Determination of the Structural Basis for Selective Binding of Epstein-Barr Virus to Human Complement Receptor Type 2

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Summary

Epstein-Barr virus (EBV) is an oncogenic herpesvirus that selectively infects and immortalizes human B lymphocytes. One determinant of this narrow tropism is human CR2, the only viral receptor within the superfamily of proteins that contain short consensus repeats (SCRs). Human CR2 serves as a receptor for both C3dg and the gp350/220 glycoprotein of EBV, and binds the monoclonal antibody (mAb) OKB7, which blocks binding of both ligands to the receptor. In contrast, although murine CR2 is capable of binding human C3dg and this interaction can be blocked with the mAb 7G6, it does not bind OKB7 or EBV. We have determined the structural basis for absolute specificity of EBV for human CR2 through characterization of a panel of 24 human-murine chimeric receptors, all of which bind human C3dg. The results indicate that preferential binding of EBV to human CR2 is not due to unique amino acids that are capable of binding the virus, but reflects a distinct receptor conformation that can be achieved in murine CR2 with single amino acid substitutions in two discontinuous regions of the primary structure: replacement of proline at position 15 with the corresponding serine from human CR2, and elimination of a potential N-linked glycosylation site between SCR-1 and SCR-2. Furthermore, species-specific binding of EBV, OKB7, and 7G6 can all be manipulated through substitutions among residues 8–15, suggesting that this octapeptide is part of a structural determinant that is critical for binding of both viral and natural ligands to CR2.

Viral tropism is a multideterminant process whereby a particular virus is able to bind, penetrate, replicate, and otherwise sustain its life cycle within a restricted host. Several tactics for such subterfuge involve the capacity of animal viruses both to evade as well as to exploit the host immune system. One of the best-described examples of a viral strategy to avoid neutralization and permanent eradication by the humoral immune response is that of picornaviruses. Protomers that comprise the protein coats of rhinovirus and poliovirus contain canyons with dimensions such that they can potentially interact with loops from their respective receptors, although they are inaccessible to host neutralizing antibodies (1). Exploitation of the host immune system, on the other hand, is a theme shared by several animal viruses, including HIV and rhinovirus, which bind CD4 (2, 3) and intercellular adhesion molecule 1 (ICAM-1)[1] (4, 5), respectively.

EBV, a lymphocryptovirus, demonstrates narrow tropism that includes infection and efficient immortalization of human B lymphocytes (6). EBV is also an oncogenic herpesvirus that infects nearly all humans and is intimately involved in the pathogenesis of Burkitt's lymphoma and other B cell lymphomas that arise during states of impaired immune surveillance such as AIDS and post-transplant immunosuppression. Members of the family *Herpesviridae*, such as EBV, have adopted a form of stealth in which they express only a small subset of viral genes after infection, and in ways that are not entirely understood, avoid immune surveillance by establishing latency (7, 8). EBV also exploits the human immune system to gain entry to B cells. Preferential infection of human B lymphocytes by EBV appears to be multideterminant, and includes their unique capacity to bind the virus specifically via complement receptor type 2 (CR2; CD21) (9, 10).

Human CR2 is a 145-kD transmembrane phosphoprotein that serves as a receptor for both the C3dg and iC3b proteins of the complement system (11), as well as for the gp350/220 envelope glycoprotein of EBV (12, 13). However, whereas CD4 (14), ICAM-1 (15, 16), and the poliovirus receptor (17)

[1] *Abbreviations used in this paper:* ICAM-1, intercellular adhesion molecule 1; SCR, short consensus repeat.

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are members of the Ig superfamily, CR2 is unique in that it is the only viral receptor so far identified that is a member of the large family of proteins containing short consensus repeats (SCRs) (18, 19). The SCR is comprised of ~60 amino acids including four invariant cysteines that are disulfide bonded in a cys-1 to cys-3 and cys-2 to cys-4 pattern (20). This results in a three-dimensional structure consisting of a hydrophobic core surrounded by three β strands on one face and two β strands on the other (21). The SCR motif has so far been identified in >30 mammalian proteins involved in the immune response, inflammation, and tissue repair, including the IL-2 receptor (22), Endothelial leukocyte adhesion molecule 1 (23), and mouse lymph node homing receptor (24, 25).

The extracellular domain of human CR2 is comprised entirely of 15 or 16 SCRs that are the products of alternative splicing (26, 27), and it has been determined that SCR-1 and SCR-2 are both necessary and together sufficient to bind both gp350/220 and C3dg (28). In contrast, although murine CR2 is also comprised of 15 SCRs (29–31) and is able to bind not only murine C3dg, but also human C3dg, EBV is specific for the human receptor (32). This suggests that residues critical for EBV binding might not be required for binding natural ligand to the human receptor. In addition, it presents a unique opportunity to define these distinct requirements and to identify the critical residues that render human and murine B lymphocytes distinct in their capacity to bind EBV.

Materials and Methods

Isolation of Murine CR2 cDNA. Full-length human CR2 cDNA was radiolabeled and used to probe a λ gt10 mouse spleen cDNA library (generously provided by Lloyd Klickstein, Center for Blood Research, Boston, MA) at high stringency. Seven positive plaques were identified among 10^6 screened. The DNA from each positive clone was purified and used as a template in a PCR (33) in which the upstream primer 5'HMCR2.1 and the downstream primer 3'HMCR2.5 represented the 5' and 3' boundaries of SCR-1 and SCR-2 of mouse CR2, respectively. Amplification of four of the seven purified cDNA clones produced the expected 419-bp fragment and one of these, λMCR2.4, was cloned into the XmaI and XhoI sites of pBS KS$^+$ (Stratagene, La Jolla, CA) to produce pBS.MCR2.1. The nucleotide sequence of both strands was determined by the dideoxy method (34) and found to correspond exactly with that determined previously (29–31).

Antibodies. HB5 (IgG2a) and OKB7 (IgG2a) (Ortho Pharmaceuticals, Raritan, NJ) are mouse mAbs specific for human CR2 (10, 11). 7G6 (IgG2b) is a rat mAb that recognizes murine CR2 (35). Murine mAb 9245 (IgG2a) (DuPont Co., Wilmington, DE) is specific for the EBV gp350/220 envelope glycoprotein. RPC5.4 (IgG2a) (American Type Culture Collection, Rockville, MD) is a mouse mAb that that was used as a nonspecific control. Rabbit polyclonal anti-C3d serum A063 (Dako Corp., Carpinteria, CA), DTAF-conjugated goat F(ab')$_2$ anti–mouse IgG, DTAF-conjugated goat anti–rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and FITC-conjugated goat F(ab')$_2$ anti–rabbit IgG (Accurate Chemical and Scientific, Westbury, NY) were purchased.

