

Human complement component C8

Molecular basis of the β -chain polymorphism

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Abstract

The β -chain of human complement component C8 exhibits a structural genetic polymorphism: using isoelectric focusing two major allotypes can be identified (C8B B ('basic') and C8B A ('acidic')). In the present report we describe a sequence polymorphism of the C8B gene (codon 63: AGA \rightarrow GGA) and demonstrate that the resulting amino acid substitution (Arg \rightarrow Gly) consistently differentiates between the two common charge variants of the C8 β chain; the C8B B allotype is characterized by an Arg and the C8B A allotype by a Gly residue in position 63 of the C8 β polypeptide chain.

Key words: Complement; Component C8; Polymorphism; Isoelectric focusing; Thrombospondin

1. Introduction

Complement component C8 plays an important role in the formation of the membrane attack complex (MAC) of the complement system. The MAC is a high-molecular amphiphilic heteropolymer that is assembled from five hydrophilic precursor proteins, the terminal complement components C5, C6, C7, C8, and C9; upon proteolytic cleavage of C5 to C5a and C5b, the sequential addition to C5b of C6, C7, C8, and C9 results in the formation of the C5b-9 complex, which mediates the damage to biological membranes associated with complement-dependent cytotoxicity [1].

Human C8 is a plasma glycoprotein with a molecular weight of 151,000, consisting of equimolar amounts of three nonidentical polypeptide chains: the α -chain ($M_r = 64,000$), the β -chain ($M_r = 64,000$), and the γ -chain ($M_r = 22,000$); these are arranged asymmetrically as a disulfide-linked α - γ -dimer that is noncovalently associated with the β -chain [2–4]. Within the process of MAC formation it is the β -chain which mediates the interaction of C8 with the C5b-7 complex; C9 associates through interaction with a C9-specific site on the α -chain [4]. The three chains of C8 are encoded by three separate

genes: C8A and C8B, the loci coding for C8 α and C8 β , are closely linked to one another and have been localized to chromosome 1p32 [5–7]; C8G, the gene encoding the γ -chain, has been assigned to chromosome 9q [5].

Structural polymorphisms as well as genetically determined deficiency states have been found for both the α - γ complex and the β -chain [8–10]. The polymorphism of the C8 α - γ subunit (= C8A (previously C81) polymorphism) has been studied rather extensively [8,11–14]. In contrast, the genetic variability of the C8 β chain has been investigated to a lesser extent, probably because of methodological difficulties (discussed in [12,13]). The β -chain polymorphism (= C8B (previously C82) polymorphism) was first described by Alper et al. [9] and later studied by Rogde and co-workers [11,12]. In addition, data on C8 β polymorphism in Japanese has been published in abstract form [15]. For the detection of C8 β phenotypes plasma or serum proteins have been separated by isoelectric focusing (IEF) in polyacrylamide gels; specific protein identification was achieved either with a hemolytic overlay containing C8 β deficient serum [9] or by means of an immunoblotting procedure [11,12,15]. Family studies demonstrated that C8 β variants are inherited as autosomal codominant traits [9,11]. Two major alleles, C8B*B ('basic') and C8B*A ('acidic'), and two rare variants have been identified; allele frequencies of approximately 0.95 (C8B*B) and 0.04 (C8B*A) were reported for Caucasians and also for Japanese [9,11,15].

The β -chain of human C8 consists of 537 amino acids; the complete primary structure was derived independently by two groups from the sequence of C8 β cDNA

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Abbreviations: MAC, membrane attack complex; IEF, isoelectric focusing; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; BSA, bovine serum albumine; bp, base pair; RFLP, restriction fragment length polymorphism; TSR, thrombospondin repeat.

clones [16,17] (see also EMBL accession nos. X04393, M16973). A comparison of the two cDNA sequences suggests the presence of either an A or a G in the first position of codon 63, corresponding to an arginine to glycine substitution. We postulated that such a type of amino acid exchange (positively charged→neutral) could explain the different isoelectric points of the two common β -chain allotypes C8B B and C8B A. Using polymerase chain reaction (PCR) we established a simple method for direct investigation of the nucleotide sequence in codon 63; this method allowed us to demonstrate that codon 63 is indeed polymorphic and to examine this DNA polymorphism to account for the C8 β protein polymorphism.

