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Immunology

Identification of a partial cDNA clone for the C3d/Epstein–Barr virus receptor of human B lymphocytes: Homology with the receptor for fragments C3b and C4b of the third and fourth components of complement

(complement receptor/virus receptor/gene family/tryptic peptides)

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ABSTRACT **Human complement receptor type 2 (CR2) is the B-lymphocyte receptor both for the C3d fragment of the third component of complement and for the Epstein–Barr virus. Amino acid sequence analysis of tryptic peptides of CR2 revealed a strong degree of homology with the human C3b/C4b receptor, CR1. This homology suggested that CR1 gene sequences could be used to detect the CR2 sequences at conditions of low-stringency hybridization. Upon screening a human tonsillar cDNA library with CR1 cDNA sequences, two clones were identified that hybridized at low, but not at high, stringency. Redundant oligonucleotides specific for CR2 sequences were synthesized and used to establish that the two cDNA clones weakly hybridizing with the CR1 cDNA contained CR2 sequences. One of these CR2 cDNA clones hybridized to oligonucleotides derived from two distinct CR2 tryptic peptides, whereas the other, smaller cDNA clone hybridized to oligonucleotides derived from only one of the CR2 peptides. Nucleotide sequence analysis of the CR2 cDNA confirmed that the site of oligonucleotide hybridization was identical to that predicted from the peptide sequence, including flanking sequences not included within the oligonucleotide probes. The CR2-specific cDNA sequences identified a poly(A)$^+$ RNA species of 5 kilobases in RNA extracted from human B cells but did not hybridize to any RNA obtained from the CR2-negative T-cell line HSB-2, thus confirming the appropriate size and tissue-specific distribution for the CR2 mRNA. The striking peptide sequence homology between CR2 and CR1 and the cross-hybridization of the CR2 cDNA with the CR1-specific sequences allow the placement of CR2 in a recently defined gene family of C3- and C4-binding proteins consisting of CR1, C4-binding protein, factor H, and now, CR2.**

The C3d/Epstein–Barr virus (EBV) receptor (complement receptor type 2, CR2) of human B lymphocytes is a M_r 145,000 glycoprotein (1–3) comprised of a single polypeptide of M_r 110,000 and 8–11 N-linked oligosaccharides (4). It is expressed by B lymphocytes (5, 6) and follicular dendritic cells of the spleen (7). CR2 recognizes the d region of the C3 component of complement (5, 6) and also serves as the membrane receptor for the human herpesvirus EBV (8, 9), which induces polyclonal activation and immortalization of B lymphocytes and is associated with Burkitt lymphoma and nasopharyngeal carcinoma. CR2 may have a role in physiologic B-cell activation (10, 11) and is phosphorylated when B cells are treated with phorbol 12-myristate 13-acetate or antibody to surface immunoglobulin (12).

The recent purification of CR2 permitted the demonstration (13) of significant similarities between its amino acid composition and that of three other proteins of the complement system: factor H, C4-binding protein, and the C3b receptor (complement receptor type 1, CR1). Factor H and C4-binding protein are plasma proteins and CR1 is a membrane glycoprotein found on erythrocytes, most leukocytes, and glomerular podocytes (14). These proteins share certain functions, such as binding to C4b and/or C3b and serving as cofactors for cleavage of C4b or C3b by a protease, factor I, and are encoded by apparently linked genes (15), which may be located on chromosome 1, based on the recent assignment of CR1 to this chromosome (16). In addition, they share a 60 amino acid consensus repeat having conserved residues at 11–14 positions (16–18). This unusual consensus repeat has also been identified in the Ba and C2b fragments of factor B and C2 (19, 20), respectively, of the complement system, the interleukin-2 receptor (21) and the plasma protein β_2-glycoprotein I (22).

The present study provides direct evidence for the placement of CR2 in this multigene family. CR2 tryptic peptides showed strong sequence homology to CR1, and CR2 cDNA clones were identified in a human tonsillar cDNA library by cross-hybridization with CR1 cDNA probes.