Immunofluorescence and Flow Cytometry. 48 h after transfection, COS cells were harvested with PBS containing 0.5 mM EDTA and washed with PBS containing 0.1% BSA and 0.02% sodium azide. Replicate samples of cells from each transfection were assayed for the capacity to bind HB5, OKB7, 7G6, C3dg, or EBV. Studies with HB5, OKB7, and 7G6 were performed by sequential incubation with 4.0 μg/ml of the primary antibody followed by 4.5 μg/ml DTAF-conjugated goat F(ab')$_2$ anti–mouse IgG (for HB5 and OKB7) or 4.5 μg/ml DTAF-conjugated goat anti–rat IgG (for 7G6). C3dg binding was determined by sequential incubation of cells with 3 μg/ml pC3dg, followed by 1:100 rabbit serum raised against human C3d, and finally 1:100 FITC-conjugated goat F(ab')$_2$ anti–rabbit IgG. EBV binding was determined by resuspension of COS transfectants in 50 μl purified EBV in RPMI followed by sequential incubation with 1.0 μg/ml anti-gp350, and 4.5 μg/ml DTAF-conjugated goat F(ab')$_2$ anti–mouse IgG.

K562 stable transfectants were directly labeled with 7.0 μg/ml FITC-conjugated HB5, and sorted once with a FACStar® (Becton Dickinson & Co., Mountain View, CA) to obtain the brightest 5–10%. These populations of K562 transfectants were expanded in cultures containing 200 μg/ml hygromycin B (Calbiochem-Behring Corp., San Diego, CA) and assayed for the capacity to bind HB5, OKB7, 7G6, and EBV in the same manner as described above for COS cells.

Radioimmunoassays. Polymerized iC3b (piC3b) was radiolabeled to a specific activity of 10^6 cpm/μg with the iodobead method. Affinities for ^{125}I-p(iC3b) were determined by incubation of 5 × 10^6 K562 transfectants at 0°C for 60 min with increasing concentrations of ^{125}I-p(iC3b) in the presence or absence of OKB7 for CR2.H or 7G6 for CR2.M. Competition assays were performed by incubation of 5 × 10^6 K562 transfectants at 0°C for 30 min with 10.0 μg/ml 7G6, OKB7, or RPC5.4, after which 3 μg/ml ^{125}I-p(iC3b) was added for an additional 60-min incubation. Replicate samples for all RIAs were centrifuged through 0.3 ml of a 3:1 mixture of dibutylphthalate/dinonylphthalate in 0.4-ml polypropylene microfuge tubes for 30 s at 8,000 rpm at room temperature. The tubes were cut, cell bound and free ^{125}I were determined, and the mean cpm was calculated for each replicate pair.

Purification and Polymerization of Ligands. Polymerized C3dg (pC3dg) was prepared as described (28). Analysis of pC3dg by sucrose gradient ultracentrifugation demonstrated an average molecular weight of 450,000, consistent with polymers ranging from tetramers to 20-mers. Purified C3b, factor H, and factor I were used to make iC3b, which was crosslinked with an 80-fold molar excess of glutaraldehyde for 2 h at room temperature, and then quenched with 1 M Tris-HCl, pH 7.5. Polymers were separated over gradients of 7.5–40% sucrose in PBS (wt/vol) centrifuged at 200,000 *g* for 12 h at 4°C. Fractions containing tetramers or larger polymers of polymerized iC3b (piC3b) were pooled, dialyzed, and stored in PBS at 0°C. The B95-8 strain of EBV was prepared as described (36), and used as 1,000-fold concentrated culture supernatant in RPMI.

Cell Lines and Transfections. COS cells were maintained in DMEM with D-glucose at 4,500 mg/liter supplemented with 10% bovine calf serum (Hyclone Laboratories, Logan, UT) and 2 mM glutamine. K562 cells and Raji B lymphoblastoid cells (American Type Culture Collection) were grown in RPMI 1640 supplemented with 10% bovine calf serum and 2 mM glutamine. Plasmids were transfected into COS cells for transient expression using the DEAE-dextran procedure (37). Each of the plasmids encoding a chimeric CR2 cDNA was cotransfected with plasmid p141 (38) into K562 cells through lipofection as described previously (39). Stable transfectants were selected for and maintained in 200 μg/ml hygromycin B.

Construction of Human-Murine Chimeric CR2 cDNA Fragments. Cassettes comprised of cDNA encoding SCR-1 and SCR-2 of human CR2, SCR-1 and SCR-2 of murine CR2, or two-SCR chimeric fragments encoding a portion of each of these regions were

created through PCRs (33). All of the two-SCR products included unique XmaI and XhoI sites at the 5′ and 3′ ends, respectively. The cassette encoding SCR-1 and SCR-2 of human CR2 was created through PCR using pi15 as a template and oligonucleotides 5′HMCR2.2 and 3′HMCR2.3 (see Table 2) as upstream and downstream primers, respectively. The cassette encoding SCR-1 and SCR-2 of murine CR2 was created in similar fashion as described above (isolation of murine CR2 cDNA).

The two-SCR chimeric fragments were created by taking advantage of short stretches of nucleotides that are conserved between human CR2 and murine CR2 within SCR-1 and SCR-2. This enabled design and synthesis of a panel of oligonucleotides for use in a two-step PCR strategy as follows (Tables 1 and 2). First, an upstream primer that adds a unique XmaI restriction site to the 5′ end of the fragment and a downstream primer that encodes several amino acids that are identical between the two species are used to amplify a segment of the first two SCRs of either human or murine CR2. The double-stranded product of this reaction is then used as an upstream primer together with a downstream primer that adds a unique XhoI restriction site to the 3′ end of the fragment, now using CR2 cDNA from the other species as template in a PCR. The final product is a chimeric two-SCR fragment flanked by unique restriction sites. In some cases the template was neither wild-type human CR2 nor wild-type murine CR2, but rather one of the chimeric fragments (Table 2). The nomenclature used for the chimeric fragments and for the constructs containing them consists of the prefix pi followed by a numerical designation, which refers to the amino acids that are murine. The construct pi16-127 was not created by PCR but rather was the product of a ligation between the 5′ XmaI-SphI fragment of 16-127/Y and the 3′ SphI-XhoI fragment of pi36-127. The construct piPS was not created by PCR but rather was the product of a ligation between the 5′ XmaI-SphI fragment of piPS/Y and the 3′ SphI-XhoI fragment of pi36-127.

