DECAY-ACCELERATING FACTOR

Genetic Polymorphism and Linkage to the RCA (Regulator of Complement Activation) Gene Cluster in Humans

BY JAVIER REY-CAMPOS,* PABLO RUBINSTEIN,* AND SANTIAGO RODRIGUEZ DE CORDOBA**

From the *Department of Immunogenetics, The Lindsley F. Kimball Research Institute of The New York Blood Center, New York 10021; and the °Centro de Investigaciones Biologicas, Consejo Superior de Investigaciones Cientificas (CSIC), Madrid, Spain

Classical genetic studies have shown that the human complement components C2, C4, and factor B are encoded by closely linked genes within the MHC. This is also the case in all other mammalian species studied and, for this reason, these genes are referred to as the class III genes of the MHC (1, 2). In the mouse, the C3 locus has been mapped 10–12 cm telomeric to the MHC (3) but in man C3 maps on a different chromosome (4). Similarly, we have shown that the genes encoding the regulatory complement components C4-binding protein (C4bp), C3b/C4b receptor (CR1) and factor H (H) are closely linked in humans (5, 6). We have designated this linkage group as regulator of complement activation (RCA)¹ and have shown that it segregates independently of the class III gene cluster located on human chromosome 6 (6). Recently, in situ hybridization experiments using a CR1 cDNA probe have provided a chromosomal location for the RCA gene cluster on the long arm of human chromosome 1 (7). C2, factor B, C4bp, H, and CR1 are all members of a newly recognized family of proteins that bind C3b and/or C4b and show a particular structural organization based on the presence of internal repeat units of ~60 amino acids that share a framework of highly conserved residues (8). These homologies support the concept that these proteins originated from a common evolutionary ancestor. The demonstration, however, of the existence of two separate linkage groups, one encoding the components of the C3-convertases, C2, C4, and factor B, and the other encoding the regulators of the C3-convertases, C4bp, H, and CR1, suggests that the encoding of these proteins in two separate gene clusters may relate to their specific roles in the complement cascade. How the class III and the RCA gene clusters developed through the evolution of the vertebrate animals remains, however, to be determined.

The decay-accelerating factor (DAF) is a membrane glycoprotein similar to C4bp, CR1, and H in that it binds C3b or C4b inhibiting the formation of the C3-convertases (9, 10). DAF, however, does not act as a cofactor for the cleavage

¹ Abbreviations used in this paper: DAF, decay-accelerating factor; RCA, regulator of complement activation; RFLP, restriction fragment length polymorphism.
of C3b and C4b by factor I (11). Erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH), an acquired disease characterized by intermittent hemolytic anemia, have been shown to be deficient in DAF (12, 13). Recently, the cDNA encoding DAF has been cloned and sequenced, demonstrating the presence of four of the internal repeat units characteristic of the C3b/C4b binding proteins (14). DAF, therefore, shares functional and structural characteristics with the subgroup of C3b/C4b binding proteins, C4bp, CR1, and H, that function as regulators of the C3 convertases. To analyze the genetic relationships of DAF with C4bp, CR1, and H, we have searched for and found restriction fragment length polymorphisms (RFLPs) for the DAF gene. We demonstrate in this report that, in humans, the gene encoding DAF is closely linked to the genes encoding CR1, C4bp, and H in the RCA gene cluster.

Materials and Methods

**RCA Allotyping.** Allotyping for protein variants of CR1, C4bp, and H were performed using erythrocytes and neuraminidase-treated EDTA-plasma or serum samples as previously described (15, 16). Additional discrimination between otherwise indistinguishable CR1 variants was obtained through the analysis of previously described RFLPs for the CR1 gene (17).

**RFLP Analysis.** The cloning of cDNAs encoding human DAF has revealed the existence of two classes of DAF mRNAs that apparently represent an alternative splicing event involving the deletion of a 118-bp-long intron (14). Sequence analysis of this fragment showed an 80% homology with the \textit{Alu} family consensus sequence (14). To avoid the background hybridizations due to \textit{Alu} sequences in the southern blot analysis of genomic DNA, we have used a 2.1 kb DAF cDNA probe lacking the 118 bp intron. This DAF cDNA probe was a generous gift of Dr. I. Caras (Genentech, Inc., South San Francisco, CA). In the analysis of RFLPs of the CR1 gene, we have used the CR1-1 cDNA probe kindly provided by Dr. D. T. Fearon (Department of Medicine, Harvard Medical School, Boston, MA).