2. Materials and methods

2.1 Blood samples

EDTA anticoagulated venous blood samples were drawn from 82 unrelated healthy German individuals. Plasma was obtained by centrifugation and leucocyte DNA was isolated as described by Miller et al. [18]. In selected cases (heterozygous genotype) blood samples were also collected from family members. Plasma samples from three individuals with a homozygous C8 β deficiency were kindly provided by Dr. W. Nürnberger.

2.2 Polymerase chain reaction (PCR)

The PCR was performed using the following primers: C8B-63-F (5'-GCTCCAGCCCTCTCAGTTC-3') corresponding to positions 129–147 of the C8 β cDNA sequence reported by Haeffliger et al. [16] and C8B-63-R (5'-AAAGCCTTCACATCGCACTT-3') complementary to positions 237–218. Standard PCR was carried out in a 50 μ l volume containing 80 ng genomic template, 20 pmol of each primer, 200 μ M of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.01% gelatine and 2.5 U Taq-Polymerase (Perkin-Elmer Cetus). Samples were processed in a Perkin-Elmer Cetus DNA Thermal Cycler through 30 temperature cycles consisting of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, followed by a final extension step of 10 min at 72°C. An aliquot (10 μ l) of the PCR product was digested using 5 U/sample of *Acl*I (New England Bio Labs) according to the manufacturer's instructions. Fragments were separated on 10% polyacrylamide gels and visualized by silver-staining [19].

2.3 Cloning and sequencing of PCR products

PCR products from two heterozygous individuals were cloned into pUC 18 *Sma*I/BAP vector (Pharmacia). Lysates from single colonies were used as template for PCR with primer pair C8B-63-F/C8B-63-R; subsequent *Acl*I digestion of the PCR product allowed the identification of clones containing different alleles. From selected colonies a hemibiotinylated PCR product was generated by using one biotinylated vector primer and one normal vector primer. The PCR product was incubated with streptavidine Dynabeads (Dynal Ltd.) and magnetic beads were collected with a magnetic concentrator. After washing and denaturing both strands of DNA were sequenced using Sequenase Version 2.0 Kit (US Biochemicals).

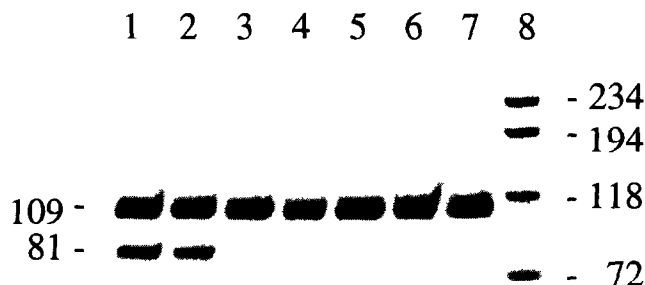
2.4 Analysis of the C8 β protein polymorphism

C8 β protein phenotypes were determined by methods comparable to those previously used for C7 typing [20,21]. Prior to focusing, all plasma samples were pretreated with *Clostridium perfringens* neuraminidase (Sigma, type V). 15 μ l of the enzyme solution (30 U/ml 1 M potassium phosphate buffer, pH 7.0) were added to 90 μ l plasma; the mixture was incubated overnight at room temperature. The desialized samples (25 μ l) were subjected to IEF in thin-layer polyacrylamide gels (110 \times 240 \times 0.8 mm) containing 2.8% (w/v) Ampholine carrier ampholytes (LKB/Pharmacia; pH 3.5–10, pH 5–8, pH 7–9, pH 8–9.5 (3:2:3:2)) and, according to Alper et al. [9], 3 M urea. IEF along the short axis