Creation of Expression Vectors. Plasmid pi15 contains a full-length human CR2 cDNA within the eukaryotic expression vector CDM8 (40). Plasmid piCR2.H was created through oligonucleotide-directed mutagenesis of pi15 such that a silent G to C transversion was incorporated into the third position of a proline codon within the human CR2 leader peptide, seven residues NH_2 terminal to cysteine 1 of SCR-1. This resulted in creation of a unique XmaI restriction site located ~400 bp upstream from the unique XhoI restriction site already present in pi15, and facilitated unidirectional cloning of the 5′-XmaI- and 3′-XhoI-restricted two-SCR cassettes described above. The entire sequence of both strands of each insert was confirmed through dideoxy sequencing.

Table 1. *Oligonucleotides Used in This Study*

Name	5′-3′ Sequence
5′HMCR2.1	GCACCCGGGGTCCTCGGGATTTCTTGTGACCCTCCTC
5′HMCR2.2	GCACCCGGGGTCCTCGGGAT
5′HMCR2.3	AATAAAACCATTTCTTGCTCAGATCCCATAGTACCAGG
5′HMCR2.4	TCTAGAACTAGTGGATCCCC
5′HMCR2.5	AGACATGGTGATTCTGTG
5′HMCR2.6	GCTCCTCCTATATGTGAATATTTCAATAAA
5′HMCR2.7	TGGGATAAACCTGCTCCTATATGTGAATCTGTGAATAAAACCATTTCTTGCTCA GATCCCATAGTACCAGGAGGA
5′HMCR2.8	AATAAATATTCTTCTTGCTCAGATCCCATA
5′HMCR2.9	TGGGATAAAGCTCCTCCTAAATGTGAATATTTCAATAAATATTCTTCTTGCCCT GAGCCCATAGTACCAGGGGGA
5′HMCR2.10	GTGAATAAATATATTTCTTGC
3′HMCR2.1	TTTTCTCCAATGAGGCGG
3′HMCR2.2	TTTATTCACAGATTCACAT
3′HMCR2.3	CCAACATTCTCACTTGTGTG
3′HMCR2.4	ATCACCATGTCTGAATGGTGCCTTGGATCCTTTATTCATGAATCCCCCTGGTAC
3′HMCR2.5	ACACTCGAGAGGGAAATCACTCTCACA
3′HMCR2.6	AGAATAATAGGGTTTCCGAGCATTTTTGACTTCAGGAGGAG
3′HMCR2.7	ACAGTTCCAGGAACTATGGGAAGAGAATAATA
3′HMCR2.8	AATGAGGCGGTAGCTAGGTGAACAAGTGTACCTCAGAACAGTTCCA
3′HMCR2.9	GCATTTTTGACAGGAGGAGGAGGGTC
3′HMCR2.10	CGAGCATTTTTGATTTCAGGAGGAGG
3′HMCR2.11	GGTTTCCGAGCATTTAGGACTTCAGGAGG
3′HMCR2.12	GAATAATAGGGTTTCCGGCCATTTTTGACTTC
3′HMCR2.13	GAATAATAGGGAATCCGAGCATTTTT
3′HMCR2.14	AGAGAATAATAACTTTTCCGAGCATT

Table 2. *Strategies for Construction of 2-SCR Chimeric CR2 Fragments*

	First PCR			Second PCR		
Chimera	5′ Oligo	3′ Oligo	Template	5′ Oligo	3′ Oligo	Template
pi3-36	5′HMCR2.1	3′HMCR2.1	piCR2.M	PCR #1	3′HMCR2.3	piCR2.H
pi36-66	5′HMCR2.2	3′HMCR2.2	pi36-127	PCR #1	3′HMCR2.3	piCR2.H
pi66-74	5′HMCR2.3	3′HMCR2.3	piCR2.H	5′HMCR2.2	PCR #1	piCR2.H
pi74-90	5′HMCR2.4	3′HMCR2.4	piCR2.H	PCR #1	3′HMCR2.3	piCR2.H
pi90-127	5′HMCR2.5	3′HMCR2.5	pi36-127	5′HMCR2.2	PCR #1	piCR2.H
pi3-62	5′HMCR2.6	3′HMCR2.3	piCR2.H	5′HMCR2.1	PCR #1	piCR2.M
pi60-74	5′HMCR2.7	3′HMCR2.3	piCR2.H	5′HMCR2.2	PCR #1	piCR2.H
pi71-127	5′HMCR2.8	3′HMCR2.5	piCR2.M	5′HMCR2.2	PCR #1	piCR2.H
pi3-60/74-127	5′HMCR2.9	3′HMCR2.5	piCR2.M	5′HMCR2.1	PCR #1	piCR2.M
pi3-7	5′HMCR2.1	3′HMCR2.5	piCR2.H	None	None	None
pi7-16	5′HMCR2.4	3′HMCR2.6	piCR2.H	PCR #1	3′HMCR2.3	piCR2.H
pi16-24	5′HMCR2.4	3′HMCR2.7	piCR2.H	PCR #1	3′HMCR2.3	piCR2.H
pi24-36	5′HMCR2.4	3′HMCR2.8	piCR2.H	PCR #1	3′HMCR2.3	piCR2.H
pi16-127	None	None	None	None	None	None
pi3-127/Y	5′HMCR2.10	3′HMCR2.5	piCR2.M	5′HMCR2.1	PCR #1	piCR2.M
pi16-127/Y	5′HMCR2.4	3′HMCR2.7	pi16-24	PCR #1	3′HMCR2.5	pi3-127/Y
piEP/Y	5′HMCR2.4	3′HMCR2.9	piCR2.M	PCR #1	3′HMCR2.5	pi3-127/Y
piVI/Y	5′HMCR2.4	3′HMCR2.10	piCR2.M	PCR #1	3′HMCR2.5	pi3-127/Y
piKL/Y	5′HMCR2.4	3′HMCR2.11	piCR2.M	PCR #1	3′HMCR2.5	pi3-127/Y
piAG/Y	5′HMCR2.4	3′HMCR2.12	piCR2.M	PCR #1	3′HMCR2.5	pi3-127/Y
piKI/Y	5′HMCR2.4	3′HMCR2.13	piCR2.M	PCR #1	3′HMCR2.5	pi3-127/Y
piPS/Y	5′HMCR2.4	3′HMCR2.14	piCR2.M	PCR #1	3′HMCR2.5	pi3-127/Y
piPS	None	None	None	None	None	None

