Molecular Basis of Polymorphisms of Human Complement Component C3

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Summary

C3 exhibits two common allotypic variants that may be separated by gel electrophoresis and are called C3 fast (C3 F) and C3 slow (C3 S). C3 F, the less common variant, occurs at appreciable frequencies only in Caucasoid populations (gene frequency = 0.20). An increased prevalence of the C3 F allele has been reported in patients with partial lipodystrophy, IgA nephropathy, and Indian childhood hepatic cirrhosis. Studies of the genomic organization of the human C3 gene led to the identification of a single change (C to G) between C3 S and C3 F at nucleotide 364 in exon 3. This leads, at the translation level, to the substitution of an arginine residue (positively charged) in C3 S for a glycine residue (neutral) in C3 F. This substitution results in a polymorphic restriction site for the enzyme HhaI. The resulting restriction fragment length polymorphism (RFLP) was investigated using genomic DNA, amplified using the polymerase chain reaction; there was absolute concordance between the genomic polymorphism and the distribution of C3 S and C3 F in 50 normal subjects. The molecular basis of a second structural polymorphism, defined by the monoclonal antibody HAV4-1, was also characterized. The polymorphic determinant was identified at codon 314 in the exon 9 of the β chain where a leucine residue (HAV 4-1+) is substituted for a proline residue (HAV 4-1-). Identification of the amino acid sequences of these polymorphic variants will facilitate characterization of possible functional differences between different allotypes of C3. Three RFLPs (BamHI, EcoRI, and SstI) were located to introns in the C3 gene. There was no allelic association between these three RFLPs, or between the RFLPs and the C3 F/S polymorphic site. Genetic equilibration of these polymorphisms has occurred within a gene of 41 kb.

The third component of complement, C3, occupies a central position in the complement cascade and is quantitatively the major protein of the complement system, present in plasma at a concentration of ~1 g/liter. Its activation results in the release of several split fragments with potent chemotactic, opsonic, and anaphylatoxic properties. The proteolytic cleavage of C3 is also required for the formation of the cytolytic membrane attack complex.

Human C3 exhibits genetic polymorphism that was first described by Weime and Demeulenaere (1) and Alper and Propp (2). The genetic variants of the protein are inherited as autosomal codominant traits and are characterized using prolonged agarose gel electrophoresis of fresh serum. In this way, two common polymorphic forms have been found, designated C3 F and C3 S. The C3 S allele is most common in all races in humans (2). The C3 F allele is relatively frequent in Caucasoids, less common in America Negroes (2), and extremely rare in Orientals (3, 4). More than 20 rare allotypes have been characterized (5) by variations in their relative electrophoretic mobilities.

An additional common structural polymorphism was identified by Koch and Behrendt (6) based on the reactivity of human C3 with a mouse mAb (HAV 4-1) that detected a genetic variation not associated with any charge difference. The two polymorphic systems, C3 S/F and HAV 4-1, are closely related as HAV4-1 usually, but not always, reacts with the C3 F allotype. RFLPs have also been detected in the C3 gene (7, 8), but none distinguish between C3 S and C3 F (9). In humans, the only known functional difference between the C3 S/F allotypes is binding to monocyte-complement receptors (10), while in mice, C3 allotypes affect the degree of activation of complement by zymosan (11). Further, an increased prevalence of the C3 F allotype has been found in patients with several diseases, including Indian childhood cirrhosis (12), IgA nephropathy (13, 14), and partial lipodystrophy (15). The mechanism that is responsible for these as-
Specific Oligonucleotide Hybridization. Two 21-mer oligonucleotides, Sequencing and RFLP. Two 18-mer oligonucleotides (5'ATCC-, EX9b (5'CTGAGTGCAAGATGACGGTGG 3'), complementary oligo EX9a (5'ATTGAGGATGGCTCGGGGGAG 3') and oligo followed by autoradiography. Absolute ethanol. The kinased DNA fragments were analyzed by separated through a G-50 Sephadex column and precipitated with Hhal. Cleaved fragments were then kinased using γ-[32P]ATP, ng of amplified DNA was digested with the restriction endonuclease Biochemical Corporation, Cleveland, OH). In RFLP studies, 60-80 

Materials and Methods

Serum and DNA Preparation. Fresh-frozen serum and EDTA plasma were obtained from blood samples of 42 Caucasians and three Chinese. At the same time, genomic DNA was isolated from whole blood. Genomic DNA samples from five subjects who were homozygous C3 FF were kindly donated by D. Whitehouse (Galton Laboratory, University College, London).

C3 Allotyping and Immunoblotting. C3 allotyping was performed on plasma samples by means of high voltage agarose gel electrophoresis as described by Teisberg (18). Immunoblotting was carried out as reported by Koch et al. (19).