of the gel was carried out for 5½ h, including 30 min prefocusing; the settings for the power supply were 1400 V, 40 mA and 13 W. The focused proteins were transferred onto a nitrocellulose membrane (0.45 μ m; Schleicher and Schuell BA-85) by press-blotting for 1 h [20]. After protein transfer the nitrocellulose was removed from the gel, blocked overnight at 4°C in phosphate-buffered saline (PBS; pH 7.2) containing 3% (w/v) bovine serum albumine (BSA; Sigma, fraction V) and then washed 3 \times 5 min in PBS. A goat anti-human-C8 antiserum (Cappel) and a peroxidase-conjugated rabbit anti-goat-immunoglobulin antiserum (DAKO) were used for the subsequent enzyme immunoassay. The nitrocellulose membrane was probed for 120 min at 37°C with the first antibody (diluted 1:200 in 3% BSA/PBS), washed 5 \times 5 min in PBS, exposed to the second antibody (diluted 1:500 in 3% BSA/PBS) for 120 min at 37°C, and washed again 5 \times 5 min in PBS. Finally, peroxidase activity was developed using *o*-diaminidine [21]. C8 β phenotypes were scored without knowledge of genotyping results.

3. Results

To evaluate if codon 63 of the human C8B gene is indeed polymorphic, as suggested by the difference between the two published C8 β cDNA sequences [16,17], we used PCR to amplify a 109-base pair (bp) segment of the C8B gene containing the putatively polymorphic site. Primers were designed from the C8 β cDNA sequence assuming homology of the C8B gene structure with the known exon–intron organization of complement C9 gene [22]. Amplification of genomic DNA resulted in a single DNA fragment of the expected size. The sequence difference noted in the cDNA sequences is distinguishable by a change in a recognition site for restriction endonuclease *Acl*I. As can be seen from Fig. 1, digestion of the 109-bp PCR product with *Acl*I resulted in a restriction fragment length polymorphism (RFLP), depending on the absence or presence of the polymorphic *Acl*I re-



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Fig. 1. Restriction fragment length polymorphism of complement C8B gene as detected by *Acl*I digestion of a 109-bp PCR product encompassing codons 35–71. Lanes 1 and 2, genotype C8B*1/C8B*2; lanes 3 to 7, genotype C8B*1/C8B*1; lane 8, molecular weight marker (Φ X174 DNA-*Hae*III digest).

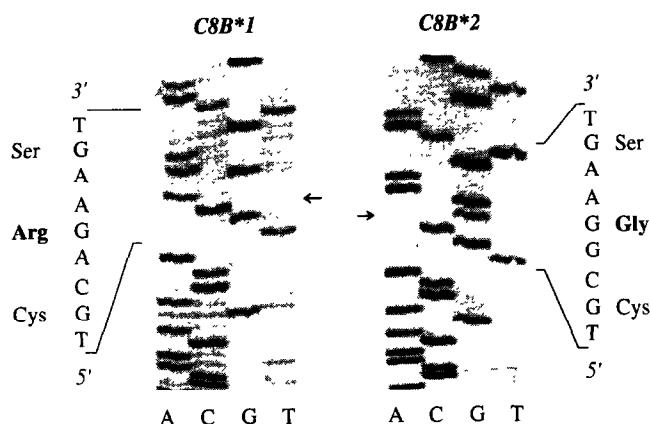


Fig. 2. Autoradiograms of the regions of sequencing gels showing the single nucleotide substitution (A→G) that determines the presence of either Arg or Gly at position 63 of the mature C8β chain. Sequence analysis of both alleles from an individual with genotype *C8B*1/C8B*2* (and protein phenotype C8B AB).

striction site, either a fragment of 109 bp (allele *C8B*1*) or two fragments of 81 bp and 28 bp (allele *C8B*2*) are produced. Autosomal codominant inheritance of alleles was demonstrated in four two-generation families each with at least two children. The existence of a nucleotide sequence variation in codon 63 was confirmed by sequencing both alleles from genotypically heterozygous individuals (Fig. 2). The polymorphism changes codon 63 from AGA (encoding Arg; *AciI* site absent) to GGA (encoding Gly; *AciI* site present). Typing 82 unrelated German Caucasians we observed 74 individuals homozygous for *C8B*1* and 8 individuals with the heterozygous *C8B*1/C8B*2* genotype. The allele frequencies were 0.951 (*C8B*1*) and 0.049 (*C8B*2*).