Results

The Ligand Binding Site of Murine CR2 Is Located at the NH_2 Terminus of the Receptor. SCR-1 and SCR-2 of human CR2 have the same affinity for both human C3dg and gp350/220 as does the entire protein, and are together sufficient to bind the mAb OKB7, which blocks binding of both ligands to the receptor (28). We anticipated that the NH_2-terminal pair of SCRs of murine CR2 would also be necessary and sufficient for binding human C3dg because the extracellular domains of both human and murine CR2 are comprised entirely of 15 SCRs, the amino acid sequences of these homologous proteins are 61% identical within the first two SCRs, and murine B lymphocytes are capable of binding human C3dg (29–31). This hypothesis was tested by substituting a cDNA fragment encoding SCR-1 and SCR-2 of murine CR2 for sequence encoding the homologous pair of SCRs of human CR2 in the eukaryotic expression vector piCR2.H (Fig. 1). This resulted in creation of piCR2.M, in which amino acids from cysteine 1 of SCR-1 through cysteine 4 of SCR-2 represent murine CR2, and the remainder

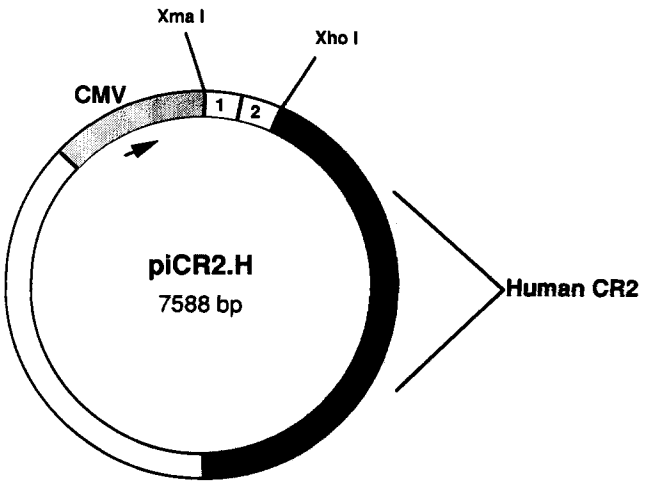

Figure 1. Expression construct piCR2.H. Transcription of wild-type full-length human CR2 is driven by the CMV immediate early promoter, and the first two SCRs are flanked by unique restriction sites such that they can be replaced with two-SCR cassettes encoding murine or chimeric binding sites.