Southern Blot Analysis. 8-μg aliquots of genomic DNA were digested to completion with restriction endonucleases (EcoRI, SstI, and BamHI) (Gibco-BRL, Uxbridge, Middlesex, UK) according to the manufacturer's suggested conditions. The DNA samples were then subjected to electrophoresis on a 0.8% agarose gel and blotted onto Hybond-N membranes (Amersham, Buckinghamshire) (20). The blots were hybridized with a cDNA probe for C3, PC3.11 (16) (kindly provided by Dr. G. Fey, Scripps Clinic, La Jolla), which was labeled by the random primer method (21). The filters were then washed to high stringency (0.2x SSC + 0.1% SDS at 65°C). The same blots were then stripped and hybridized at 95°C with oligo 2 (5'CTGCTCGGAGGTCTGC3', complementary to nucleotides 993–1009; A at 1001). The washes were carried out as described above. Both oligonucleotides were end-labeled using γ-[32P]ATP and T4 polynucleotide kinase (Gibco-BRL) to a specific activity of 10⁸ cpm/μg.

PCR Amplification and Sequence of 5' Flanking Region of the C3 Gene. Two 21-mer oligonucleotides, oligo A (5'TCTGACTCC-CAGCCTACAGAG 3') and oligo B (5'GGAGGTGGGTTAG-TAGCAGGA 3'), complementary to nucleotide positions 993–1009; A at 1001), were used as primers to amplify genomic DNA from a normal individual allotyped C3 SS and another of allotype C3 FF. The DNA was denatured at 94°C for 1 min, annealed at 50°C for 1 min, and extended at 72°C for 1 min. Amplified fragments were ligated in M13 vector and sequenced as described above.

Results

Molecular Basis for C3 F and C3 S and C3 Alloype by RFLP. In the course of determining the genomic organization of the β chain of the human C3 gene (17), a single base change (C to G) was identified in one of the genomic clones (clone 6) at nucleotide 364, in exon 3, when compared with the published cDNA sequence (16). This resulted, at the translation level, in the substitution of an arginine residue (positively charged), for a glycine residue (neutral). We therefore postulated that this charged amino acid change could explain the different electrophoretic mobility of C3 S and C3 F. To test this hypothesis, we used PCR to amplify this region from genomic DNA of six unrelated individuals of known C3 allotype (two C3 FF, two C3 SS, and two C3 FS). Sequencing of the amplified DNA demonstrated absolute concordance of this nucleotide polymorphism with the C3 S and C3 F alleles (Fig. 1).

This nucleotide substitution generated also an RFLP with the restriction endonuclease Hhal, which cleaves at the sequence GCCG in the C3 S allele (nucleotide positions 363–366), but not GGGC in the C3 F allele. We therefore used the PCR to amplify this region from the genomic DNA of 50 selected normal subjects with known C3 alleles (7 C3 FF, 30 C3 SS, and 13 C3 FS). Digestion of the 286-bp amplified fragments with Hhal resulted in the generation of a single uncleaved band from each C3 F allele and of two bands of 248 and 38 bases, respectively, from each C3 S allele (Fig. 1).

1 Abbreviations used in this paper: ASO, allele-specific oligonucleotide; TMACI, tetramethylammonium chloride.

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Figure 1. Charged amino acid difference between C3 F and C3 S. The nucleotide guanine at position 364 in C3 F individuals was substituted for cytosine in C3 S individuals. This results in the substitution of glycine, a neutral amino acid for arginine, a positively charged amino acid. The autoradiograph is read from the bottom upwards, and the lanes reading from left to right represent guanine, adenine, thymine, and cytosine (G, A, T, and C, respectively). The polymorphic nucleotide and codon is boxed to the left of the lanes with the translated amino acid in bold (glycine [G] and arginine [R]).

2). C3 allotyping determined by this method again concurred absolutely with that derived by conventional high-voltage agarose gel electrophoresis.