MATERIALS AND METHODS

Protein Purification and Analysis. CR2 was purified from detergent lysates of the B-lymphoblastoid cell lines SB and Raji, by monoclonal antibody affinity chromatography (13). The purified receptor was homogeneous, when analyzed by polyacrylamide gel electrophoresis in the presence of Na-DodSO$_4$, and had a blocked amino terminus (13). Detergent was removed by extraction of lyophilized CR2 with 80% acetone and washing of the precipitated protein with methanol. CR2 (1.4 nmol) was sequentially reduced by incubation with 5 mM dithiothreitol in 0.1 ml of 0.5 M Tris Cl, pH 8.2/5 mM ethylenediaminetetraacetate/7 M guanidine hydrochloride/0.2% Zwittergent 3-14 (Calbiochem) and carboxymethylated by incubation with 250 μCi of iodo[2-^{14}C]acetic acid (Amersham, 56 Ci/mmol; 1 Ci = 37 GBq) followed by 5 μmol of unlabeled iodoacetic acid. After precipitation and washing

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Abbreviations: C3b, major cleavage fragment of the third component of complement (C3); C4b, major cleavage fragment of C4; CR1, complement receptor type 1 (the C3b/C4b receptor); CR2, complement receptor type 2 (the C3d receptor); EBV, Epstein–Barr virus; kb, kilobase(s).

with 1:1 chloroform/methanol, the CR2 was resuspended in 0.1 M NH_4HCO_3 with 0.2% Zwittergent 3-14 and digested for 24 hr at 37°C with 67 pmol of L-1-tosylamido-2-phenylethyl-chloromethyl ketone-treated trypsin. The tryptic peptides were resolved by chromatography on a Vydac phenyl column, employing a gradient of 0–80% acetonitrile. Tryptic peptides were sequenced by automated Edman degradation performed with an Applied Biosystems 470A sequencer (13).

Screening of the Tonsillar cDNA Library. The human tonsil cDNA library was prepared as described (23) except that the cDNA was not size-selected prior to cloning in λgt11. Nitrocellulose filters containing recombinant clones (400,000) were hybridized overnight at 37°C with a mixture of two homologous CR1 cDNAs, CR1-1 and CR1-2 (23), that had been labeled with ^{32}P by nick-translation (24). After hybridization, the filters were washed at 37°C (low stringency) or 60°C (high stringency) in 0.2× NaCl/Cit (1× is 0.15 M NaCl/15 mM Na citrate).

Oligonucleotide Hybridization. Redundant oligonucleotide probes were synthesized with an Applied Biosystems 380A DNA synthesizer. Oligonucleotides were labeled to specific activities of 2–8 × 10^8 cpm/μg with [γ-^{32}P]ATP (New England Nuclear) by T4 polynucleotide kinase (New England Biolabs) (25). Southern blots containing phage cDNA inserts or fragments of cDNA inserts that had been subcloned in pBR322 were incubated with labeled oligonucleotides in 6× NaCl/Cit/0.1% $NaDodSO_4$/0.05% $NaPP_i$ for 1 hr at 65°C. The water bath containing the filters was turned off and allowed to come to room temperature overnight. Blots were washed at 45°C with this buffer (T_{dmin} of the oligonucleotides ranged from 46 to 52) (26).

RNA and DNA Blot Analysis. Phage and plasmid DNAs were examined by hybridization according to the method of Southern (27). Poly(A)$^+$ RNA from cell lines SB and HSB-2 was analyzed by blot hybridization (28) with subcloned fragments of λ4.11 and with a cDNA probe for actin (29).