To investigate the relationship between the *AciI* RFLP and C8β protein polymorphism plasma samples from the 82 unrelated genotyped German individuals were subjected to isoelectric focusing in polyacrylamide gels containing 3 M urea and C8 protein bands were identified by immunoblotting. IEF in the presence of urea causes dissociation of C8 into α-γ and β, thus facilitating the separate analysis of α-γ- and β-subunits [9]. The IEF conditions employed in the present study allowed an unequivocal typing of C8β phenotypes (Fig. 3). C8β bands are located in the more cathodal region of the gel. The specificity of the detection system is clearly supported by the observation that plasma samples from individuals with a genetically determined homozygous C8β deficiency did not show any bands in this region. In accordance with observations of other authors [11,12,15] we also encountered immunoreactive patterns (originating from the α-γ-subunit) in the more anodal region of the gel; however, the IEF conditions used here for β-chain typing did not allow a clear typing of the α-γ-pattern. As can be seen in Fig. 3, two different structural C8β phenotypes were observed: C8B B and C8B AB. There was an absolute concordance between C8β protein

typing and DNA typing. All 74 individuals with genotype *C8B*1/C8B*1* (Arg63/Arg63) were typed as C8B B. The 8 individuals with heterozygous *C8B*1/C8B*2* genotype (Arg63/Gly63) were all C8B AB.

4. Discussion

In the present study we describe a sequence polymorphism in codon 63 of the C8β gene. Allele *C8B*1* is characterized by the nucleotide sequence AGA coding for an arginine residue, allele *C8B*2* by the nucleotide sequence GGA coding for a glycine residue. This variation is easily detected as an RFLP after *AciI* digestion of an amplified 109-bp portion of the C8β gene. During the preparation of this manuscript, the exon-intron organization of the C8β gene has been reported, demonstrating that it is indeed highly homologous to the structure of the C9 gene [23]. The nucleotide sequence amplified with our primers C8B-63-F and C8B-63-R is completely located within exon 3 of the C8β gene.

The method described here for C8β protein typing allows an unequivocal identification of phenotypes; it will be valuable for further population screening and in particular for the identification of rare electromorphs. The separate analysis of the β-chain polymorphism, possible due to IEF in the presence of urea [9], is easier than the typing of banding patterns produced by complete C8 molecules (IEF in the absence of urea) [11,12] where pattern variation reflects the genetic variability of both the α-γ- and the β-subunit of C8. The allele frequencies that we encountered in 82 unrelated German individuals are in excellent agreement with frequencies previously reported for U.S. Whites and Norwegians [9,11].

Investigating DNA and plasma samples from 82 unre-

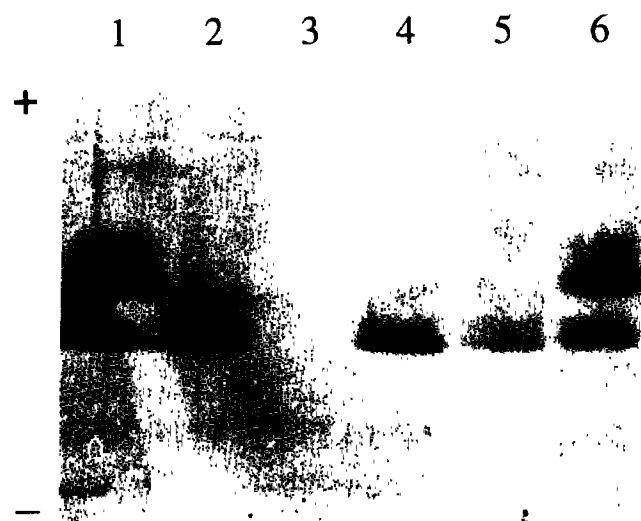


Fig. 3. C8β protein phenotypes observed in the present study. Lanes 1 and 6, C8B AB; lanes 2, 4 and 5, C8B B; lane 3, sample from an individual with homozygous C8β deficiency. In the heterozygous pattern the A band is consistently stronger than the B band.