Identification of the Polymorphic Site Defined by the mAb HAV 4-1. A second polymorphic determinant on human C3 was detected by immunoblotting with the mAb HAV 4-1 (6). C3 S alleles are commonly HAV 4-1 negative, while C3 F alleles are commonly HAV 4-1 positive. We studied 45 normal individuals (three Chinese and 42 Caucasoids with the following gene frequency: C3 S = 0.81; C3 F = 0.19) by immunoblotting. We found two C3 S HAV 4-1-positive alleles, but no C3 F HAV 4-1-negative allele. Comparison of the available DNA sequences (16, 17) spanning the coding region from nucleotide positions 661-1179 of the cDNA sequence (previously identified by Koch and coworkers [23] as the area containing the polymorphic determinant) revealed a T to C transversion at nucleotide 1001, in the exon 9 of the β chain, which resulted in the substitution of a leucine residue by proline at amino acid position 314 (Fig. 3). The segregation of this polymorphic amino acid with mAb HAV 4-1 reactivity was confirmed by sequencing exon 9 from PCR-amplified genomic DNA of six normal individuals with known HAV 4-1 polymorphism (three C3 SS HAV 4-1 negative; two C3 FF HAV 4-1 positive; and one C3 SS HAV 4-1 positive). The subject heterozygous for the HAV 4-1 polymorphism (C3 SS HAV 4-1 positive) possessed both sequences. We extended the analysis of this polymorphism by the use of two allele-specific oligonucleotide (ASO) probes on PCR-amplified DNA in 45 normal subjects (Fig. 4). All were C3 allotyped as well as studied by immunoblotting with mAb HAV 4-1. The results of the ASO hybridization experiments were concordant with those derived by the immunoblotting of C3.

Sequencing of 5' Flanking Region of C3 F and C3 S. The 5' flanking region sequences from one C3 FF and one C3 SS individual were amplified by PCR, sequenced, and compared. No differences were found up to 442 bases upstream from the signal peptide.

Localization of Three RFLPs within the C3 Gene. Three different RFLPs of the C3 gene were identified and localized using probes for different regions of the C3 gene. We analyzed the frequency of these RFLPs in the same 45 normals who had been alltyped by agarose gel electrophoresis and immunoblotting (Table 1). An EcoRI RFLP using the C3 cDNA probe pC3.11 revealed two allelic fragments of 5.2 and 5.6 kb (24), and was localized to intron 19 of the C3 α chain by hybridization with subfragment probes from the cDNA (data not shown). The second RFLP, detected by the restriction enzyme SstI, was originally described by Davies et al. (7). This polymorphism gave two allelic fragments of 12 and 9+3 kb in length, respectively, and was localized to
intron 27 of the C3 α chain, ∼6 kb 3' to the EcoRI polymorphism, using a pC3.11 subfragment probe spanning exons 28–29 (17). A third RFLP was detected with the restriction enzyme BamHI using a C3 cDNA probe spanning exons 11–14, and showed two allelic fragments of 2.8 and 2.5 kb. This polymorphism was localized to an intron of the C3 β chain, between exons 12 and 15.

From the genomic structure of the human C3 gene (17), we have determined that the physical distances between the EcoRI and SstI RFLPs in the α chain and the C3 S/F polymorphism in the β chain is ∼15 and ∼19 kb, respectively (Table 2 and Fig. 5). The BamHI RFLP in the β chain is <10 kb from the C3 S/F polymorphic site. The cosegregation of these RFLP markers with the protein variants was analyzed in the panel of 45 normal individuals, and 29 were informative. The data are summarized in Table 2. From the observed gene frequencies of the individual polymorphisms, the expected frequency of individual C3 haplotypes was calculated (Table 2). This revealed near random association between alleles at the C3 S/F polymorphic site and other sites in the α and β chain within the human C3 gene. Although not statistically significant, there was a trend towards allelic association between the EcoRI 5.2-kb allele and the SstI 12-kb allele (data not shown).

Discussion

The two common C3 variants, C3 S/F, may be separated on the basis of their differing electrophoretic mobility through agarose, which implies variation in surface charge between the two allotypes. The very small difference in pI (0.05) between C3 S and C3 F, and their similar migration in PAGE (25), suggests that the variation in charged amino acids between the two allotypes is limited to the substitution of only one or two residues.

In studying the genomic structure of the β chain of the human C3 gene, a C to G substitution was detected in the sequence of one of the clones (clone 6)(17) when compared with the published cDNA. The finding of a resultant charged amino acid change led us to postulate the hypothesis that this may be the molecular basis of the difference between the C3 S and C3 F allotypes. Conveniently, the nucleotide change resulted in the predicted loss of a restriction site for HhaI endonuclease, and we took advantage of this to measure the concordance of the molecular and protein polymorphisms. There was 100% concordance between C3 S/F allotypes in 50 individuals, determined by high-voltage agarose gel electrophoresis, and genomic polymorphism determined using HhaI. This finding suggested that the single nucleotide change may be the molecular basis for the variation between C3 S and C3 F, and this was supported by our other findings.