RESULTS

Tryptic Peptides of CR2 Show Sequence Homology with CR1. The amino acid sequences of seven tryptic peptides of CR2 were compared with those of CR1 which were derived from nucleotide sequences of CR1 cDNA (Table 1) (16). These CR2 peptides were divided into four groups based not only on their homologies with distinct CR1 sequences but also on their similarities with each other. For example, the two CR2 peptides in group I, 279 and 310, differed by only one position over an eight amino acid stretch, and the two peptides in group II, 278 and 213, were identical over 10 amino acids. Although all four groups of CR2 peptides demonstrated some similarities to CR1 sequences, the resemblance was most remarkable for group I, where the homologies were in the range of 70%, and rose to almost complete identity when only residues that were conserved in the CR1 repeats were considered. Furthermore, the positions of tryptophan, proline, and cysteine in these peptides could be aligned with the conserved residues of the 60 amino acid consensus repeat shared by the family of C3b/C4b-binding proteins (17–20).

Identification of CR2 cDNA by Cross-Hybridization with CR1 cDNA. The finding of amino acid sequence homology between CR2 and CR1 provided the rationale for attempting to identify CR2 recombinant clones in a tonsillar cDNA library by hybridization with CR1-specific cDNA probes at low stringency. The two CR1 cDNA probes, CR1-1 and CR1-2, are 0.8 and 0.9 kilobases (kb) long, respectively, and are fragments of a single CR1 cDNA insert. Each represents a portion of two long homologous repeats that have 87% nucleotide homology (16, 23) and encode the amino acid sequences shown in Table 1.

Five recombinant clones of a total of 400,000 in the cDNA library were identified by CR1-1 and CR1-2 after hybridization and washing at 37°C in 0.2× NaCl/Cit (low stringency). The three strongly hybridizing clones, λ1.11, λ3.11, and λ7.11, remained positive when the filters were washed at 60°C in 0.2× NaCl/Cit (high stringency), whereas the two weakly positive clones, λ4.11 and λ5.11, lost their specific hybridization. DNA from these clones was digested with *Eco*RI and examined by Southern blot analysis. The inserts from λ1.11, λ3.11, and λ7.11 were each comprised of a single restriction fragment that hybridized with the CR1 cDNA probes under conditions of low and high stringency (Fig. 1 *a–c*). In contrast, the inserts from λ4.11 and λ5.11 contained three restriction fragments, two of which, 1.45 and 0.7 kb long, were common to both clones (Fig. 1*a*). The 1.45-kb fragment of both λ4.11 and λ5.11 and the 1.6-kb fragment of λ4.11 hybridized to the CR1 cDNA probes under conditions

Table 1. Comparison of amino acid sequences of tryptic peptides of CR2 with homologous sequences of CR1

CR2 peptide or CR1 region	Amino acid sequence
Group I	
CR2 279	Gly-Thr-**Trp**-Ser-Gly-Pro-Ala-**Pro**-Leu-**Gln/Cys**-Lys
CR2 310	Thr-Gly-Thr-**Trp**-Ser-Gly-Pro-Ala-**Pro**-Arg
CR1a	Gly-Pro-Ala-**Pro**-Gln-**Cys**-Ile
CR1b	Val-Gly-Ile-**Trp**-Ser-Gly-Pro-Ala-**Pro**-Gln-**Cys**-Ile
CR1c	Asn-Gly-Val-**Trp**-Ser-Ser-Pro-Ala-**Pro**-Arg-**Cys**-Gly
Group II	
CR2 278	Gln/Cys-Val-Ile-Ala-Gly-Gln-Gly-Val-Ala-**Trp**-Thr-Lys
CR2 213	Ile-Val-Ile-Ala-Gly-Gln-Gly-Val-Ala-**Trp**-Thr
CR1d	Ser-Asp-Pro-Gln-Gly-Asn-Gly-Val- * -**Trp**-Ser
Group III	
CR2 314	Asp/Gln-Ser-Leu-Gln-Glu-Leu-**Pro**-Ala-Gly-Ser-Arg
CR1e	Lys-**Trp**-Glu-Pro-Glu-Leu-**Pro**-Ser-Cys-Ser-Arg
Group IV	
CR2 313	Val-Lys-Glu-**Pro**-Pro-Xaa-Ile-Leu
CR1f	**Cys**-Gln-Pro-**Pro**-Pro-Glu-Ile-Leu

CR2 sequences were determined by amino acid sequence analysis of HPLC-purified tryptic peptides of CR2, whereas CR1 sequences were derived from nucleotide sequences of CR1 cDNA (16). Underlined residues indicate sequence homologies between CR1 and CR2. Residues in boldface align with consensus sequence. Slash (/) indicates ambiguity. Xaa indicates unknown amino acid assignment. Asterisk in CR1d sequence shows gap inserted to maximize homology.