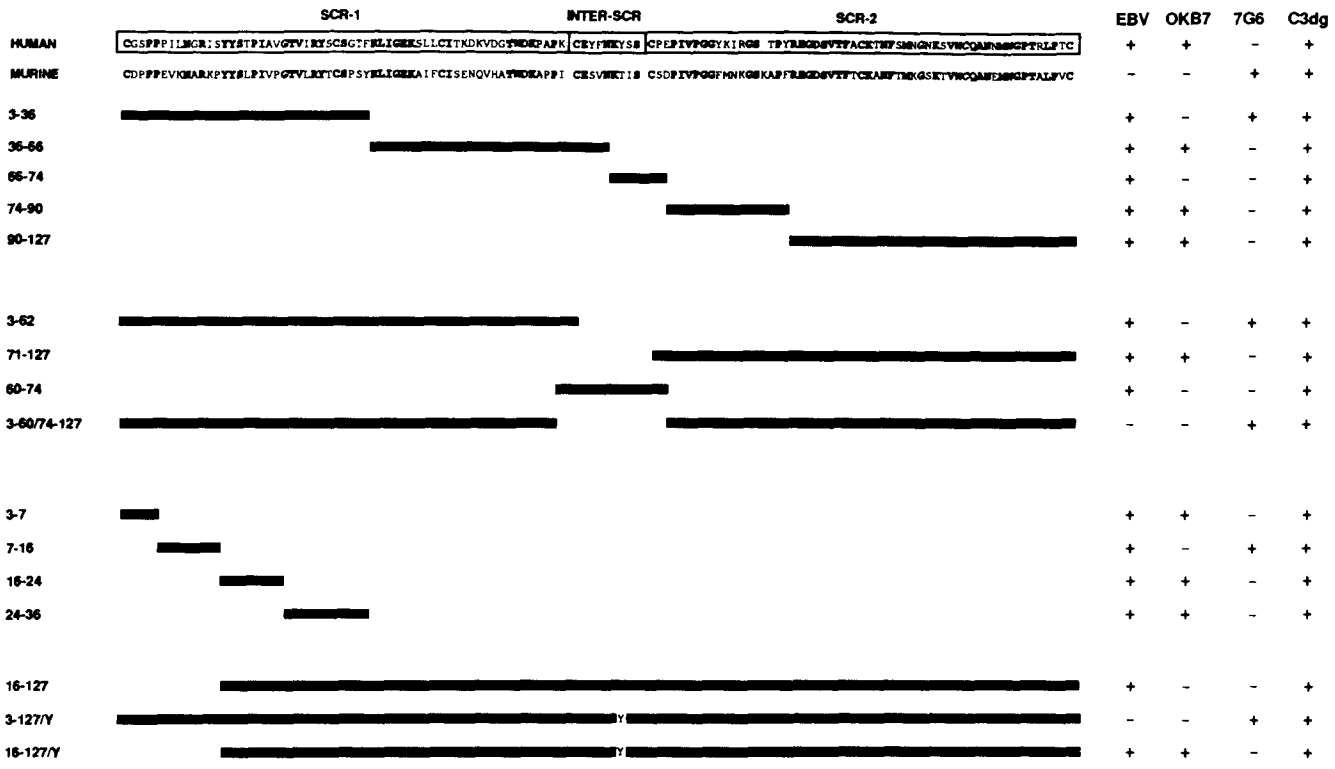

Figure 2. Binding of ligands and mAbs to human-murine chimeric mutants of CR2. Shown at the top are amino acid sequences of the first two SCRs of CR2.H (HUMAN) and CR2.M (MURINE) in which identical residues are in bold and a single gap has been introduced into the human sequence to maximize alignment. Below are schematic representations of the first two SCRs of human-murine chimeric mutants in which the murine portion of each receptor is indicated by a black bar. The numerical designations shown on the far left indicate the boundaries of the murine sequence within each chimeric fragment, in which the first and last (eighth) cysteine residues are amino acids 3 and 127, respectively, of the mature murine protein (the residues that define the boundaries of each chimeric substitution are identical between the two species). Shown to the right of each construct are the results of immunofluorescence analyses of the capacity of COS cells expressing each of the recombinant receptors to bind the anti-human CR2 mAb OKB7, the anti-murine CR2 mAb 7G6, and the CR2 ligands pC3dg and EBV. Absence of binding indicates no positives among at least 2,000 cells counted. A positive result indicates at least 10% brightly stained cells as determined by fluorescence microscopy. Wild-type K562 cells do not specifically bind any of the antibodies or ligands.

of upstream and downstream coding sequence is from human CR2.

Receptors encoded by piCR2.H and piCR2.M were characterized through expression transiently in COS cells and stably in K562 cells. COS cells transfected with either piCR2.H or piCR2.M were assessed for their capacity to bind polymerized human C3dg (pC3dg), EBV, and mAbs OKB7 (41) and 7G6 (35), which are specific for and block ligand binding to human CR2 (10) and murine CR2 (42), respectively. As predicted, indirect immunofluorescence demonstrated binding of human C3dg to COS cells bearing either CR2.H or CR2.M, whereas EBV and OKB7 were specific for cells bearing human CR2, and 7G6 bound only to cells transfected with piCR2.M (Fig. 2).

K562 stable transfectants bearing CR2.H or CR2.M were indirectly labeled with EBV, OKB7, or HB5 (which recognizes an epitope within SCR-3 and SCR-4 of human CR2) (28), and analyzed by flow cytometry. Consistent with the results obtained by transient expression in COS cells, K562 cells bearing CR2.H bound EBV, OKB7, and HB5, whereas CR2.M transfectants stained only with HB5, which recognizes a human sequence that is COOH-terminal to the ligand binding site and common to both constructs (Fig. 3 and Table 3). These stable transfectants were then used to determine the relative affinities of murine and human CR2 for human ligand. First, replicate samples of K562 cells bearing CR2.H or CR2.M were incubated with 3 μg/ml ^{125}I-p(iC3b) in the presence of either OKB7 or 7G6, respectively. As shown in Table 4, both CR2.H and CR2.M bound human piC3b specifically, these interactions could be blocked with OKB7 and 7G6, respectively, and there was no cross-species reactivity of these antibodies. These findings were extended to a quantitative assessment of binding ^{125}I-p(iC3b) to the two receptors. Replicate samples of K562 cells bearing CR2.H or CR2.M were incubated with incremental concentrations of ^{125}I-p(iC3b) in the presence or absence of OKB7 or 7G6, respectively, after which bound and free ligand were separated by centrifugation through dibutyl/dinonylphthalate. Specific competition of the radiolabeled ligand with each species-specific antibody was observed (Fig. 4). Scatchard anal-