Southern blot analyses were performed using genomic DNA digested with either Eco RI, Bam HI, or Hind III restriction endonucleases (BRL, Bethesda, MD). 6 µg of digested DNAs were electrophoresed for 30–40 h using 0.7% agarose gels in TAE buffer (Tris acetate, 40 mM; pH 7.2; EDTA 1 mM) and, after acid treatment, were transferred onto Biotrace-RP nylon membranes (Gelman Sciences Inc., Ann Arbor MI) in 0.4 M NaOH.

The membranes were hybridized with \(^{32}P\)-labeled probes for 40 h at 65°C. Both the prehybridization and hybridization solutions were: 5× SSC, 1% SDS, 0.5% nonfat milk powder, and 200 µg/ml of denatured salmon testes DNA. The blots were washed at 65°C in 0.5× SSC, 1% SDS and exposed at −70°C on Kodak XAR-5 film using Cronex Lightning Plus intensifying screens (DuPont Co., Wilmington, DE).

Results and Discussion

**Bam HI and Hind III RFLPs for DAF Gene.** Southern blot analysis of human genomic DNAs digested with either Bam HI or Hind III restriction endonucleases and hybridized with the 2.1 kb DAF cDNA probe (see Materials and Methods) revealed the existence of genomic variants. The hybridization patterns obtained with Bam HI disclosed two allelic fragments of 30 (allele S) and 35 kb (allele L), and two invariant bands of 19 and 12 kb. The three expected Bam HI patterns are illustrated in Fig. 1. Individuals PED-3 and PED-8 are homozygotes for the S allele. PED-6 and PED-7 are homozygotes for the L allele and all the others are heterozygotes S, L.
MAPPING OF HUMAN DECAY-ACCELERATING FACTOR

FIGURE 1. Bam HI RFLP for the DAF gene. Southern blot analysis of family PED showing the three Bam HI polymorphic patterns for the DAF gene. Alleles of the polymorphic fragment are indicated as S and L. Position of size markers corresponding to fragments generated by separate digestions of λ phage DNA with Kpn I, Hind III, Bst EII, and Sma I restriction enzymes is shown at left. Individual designations are given above the corresponding lanes.

FIGURE 2. Hind III RFLPs for the DAF gene. Southern blot analysis of family DIF. The Hind III-A polymorphic fragment alleles are indicated as Hind IIIA-1 and Hind IIIA-2. The Hind III-B polymorphic alleles are shown as Hind IIIB-1 and Hind IIIB-2, respectively. Position of size markers are indicated on the right side. Individual designations are given above the corresponding lines.

Two independent RFLPs were found in genomic DNA digested with Hind III. One of them, referred to as Hind III-A RFLP, has two allelic fragments of 9.8 (allele 1) and 7.0 kb (allele 2), originating three Hind III patterns (Fig. 2). Individuals DIF-2, DIF-5, and DIF-7 are homozygotes for the 1 allele, while DIF-9, DIF-10, and DIF-11 are homozygotes for the 2 allele, and all the others are 1, 2 heterozygotes. The second Hind III RFLP, referred as Hind III-B RFLP, corresponds to the presence or absence of a Hind III restriction site in a 13.9 kb fragment. The presence of this Hind III restriction site is designated as allele 2 and determines two fragments, of 5.5 and 8.4 kb, respectively. The absence of this site, a 13.9 kb fragment, is designated as allele 1. Homozygous for the allele 1 are illustrated by individuals DIF-3, DIF-4, DIF-7, DIF-9, DIF-
Family PED

Family DIF

**Figure 3.** Segregation of the different alleles at the CRI, C4BP, and factor H loci and DAF RFLPs in the two families. For each individual, typings have been organized so that each column represents alleles inherited within the same haplotype. Two individuals (asterisk) have inherited a C4BP, CRI/H recombinant haplotype (boxed) from one of their parents. In the description of the CRI typings, A and A' or B and B' are only to indicate that these CRI variants, indistinguishable at the protein level, can be differentiated by means of RFLP analysis, as described in Materials and Methods.

10, and DIF-11 in Fig. 2, while all other individuals in Fig. 2, showing the three bands of 13.9, 8.6, and 5.5 kb, are heterozygotes 1, 2. The third pattern, corresponding to homozygous for the allele 2 (only the 8.6 and 5.5 kb fragments) has not been found among the individuals studied here. In addition to the polymorphic fragments described above, the Hind III restriction pattern includes two invariant fragments, of 4.5 and 5.0 kb, respectively.

Variants at each of these three polymorphic sites are inherited in block defining restriction haplotypes. The use of the DAF restriction haplotypes increases our ability to unravel different genetic variants at the DAF gene and thus facilitates subsequent linkage analysis.