Koch and Behrendt (6) reported another polymorphic site, characterized by a mAb, HAV 4-1, that was closely, but not
Shown is the frequency of the different haplotypes identified in the cohort of 45 normal individuals (58 informative chromosomes). The right hand column shows the expected frequency of each haplotype calculated from the individual gene frequencies of the different polymorphisms.

| C3 F/S   | HAV 4-1 mAb | BamHI (2.5/2.8 kb) | EcoRI (5.2/5.6 kb) | SstI (9 + 3/12 kb) | No. | Observed frequency | Expected frequency |
|---------|-------------|---------------------|--------------------|---------------------|-----|--------------------|--------------------|
| 1 S     | –           | 2.8                 | 5.2                | 9 + 3               | 7   | 12                 | 10.8               |
| 2 S     | –           | 2.8                 | 5.6                | 9 + 3               | 5   | 8                  | 5.8                |
| 3 S     | –           | 2.8                 | 5.2                | 12                  | 20  | 34                 | 22.9               |
| 4 S     | –           | 2.8                 | 5.6                | 12                  | 7   | 12                 | 12.3               |
| 5 S     | –           | 2.5                 | 5.2                | 9 + 3               | 1   | 1.7                | 2.5                |
| 6 S     | –           | 2.5                 | 5.2                | 12                  | 6   | 10                 | 5                  |
| 7 S     | –           | 2.5                 | 5.6                | 12                  | 1   | 1.7                | 2.8                |
| 8 S     | –           | 2.5                 | 5.6                | 9 + 3               | 2   | 3.5                | 1.3                |
| 9 S     | +           | 2.8                 | 5.2                | 12                  | 1   | 1.7                | 6                  |
| 10 S    | +           | 2.8                 | 5.6                | 12                  | 1   | 1.7                | 3.2                |
| 11 F    | +           | 2.8                 | 5.2                | 9 + 3               | 1   | 1.7                | 0.6                |
| 12 F    | +           | 2.8                 | 5.2                | 12                  | 3   | 5                  | 1.4                |
| 13 F    | +           | 2.8                 | 5.6                | 12                  | 3   | 5                  | 0.7                |

Therefore, we sequenced exons 1–12 of the C3 gene from the genomic clone 6, which included the entire region identified by Koch and coworkers (23) as containing the HAV 4-1 epitope. Comparison of the clone 6 coding sequence with the published DNA sequences (16, 17) showed only one difference: a T to C change in exon 9 (nucleotide position 1001). This difference lies within the 20-kD fragment previously identified (23) as containing the HAV 4-1 determinant. We proceeded to study the correlation of this nucleotide change with HAV 4-1 reactivity by sequencing of genomic DNA, amplified by PCR, from individuals of known C3 S/F and HAV 4-1 allotypes and by allele-specific oligonucleotide hybridization. The sequences giving rise to the amino acid leucine (CTC) and proline (CCC) segregated completely with HAV 4-1 positivity and negativity.

These findings strongly support the hypothesis that the difference between C3 S and C3 F may be explained solely by the arginine/glycine polymorphism at position 102. In addition, no difference between C3 S and C3 F was found in the sequences of 5' flanking region of the gene up to 442 bases upstream from the signal peptide.

Three RFLPs were identified using the restriction endonucleases BamHI, EcoRI, and SstI. The polymorphic restriction sites were localized to the β chain (between exons 12 and 15) and introns 19 and 27 of the α chain, respectively. The BamHI RFLP comprised two bands of 2.5 and 2.8 kb in the β chain. Although there was strong allelic association between the C3 S/F and HAV 4-1 polymorphisms, separated by ~5 kb, there was no significant allelic association between either of these structural polymorphisms and the three RFLPs.
not between each of the RFLPs. The distances between each of the RFLPs were mapped and found to be between 6 and 10 kb of DNA. Despite these short distances, these polymorphisms have reached equilibrium in the Caucasoid population, and this may reflect the great antiquity of the C3 gene. Similar findings have been reported previously in other genes, such as the β globin (26) and the phenylalanine hydroxylase (27) genes.

Partial lipodystrophy (15), associated with autoantibody production to neoantigens in the C3bBb C3 convertase enzyme, IgA nephropathy (13, 14), and Indian childhood hepatic cirrhosis (12) have each been reported to be associated with the C3 F allele, suggesting that there may be important functional differences between the two common allotypic variants. There is one report that the C3 F allotype may have an enhanced capacity to bind to complement receptors on mononuclear cells (10). Most of the functional domains of C3, including the region mediating binding to complement receptors, have been located to the α chain (28). However, the C3 S/F and HAV 4-1 polymorphic sites reside in the β chain, suggesting that, if these polymorphisms influence binding of C3 to complement receptors, they may do so by influencing the tertiary structure of the molecule or through accessory binding sites.

In conclusion, we have defined the molecular basis of two common structural polymorphisms of C3, and these findings will assist in future investigations of relationships between structure and function of the different allotypic variants of this important protein of the complement system.

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