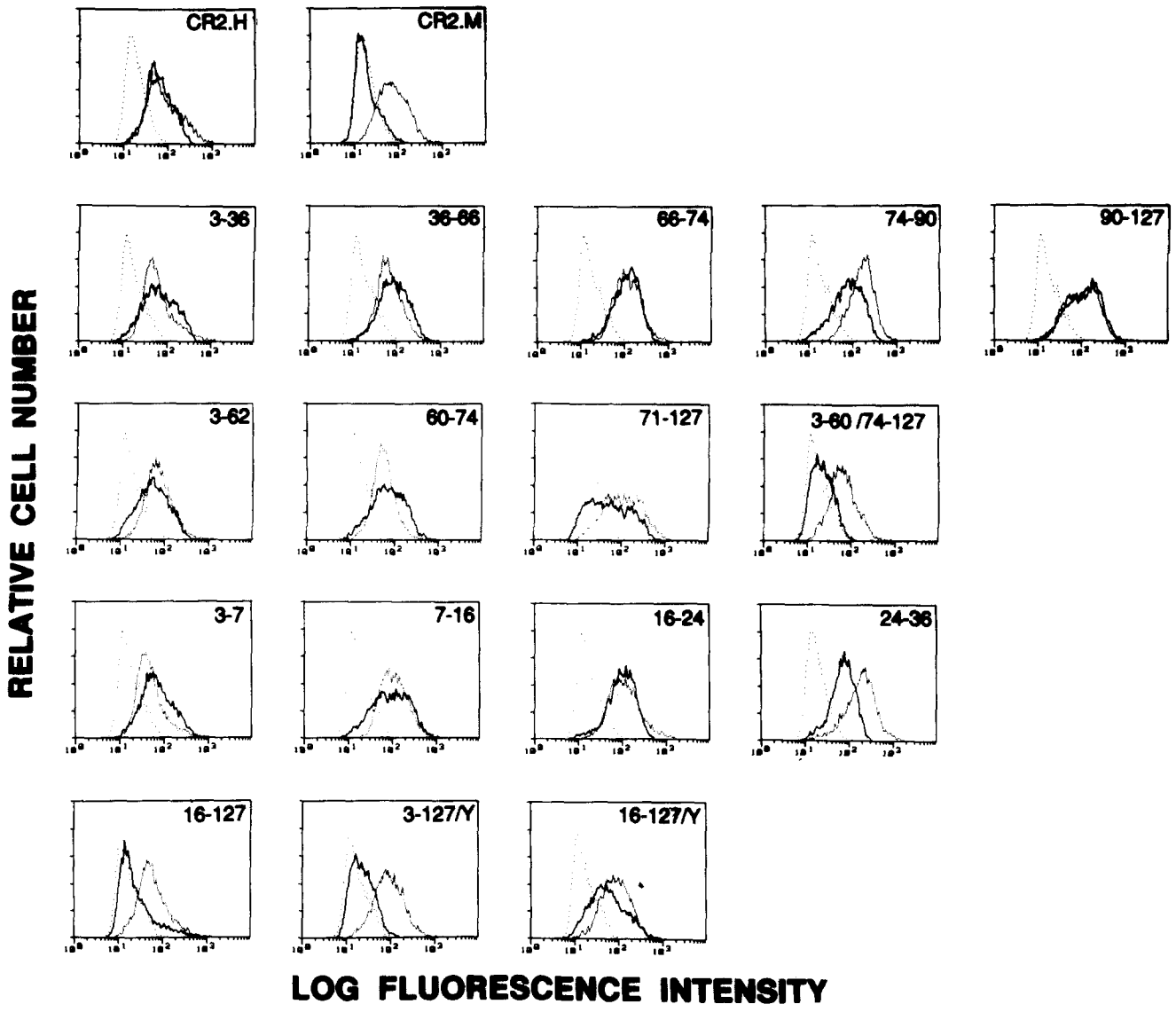

Figure 3. Flow cytofluorometric analyses of K562 cells expressing human CR2 or human-murine chimeric receptors. K562 cells stably transfected with piCR2.H or a construct encoding one of the human-murine chimeric receptors were indirectly fluorescently labeled with the anti-human CR2 mAb HB5, or with EBV. Shown in each panel are histograms in which wild-type K562 cells labeled with EBV are represented by widely spaced dotted lines, and EBV and HB5 labeling of the clone specific to each panel are represented by solid and tightly spaced dotted lines, respectively.

yses of the binding data indicated a threefold greater affinity of the human ligand for the human as compared with the murine binding site: K_d = 2.16 nM (r = −0.99) and 6.78 nM (r = −0.99) for CR2.H and CR2.M, respectively.

Selective Binding of EBV to Human CR2 Results from a Distinct Receptor Conformation Rather than from Residues Uniquely Capable of Binding Virus. We next created a series of five constructs encoding receptors that contained chimeric ligand binding sites. Cysteine 1 of SCR-1 and cysteine 4 of SCR-2 are residues 3 and 127, respectively, of the mature murine protein, and the nomenclature used for the chimeric mutants indicates which of the amino acids are murine. Thus, mutants 3-36, 36-66, 66-74, 74-90, and 90-127, (Fig. 2) represent chimeric receptors in which the substitutions are nonoverlapping and span the entire ligand binding site. These five chimeric receptors were transfected into COS cells and assayed for the capacity to bind human pC3dg, EBV, OKB7, and 7G6. The capacity of both murine and human CR2 to bind human pC3dg led us to anticipate that human-murine chimeric binding domains would also bind human pC3dg, and this proved to be a correct assumption. Surprisingly, not only did all five of the chimeric receptors bind pC3dg, but they also bound EBV specifically (Fig. 2). These observations were confirmed through stable expression of these constructs in K562 cells (Fig. 3; Tables 3 and 4). This suggested that differential binding of EBV to human and murine CR2 is not simply due to residues in the human receptor that are uniquely capable of binding EBV, but rather is the result of

Table 3. *Binding of Anti-human CR2 mAbs and EBV to K562 Cell Transfectants*

Cell line	Mean fluorescence		
	HB5	OKB7	EBV
K562 WT	9	10	10
RAJI	144	135	96
CR2.H	183	205	192
CR2.M	91	8	10
Chimeras			
3-36	91	5	88
36-66	79	77	105
66-74	152	7	128
74-90	194	145	92
90-127	144	149	146
3-62	92	6	65
60-74	59	5	89
71-127	128	123	68
3-60/74-127	77	7	14
3-7	64	64	86
7-16	144	8	126
16-24	164	196	110
24-36	253	261	95
16-127	83	5	48
3-127/Y	115	9	30
16-127/Y	112	48	97

conformational differences between the two receptors that arise from nonlinear substitutions. In contrast, specific linear sequences were found to be critical for binding OKB7 and 7G6. Two of the chimeric receptors (3-36, which contains 16 amino acids that are unique to murine CR2, and 66-74, which has four residues unique to the mouse receptor) failed to bind OKB7. Not only did the NH_2-terminal transfer of residues from the mouse to human receptor in mutant 3-36 result in loss of OKB7 binding, but it also created a 7G6 epitope and was the only substitution among these five to do so.

Four additional chimeric receptors were created and expressed in COS cells and K562 cells to determine if more extensive substitutions of murine for human sequence would eliminate the capacity to bind EBV. Chimeric receptors in which SCR-1 (3-62), SCR-2 (71-127), or the inter-SCR linker (60-74) of human CR2 were replaced with homologous murine sequence were still capable of binding EBV. Simultaneous substitution of both SCR-1 and SCR-2 of human CR2 with those from mouse while maintaining a human inter-SCR linker (3-60/74-127) did eliminate EBV binding, although this mutant still bound pC3dg and piC3b. The pattern of OKB7 and 7G6 binding to these four mutants was consistent with that observed among the first set of five chimeras described above. The receptor in this panel of four that did bind OKB7 was 71-127, which maintains human residues within the NH_2-terminal and inter-SCR regions that were determined to be critical by mutants 3-36 and 66-74, respectively. Similarly, mutants 3-62 and 3-60/74-127 contain murine residues between positions 3 and 36 and, like mutant 3-36, they reconstitute a 7G6 epitope.

Single Amino Acid Substitutions within Two Discontinuous Regions Render Murine CR2 Capable of Binding EBV. We could not eliminate EBV binding to human CR2 through simple substitution of a limited linear portion of the ligand binding site with murine residues. However, identification of amino acids within two discrete regions that were required for species-specific binding of mAbs that effectively blocked receptor-ligand interaction suggested that these regions might also account for the preferential binding of EBV to human CR2. Therefore, additional constructs were created in an attempt to define further the amino acids critical to the OKB7 and 7G6 epitopes.

The NH_2-terminal region demonstrated by chimera 3-36 to be important for binding both OKB7 and 7G6 was dissected through construction of four additional mutants: 3-7, 7-16, 16-24, and 24-36, which were expressed transiently in COS cells and stably in K562 cells, and assayed for the capacity to bind EBV, pC3dg, HB5, OKB7, and 7G6 (Figs. 2 and 3; Tables 3 and 4). All four mutants bound EBV, pC3dg, and HB5 as expected, however, only one of them, 7-16, resulted in elimination of OKB7 binding and acquisition of a 7G6 epitope. This chimeric receptor consists entirely of human CR2 sequence except for the six nonconservative substitutions contained within the mouse segment EVKNARKP, which replaces the human residues PILNGRIS.