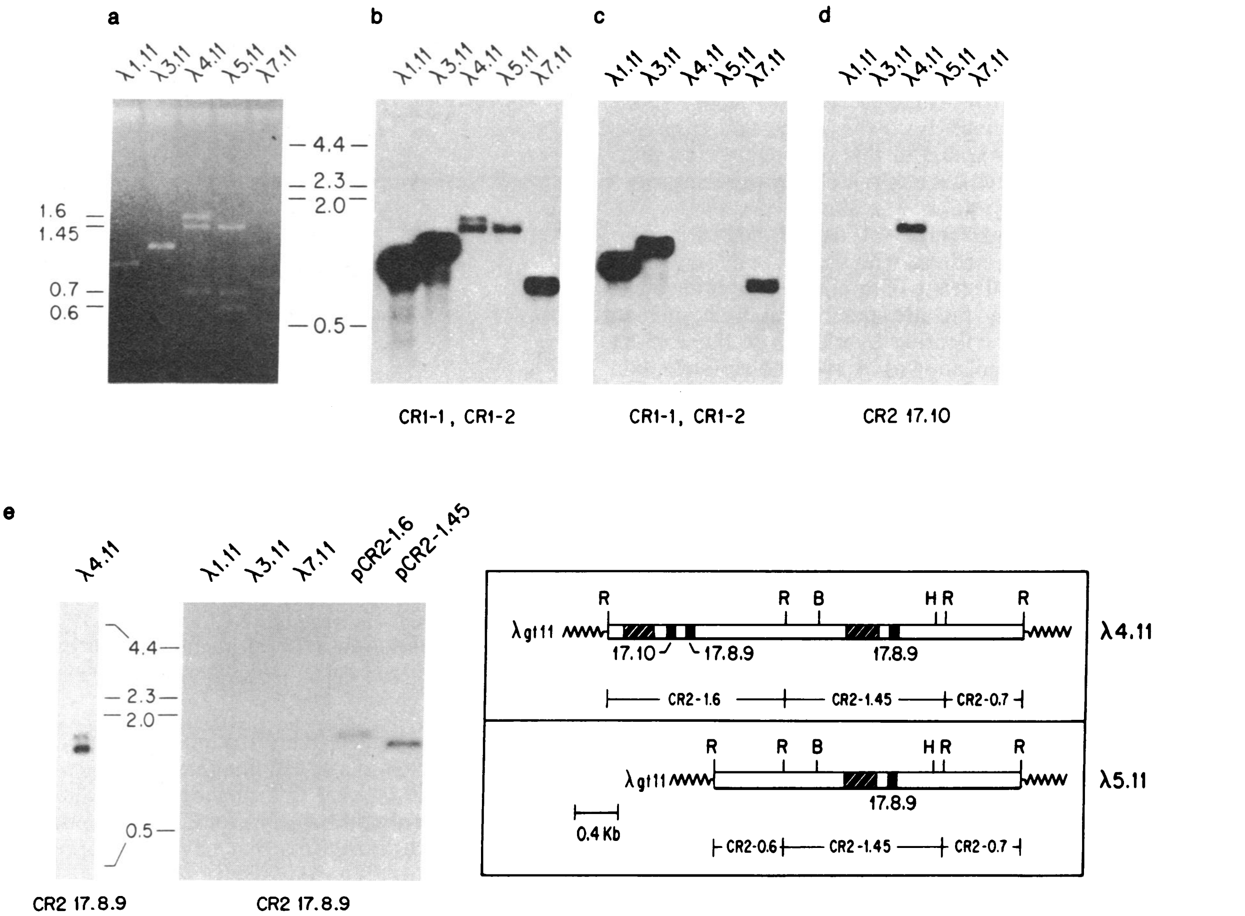

FIG. 1. Hybridization of CR1 and CR2 cDNA clones at low and high stringency with CR1 cDNA probes and with oligonucleotides derived from CR2 tryptic peptide sequences. Five recombinant clones, from a λgt11 human tonsillar library, that hybridized with CR1 cDNA sequences under conditions of low stringency were selected. DNA from each of these phage was digested with *Eco*RI and prepared for Southern blot hybridization. (*a*) Ethidium bromide-stained fragments of the three CR1 clones, λ1.11, λ3.11, and λ7.11, and the two CR2 clones, λ4.11 and λ5.11. The positions of migration of DNA standards are indicated at right and the estimated size (kb) of the *Eco*RI-generated fragments from λ4.11 and λ5.11 is indicated at left. (*b* and *c*) DNA from the gel shown in *a* was transferred to a nylon membrane, hybridized with ^{32}P-labeled CR1 cDNAs, and washed at 37°C (low stringency, *b*) and then at 60°C (high stringency, *c*). (*d*) A duplicate blot was hybridized with the CR2-specific oligonucleotide probe 17.10. (*e*) The 1.6- and 1.45-kb fragments of λ4.11, which had been subcloned into the *Eco*RI site of pBR322, and DNA from the λ1.11, λ3.11, and λ7.11 phage clones (all of which were digested with *Eco*RI) were prepared for blot analysis and probed with the CR2-specific oligonucleotide probe 17.8.9. Also shown is the hybridization of the *Eco*RI-digested λ4.11 phage with probe 17.8.9. (*Inset box*) A map of the CR2 cDNA phages λ4.11 and λ5.11. Black box represents sites of hybridization with CR2-specific oligonucleotide probes. Hatched box indicates the position of hybridization of the CR1 cDNA probes. Restriction sites: R, *Eco*RI; B, *Bam*HI; H, *Hin*dIII.

of low stringency, but the probes were removed by washing at high stringency (Fig. 1 *b* and *c*).