lated individuals we observed absolute concordance between the *AciI* RFLP and C8 β protein polymorphism. We thus conclude that the C8B B allotype is characterized by an arginine residue and the C8B A allotype by a glycine residue at position 63 of the C8 β polypeptide chain. With regard to IEF the incorporation of an electrically neutral residue (Gly) in C8B A, substituting the positively charged Arg residue of C8B B, is expected to cause a shift towards the anode. Thus, this substitution might well explain the difference between C8B B and C8B A observed in IEF gels. Aligning the two published C8 β cDNA sequences reveals that the A \rightarrow G substitution in the first position of codon 63 is the only difference within the whole coding sequence. In this context it seems important that both groups obtained the complete coding sequence from a single full-length cDNA clone, i.e. the cDNA sequence was not compiled from smaller overlapping clones. Therefore, it seems most likely that the two sequences are in fact cDNA sequences of two different alleles, namely C8B*1 and C8B*2, and that these two alleles differ exclusively in the first position of codon 63, at least with regard to the coding sequence. In the present study we demonstrated that the substitution in codon 63 consistently differentiates between C8B B and C8B A. Taking together, we propose that the Arg/Gly polymorphism in codon 63 is the molecular basis of the C8B B/C8B A protein polymorphism.

Family studies have suggested that genetic deficiency of the C8 β chain, a condition associated with a highly increased risk for meningococcal infections [10], is due to a null allele at the C8B locus [9,11,12]. In fact, it has recently been shown that probably a large proportion of C8 β deficiencies is caused by a single point mutation in codon 374 of the C8B gene (CGA (Arg) \rightarrow TGA (stop)) [24]. The occurrence of null alleles might well cause situations in which the results of C8 β protein typing and DNA typing of the *AciI* RFLP are discordant.

The C8 β chain shows extensive structural homology with the C8 α chain, with C9, and also with C6 and C7 [16,17,23,25–27]. All these proteins are typical mosaic proteins containing several distinct cysteine-rich N- and C-terminal modular segments and a central cysteine-poor region that has no discernible module structure. Starting with the N-terminus C8 β consists of a thrombospondin repeat (TSR), a low density lipoprotein receptor class A module, the central cysteine-poor region, an epidermal growth factor precursor module, and finally another TSR at the C-terminus. The polymorphic variation identified in the present report is located close to the C-terminus of the N-terminal TSR. The TSR is a sequence motif, approximately 60 amino acids long, that was first identified three times in thrombospondin, an adhesive glycoprotein secreted from the granules of activated platelets [28]. Beside its occurrence in the terminal complement components [25,26,29,30] it is also found in properdin, a regulator protein of the alternative pathway

of complement activation, and in cell surface antigens of malaria parasites [29, 31 and references therein]. On the basis of different Cys residue locations and different TSR lengths, Smith et al. [32] have subdivided TSR sequences into three groups. Group 1 includes mainly the sequences found in thrombospondin and properdin; group 2 corresponds to the N-terminal and group 3 to the C-terminal TSRs of the late complement components. The precise role of TSRs is not known but could well involve protein–protein or protein–phospholipid interactions [26,27,29]. Interestingly, it has recently been shown that the common A/B polymorphism of complement component C6 is also due to a charged amino acid substitution in the group 2 TSR of the protein [33,34]. Detailed studies of the structure–function relationships of the terminal complement components will be important to define the interactions between these proteins at the molecular level and to elucidate the eventual functional significance of polymorphic variation within certain domains.

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