Collectively, these studies indicated that it was possible to create a 7G6 epitope while destroying the OKB7 epitope of human CR2 through substitution of six amino acids with residues unique to mouse CR2 in these same positions. We next asked whether substitution of this same region of murine CR2 with human sequence would produce the opposite result, namely replacement of the 7G6 epitope with a capacity to bind OKB7. This was studied through creation and characterization of three additional mutants: 16-127, 3-127/Y, and 16-127/Y (Fig. 2).

Chimera 16-127 encodes SCR-1 and SCR-2 of murine CR2, except for eight amino acids unique to human CR2 at its extreme NH_2 terminus. As expected, this resulted in loss of 7G6 binding. However, this substitution with human residues was not sufficient to create an OKB7 epitope, which was also consistent with the earlier findings that chimeras 66-74 and 60-74 resulted in loss of OKB7 binding. These two mutants share four residues unique to murine CR2. We predicted that the substitution of threonine for tyrosine in the linker between SCR-1 and SCR-2 would be more significant because this is the third position of a potential N-linked glycosylation site in the mouse receptor (NKT), which is absent in

Table 4. *Competition of mAbs with Human p(iC3b) for Binding K562 Transfectants*

Cell line	^{125}I piC3b (percent Bound)		
	RPC5.4	7G6	OKB7
K562 WT	1	1	1
CR2.H	51	52	7
CR2.M	27	1	27
Chimeras			
3-36	36	1	36
36-66	21	17	2
66-74	51	50	52
74-90	39	35	2
90-127	48	45	9
3-62	41	2	41
60-74	17	15	17
71-127	30	27	2
3-60/74-127	12	1	12
3-7	44	41	10
7-16	27	1	27
16-24	49	47	6
24-36	67	65	32
16-127	22	19	22
3-127/Y	29	1	28
16-127/Y	31	27	17

Wild-type K562 cells, K562 cells bearing recombinant human CR2 (CR2.H), K562 cells bearing the murine CR2 ligand binding site on a human CR2 backbone (CR2.M), or K562 cells bearing chimeric receptors were incubated with 3 μg/ml ^{125}I-p(iC3b) in the presence of saturating concentrations of either anti-human CR2 (OKB7), anti-murine CR2 (7G6), or subclass-matched nonspecific mAb RPC5.4.

human CR2 (NKY). Therefore, chimeric receptor 3-127/Y was created, which eliminates the potential for glycosylation in the mouse receptor. This mutant retained the ligand and mAb binding profile of murine CR2. However, when this single amino acid substitution at position 68 was combined with the NH_2-terminal changes present in mutant 16-127 through construction of mutant 16-127/Y, an OKB7 epitope was created (Fig. 2).

It was apparent from studies of K562 cells bearing receptor 16-127/Y that its affinity for OKB7 was less than that of wild-type human CR2 (Tables 3 and 4). However, this was our initial indication that the first inter-cysteine segment and the first inter-SCR linker were together critical in forming a functional domain. This notion was confirmed through studies of EBV binding to these same three mutants.

As indicated in Fig. 2, COS cells transfected with construct pi16-127 were clearly positive when examined for the capacity to bind both pC3dg and EBV. However, when this chimera was expressed stably in K562 cells, only some of the cells were capable of binding EBV. As shown in Table 3, the mean fluorescence of 16-127 cells labeled with EBV is significantly greater than those values obtained with K562 wild-type cells and K562 cells bearing CR2.M. However, it is apparent from the histogram overlay of clone 16-127 in Fig. 3 that only a subset of these cells are positive although they all bind HB5. This is in contrast with most of the other EBV-binding clones in which the EBV histogram overlays the HB5 histogram. The 16-127 cells that appear as a shoulder on the EBV histogram in Fig. 3 are presumably those bearing the most receptors, which suggests that this chimera has a diminished capacity to bind EBV as compared with wild-type human CR2. This argument could also be made for clones 74-90 and 24-36, in which the EBV histogram is clearly positive but was reproducibly shifted to the left of the HB5 histogram. In contrast, EBV binding to K562 cells bearing receptor 16-127/Y was comparable to binding of HB5 to these same cells (Table 3; Fig. 3). Therefore, as was observed with OKB7, although the single substitution of tyrosine for threonine at position 68 between SCR-1 and SCR-2 had no effect by itself on the capacity of the murine receptor to bind EBV, when combined with human sequence between cysteine 1 and cysteine 2 of SCR-1, a viral binding site was created.

Further identification of the residue(s) between cysteine 1 and cysteine 2 of SCR-1 that were critical for differential binding of EBV, OKB7, and 7G6 to human CR2 and murine CR2 was achieved through creation and characterization of six additional chimeric receptors: EP/Y, VI/Y, KL/Y, AG/Y, KI/Y, and PS/Y (Table 5). All of these constructs contain SCR-1 and SCR-2 of murine CR2 with a substitution of tyrosine from human CR2 at position 68. In addition, each mutant contains a single amino acid from human CR2 between positions 8 and 15 inclusive. These constructs were transfected transiently into COS cells and assayed for the capacity to bind C3dg, EBV, 7G6, and OKB7 (Table 5). All six of the receptors were properly expressed as demonstrated by their capacity to bind C3dg. The results further demonstrate that substitution of a human serine for a murine proline at position 15 is sufficient to confer the capacity to bind EBV and it is the only substitution among these to do so. In addition, the single substitution of a human isoleucine for a murine lysine at position 14 is the only mutation that results in loss of the 7G6 epitope. Alternatively, although replacement of the human CR2 segment from amino acids 8–15 with murine residues results in loss of the OKB7 epitope (chimera 7-16), no single human for a murine substitution within this fragment is sufficient to restore OKB7 binding.