Initial evidence for the probable identity of λ4.11 as a cDNA clone for CR2 was provided by the hybridization of the 17.10 oligonucleotide to the 1.6-kb restriction fragment (Fig. 1*d*). This oligonucleotide is a 17-mer of 128-fold redundancy and was derived from a portion of CR2 tryptic peptide 278 (Fig. 2). Another 17-mer, 17.2.3, overlapping 17.10 by 5 nucleotides (Fig. 2), also was shown to hybridize to the 1.6-kb fragment of λ4.11 (data not shown), extending the identified sequence to 29 nucleotides and 10 amino acids. The 1.6- and 1.45-kb *Eco*RI fragments of λ4.11 were subcloned in pBR322, and the plasmid inserts were designated CR2-1.6 and CR2-1.45. The subclones, digested with *Eco*RI, and the *Eco*RI digests of λ4.11, λ1.11, λ3.11, and λ7.11 were subjected to Southern blot analysis using a third oligonucleotide,

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CR2 278 (Arg)-Gln/Cys-Val-Ile-Ala-Gly-Gln-Gly-Val-Ala-Trp-Thr-Lys

                    _______________
                         17.2.3
                                      _______________
                                           17.10

cDNA         -CGG-TGT-GTA-ATT-GCT-GGA-CAG-GGA-GTT-GCT-TGG-ACC-AAA
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FIG. 2. Identification of CR2-specific sequence in CR2 cDNA. The amino acid sequence of CR2 tryptic peptide 278 was determined by amino acid sequence analysis. CR2 peptide sequences from which the oligonucleotide probes 17.2.3 and 17.10 were derived are indicated by horizontal lines below the amino acid sequence. The cDNA sequence was determined by analysis of a CR2-1.6 *Hae* III M13 clone. The first codon (CGG) encodes Arg, which is presumed to be at the carboxyl terminus of the peptide amino-terminal to 278, and the second codon encodes Cys; the other codons are aligned beneath their respective amino acids.

