because of its ability to circulate independently in plasma yet effectively interact with target membranes as a consequence of induced conformational changes. Its quaternary structure is equally intriguing, involving both covalent and noncovalent interactions between three nonidentical subunits. Such an arrangement is rare among plasma proteins. Several contain nonidentical disulfide-linked chains but are single gene products synthesized in single-chain precursor form, for example, blood coagulation zymogen factor X and complement proteins C3, C4, and C5. Even those with chains derived from different genes generally have a common mode of interchain interaction and symmetry in their quaternary structure. Examples are immunoglobulin G and fibrinogen, both of which contain symmetrically arranged disulfide-linked chains. These proteins can all be classified as monomeric because their constituent chains are disulfide-linked (Klotz et al., 1975). In contrast, C8 can be appropriately characterized as an oligomeric protein because it contains noncovalently linked subunits. Oligomeric structures are common for intracellular proteins but not for secreted ones, particularly plasma proteins. Complement component C1 is another that contains disulfide-linked and noncovalently associated subunits but even this complex protein has considerable symmetry in its subunit structure. Thus, C8 seems

[1] S. C. Ng and J. M. Sodetz, manuscript in preparation.

to have not only a unique function but also an exceptional structure.

Our results indicate C8 may be an interesting model system for studying synthesis, assembly, and regulation of secreted, multichain proteins. The occurrence of stoichiometric amounts of α, β, and γ in the mature protein raises the possibility of gene linkage and coordinate transcriptional regulation. Translational control of intracellular subunit pools and its effect on covalent and noncovalent subunit associations would also be of interest to examine. While of general interest, such studies are particularly important to understanding C8 deficiencies because the scope of this disorder has now assumed an added dimension. Future studies must now focus not only on α–γ and β but also on how transcriptional and translational defects relate to the synthesis and interaction of all three subunits.

Added in Proof

Since submission of this paper, the N-terminus of γ was successfully deblocked by extended digestion with pyroglutamate aminopeptidase. The N-terminal sequence was found to agree with that predicted in Figure 2.

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