**Genetic Linkage Between DAF and the RCA Gene Cluster.** Because DAF resembles C4bp, CR1, and H, we were interested in determining the genetic relationships between them. C4bp and CR1 are encoded by two loci within the RCA gene cluster that appear to be very close together, since no recombinations have been observed between them and since strong positive linkage disequilibrium exists between their alleles (19). H is encoded by a third locus, H, at the RCA gene cluster, which maps 6.9 cM away from the C4BP and CR1 loci (19). As a consequence of the linkage between C4BP, CR1, and H, alleles at these loci are inherited as haplotypes (RCA haplotypes).

To determine whether the gene encoding DAF is linked to the RCA cluster, we searched for families which, in addition to being informative for the segregation of both the RCA haplotypes and the DAF restriction haplotypes, also included individuals showing recombination within the RCA gene cluster. These RCA-recombinant haplotypes would, in the event of linkage, provide information of the position of the DAF gene within the RCA gene cluster. Two families, PED and DIF, including a total of 16 offspring, were found to satisfy both requirements. The results of typing for alleles at the loci of the RCA gene cluster and the DAF RFLPs for these families are summarized in Fig. 3. The individuals PED-10 and DIF-6 each have inherited an intra-RCA recombination event between CR1/C4BP and H from their respective parents, PED-1 and DIF-5.
simplify the formal analysis of linkage, the CR1/C4BP portion of these RCA-recombinant haplotypes was used as the marker.

All 32 meioses in these families were informative for the segregation of both RCA and DAF haplotypes, and no recombination was found between them. The corresponding value for the Lod score (logarithm of odds in favor of linkage) was 8.42 at a recombinant fraction value (θ) of 0.00 (likelihood in favor of linkage of $2.6 \times 10^8$ to 1). These results demonstrate that DAF is a member of the RCA gene cluster. The two individuals (PED-10 and DIF-6) showing recombination between H and CR1/C4BP loci allowed us to determine that DAF is genetically separable from H but not from the CR1/C4BP segment of the chromosome.

The demonstration of the linkage of DAF to the genes encoding CR1, C4bp, and H supports the concept that the genes in the RCA cluster have evolved as a cluster because of selective pressures related to the specific function of these proteins. Thus, other C3b/C4b binding proteins involved in the regulation of the C3-convertases, such as gp45/70 (20), might also be encoded by genes linked to the RCA gene cluster.

The interesting possibility that proteins of the complement system with no apparent functional relationships to the regulators of the C3 convertases may also map within the RCA gene cluster has been recently raised. Thus, Weiss et al. (7), have shown that both CR1 and CR2 genes map to the same band (1q32) on human chromosome 1 using labeled cDNAs in in situ hybridization experiments. CR1 and CR2 show strong structural homology, both at the protein and DNA level, suggesting that they resulted from the divergence of relatively recently duplicated genes (7). This degree of structural homology is exceptional for the known members of the RCA complex which, in general, share only a characteristic framework of highly conserved amino acid residues (8). The linkage of CR1 and CR2, on the other hand, additionally suggests that the genes encoding the related complement receptors, CR3 and CR4, might also be linked and thus, within the RCA cluster. If they are indeed linked, RCA might, like MHC, be a supergene complex, including genes that control distinct though related functions through proteins that display varying levels of homology.

Summary

We have investigated the genetic relationships between the human decay-accelerating factor (DAF) and a group of complement components including the C3b/C4b receptor (CR1), C4-binding protein (C4bp), and factor H (H), to which DAF is structurally and functionally related. CR1, C4bp, and H were previously demonstrated to be encoded by a cluster of closely linked genes, which we have designated regulator of complement activation (RCA).

Southern blot analysis of genomic DNA using a DAF cDNA probe unraveled the existence of restriction fragment length polymorphism (RFLP) for both Bam H1 and Hind III restriction endonucleases. Segregation analysis of these polymorphic fragments in families informative for the segregation of alleles at the CR1, C4BP, and H loci (RCA-haplotypes), demonstrated that, in humans, the gene encoding DAF is located within the RCA gene cluster. No recombinants between DAF and C4BP/CR1 were encountered in 32 informative meioses. In addition, in two individuals showing recombination between the CR1/C4BP and H loci, DAF segregated with the CR1/C4BP segment. Thus, the DAF gene maps
closer to the CR1/C4BP loci than to the H gene, from which it can be separated by genetic recombination.

We thank Mrs. C. Carrier and Mr. N. Mollen for their help in obtaining the DNA samples, and Ms. T. Huima for the photographic work.

Received for publication 9 February 1987.

Note added in proof: A Brief Definitive Report on a similar topic was published by Lublin et al. (J. Exp. Med. 165:1731, 1987).

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