17.8.9, as the probe. This oligonucleotide was a 17-mer of 384-fold redundancy that was derived from the CR2 tryptic peptide, 279, containing sequence highly homologous to CR1 (Gly-Thr-Trp-Ser-Gly-Pro) (Table 1). The 1.6- and 1.45-kb fragments of λ4.11, derived from the phage and from the plasmid subclones, hybridized to this oligonucleotide (Fig. 1*e*), whereas the presumed CR1 cDNA clones were negative. The intensity of hybridization was slightly greater for the 1.45-kb than for the 1.6-kb fragment, as had occurred when these fragments were hybridized with the CR1 cDNA probes (Fig. 1 *b* and *e*). This difference of intensity of hybridization is apparently caused by the presence of multiple sites to which the 17.8.9 oligonucleotide hybridizes in the 1.45-kb fragment of λ4.11 (data not shown). A 14-mer oligonucleotide overlapping 17.8.9 by 5 nucleotides also hybridized to the 1.6- and 1.45-kb fragments (data not shown).

Partial restriction maps of the cDNA inserts from λ4.11 and λ5.11 were derived by digesting the recombinant phage with *Eco*RI, *Bam*HI, and *Hin*dIII. In λ4.11, the 1.6-kb *Eco*RI fragment appears to be 5′ to the 1.45-kb fragment, and the 0.7-kb fragment that did not hybridize with the CR1 cDNA probes or with the oligonucleotides was the most 3′ fragment (Fig. 1). The 1.45- and 0.7-kb *Eco*RI fragments of λ5.11 are identical to the similarly sized fragments of λ4.11, and the 0.6-kb fragment represented the 3′ region of the 1.6-kb fragment of λ4.11. This conclusion was supported by the observation that the latter two fragments hybridized to each other under conditions of high stringency (data not shown).

Oligonucleotide Site Hybridization. The nucleotide sequence corresponding to the 17.10 and 17.2.3 oligonucleotide probes was determined by digesting the CR2-1.6 insert with *Hae* III and ligating the fragments into the vector M13 mp18. The resulting phage were probed with the 17.10 oligonucleotide and those containing hybridizing sequences were analyzed by the dideoxynucleotide chain-termination method (30). The derived amino acid sequences matched the protein sequence obtained from the CR2 tryptic peptide, a portion of which was used for the oligonucleotide construction (Fig. 2).

Analysis of CR2 mRNA by Blot-Hybridization. Poly(A)$^+$ RNA from the CR2-positive human B-lymphoblastoid cell line SB and from the CR2-negative T-lymphoblastoid line HSB-2 were analyzed by blot-hybridization with a mixture of CR2-1.6 and CR2-1.45. A single species of 5 kb was identified in mRNA from SB cells, but not HSB-2 cells, under conditions of high stringency (Fig. 3*a*). A cDNA probe for actin hybridized to a 2-kb mRNA from both cell lines (Fig. 3*b*). Therefore, cDNA probes derived from λ4.11 identify an mRNA in the correct cell type and of a size capable of encoding the M_r 110,000 peptide of CR2.

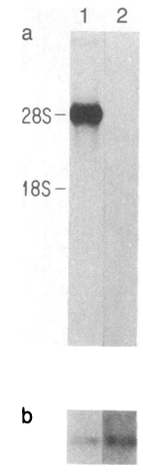

FIG. 3. Blot-hybridization analysis of mRNA from human B- and T-lymphoblastoid cell line. Poly(A)$^+$ RNA (2 μg) from the B-lymphoblastoid line SB (lane 1) or the T-lymphoblastoid line HSB-2 (lane 2) was electrophoresed in a formaldehyde-containing 1.5% agarose gel, transferred to a nylon membrane as described (28), and probed with nick-translated CR2-1.6 and CR2-1.45 probes (*a*) or with a nick-translated actin probe (*b*). Migration positions of the 28S (5 kb) and 18S (2 kb) ribosomal RNA subunits are indicated at left in *a*.

DISCUSSION

This study has identified CR2 as a member of a recently recognized family of proteins, most of which share a capacity for binding C3b and/or C4b. This assignment is based on the presence in CR2 tryptic peptides of conserved residues of a consensus 60 amino acid repeat (Table 1) that is the distinguishing structural characteristic of this protein family (17–22). In addition, the sequence of CR2 cDNA was sufficiently similar to that of cDNA of CR1, a membrane protein found recently to be a member of this family, to permit their cross-hybridization under conditions of low stringency (Fig. 1). That these clones represented CR2 cDNA rather than cDNA of CR1 or of other members of the C3b/C4b-binding protein family was proven by hybridization with CR2-specific oligonucleotide probes synthesized according to tryptic peptide sequences (Fig. 1) and by partial DNA sequence analysis of the nucleotide sequences encoding the corresponding tryptic peptides, including flanking amino acids (Fig. 2). Confirming that these cDNA clones include CR2-specific sequences was their hybridization to a single, 5-kb species of poly(A)$^+$ RNA in a B-cell line that expresses CR2 but not in a T-cell line lacking this membrane protein (Fig. 3). The observation of a 5-kb mRNA for CR2 also contrasted with the 9- and 11-kb transcripts that have been identified for CR1 (23).

Despite being a C3d- rather than a C3b-binding protein, CR2 and CR1 have previously been shown to share certain other characteristics that might have suggested a relationship between the two receptors. Both proteins are cellular receptors that are present on B cells, although CR1 has a wider cellular distribution, being present also on polymorphonuclear and mononuclear phagocytes, erythrocytes, and glomerular podocytes. Neither receptor is detectably phosphorylated in resting cells, but both receptors are phosphorylated following treatment of cells with phorbol esters (12). CR1 is phosphorylated in a tissue-specific manner, this covalent modification occurring only with phagocytic cell types and not B cells. Phagocytic activity by CR1 is associated with, although not yet shown to be dependent on, phosphorylation. The role of phosphorylation in the function of CR2 is less clear because the possible function of this receptor in augmenting proliferation of B cells has become apparent only recently (10, 31, 32).

The present studies demonstrate a structural relationship between CR1 and CR2. Sequence analysis of CR1 cDNA clones has shown (16) that this receptor, in addition to having the 60 amino acid consensus repeat in which only a few positions are conserved, is comprised of at least two highly homologous long repeats having 83% amino acid identity. Each long repeat encompasses approximately 400 amino acids and 6 or 7 shorter consensus repeats. Analysis of CR2 indicated the presence not only of conserved residues of the consensus repeat of the C3b/C4b-binding protein family, but also of repetitive amino acid sequences that were highly homologous and therefore similar to the homologous repeat sequences of CR1 (Table 1). Other evidence for the existence of homologous repeats within CR2 was the hybridization of the 17.8.9 oligonucleotide probe to the 1.6- and 1.45-kb *Eco*RI fragments of λ4.11 (Fig. 1). Finally, the CR1-like structure of CR2 was most strikingly exemplified by the cross-hybridization of the CR1 cDNA probes to CR2 cDNA sequences (Fig. 1). CR2, however, may differ from CR1 in its

overall organization, because the absence of hybridization of CR1-1 or CR1-2 to the 0.6-kb fragment of λ5.11, which represents the 3′ region of the 1.6-kb fragment of λ4.11, suggests that the homologous repeats in CR2 may not be contiguous as they are in CR1.

The full extent of the structural homology between CR1 and CR2 can be appreciated only after the sequence analyses of these receptors are completed. However, the similarities observed in this initial examination are sufficient to suggest that the receptors are evolutionarily related and that CR2 is probably more closely related to CR1 than to other members of the C3/C4-binding protein family. Therefore, it is possible that CR2 evolved from CR1 to serve a special function on B cells that CR1 could not accomplish. For example, the specificity of CR2 for the C3d region of C3 may relate to the localization of B cells to sites that are remote from sites of inflammation and complement activation. These unique functions of CR2, including that of serving as the receptor for EBV, may be addressed by experiments involving transfection of the gene encoding CR2 into cell types other than human B lymphocytes.

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