Finally, the contribution of the human tyrosine residue at position 68 was assessed through creation and characterization of chimera PS, which differs from chimera PS/Y in that position 68 is the threonine from murine CR2. The results demonstrate that the serine for proline substitution results in the capacity to bind EBV regardless of whether a tyrosine or threonine is present at position 68. These findings are consistent with those described above, in which both chimeras

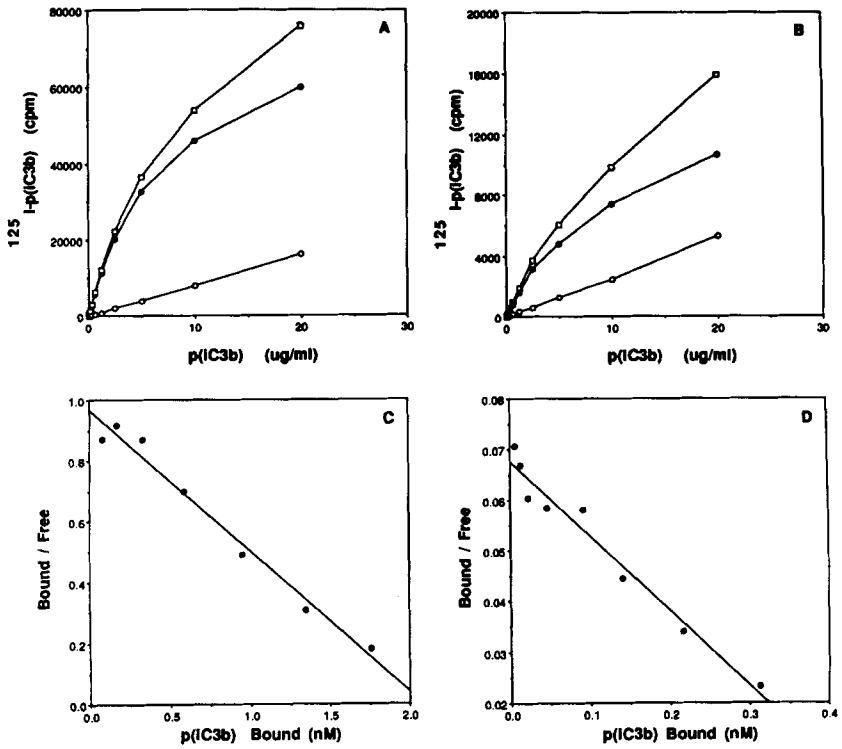

Figure 4. Saturation binding of ^{125}I-p(iC3b) to K562 cells bearing recombinant human CR2 (CR2.H) or murine CR2 (CR2.M) ligand binding sites. K562 cells bearing CR2.H were incubated with incremental concentrations of ^{125}I-p(iC3b) in the presence or absence of 10 μg/ml OKB7 (*A*), and K562 cells bearing CR2.M were incubated with incremental concentrations of ^{125}I-p(iC3b) in the presence or absence of 10 μg/ml 7G6 (*B*). Cell-bound ^{125}I-p(iC3b) was determined in replicate samples. Nonspecific binding (*open circles*) was subtracted from binding in the absence of inhibiting antibody (*open squares*) to yield specific binding (*filled circles*). Lines represent the least squares fit of all data points that are the means of replicate determinations. Scatchard analyses of ligand binding to CR2.H (*C*) and CR2.M (*D*) are shown below.

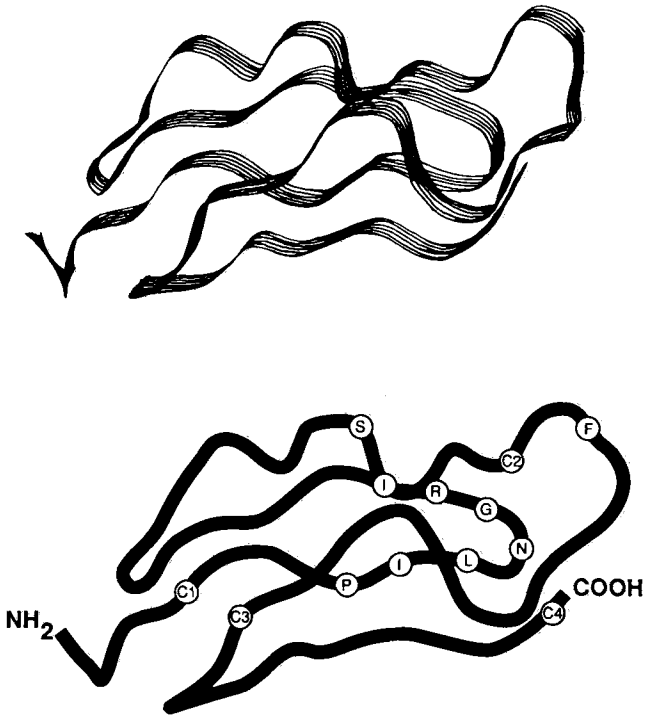

Figure 5. Schematic representation of the short consensus repeat three-dimensional structure. Shown above is the three-dimensional structure of SCR-16 from factor H (21) in which a hydrophobic core is surrounded by three β strands on one face and two β strands on the other. Shown below is the same structure on which we have indicated the positions of the four invariant cysteines, the location of the loop that contains amino acids 8–15, which correspond to amino acids PILNGRIS of human CR2, and the location of F37 of factor H, which corresponds to F35 of human CR2.

16-127 and 16-127/Y were capable of binding EBV when expressed on COS cells.

Discussion

The narrow tropism of EBV is currently a unique experimental system for several reasons. First, EBV is the only herpesvirus with a single predominant membrane glycoprotein, and the only herpesvirus for which the viral ligand and its host receptor have been defined biochemically. It is this

Table 5. *Binding of mAbs and Ligands to Chimeric CR2 Point Mutants*

Construct	Amino acid sequence 8-15	68	EBV	OKB7	7G6	C3dg
CR2.H	PILNGRIS	Y	+	+	−	+
CR2.M	EVKNARKP	T	−	−	+	+
EP/Y	PVKNARKP	Y	−	−	+	+
VI/Y	EIKNARKP	Y	−	−	+	+
KL/Y	EVLNARKP	Y	−	−	+	+
AG/Y	EVKNGRKP	Y	−	−	+	+
KI/Y	EVKNARIP	Y	−	−	−	+
PS/Y	EVKNARKS	Y	+	−	+	+
PS	EVKNARKS	T	+	−	+	+

envelope glycoprotein, gp350/220, that contains the NH_2-terminal sequence EDPGFFNVE, which has been shown to be the primary CR2 binding site for EBV and corresponds to the sequence EDPGKQLYNVE of human C3dg (43, 44). Second, whereas poliovirus, rhinovirus, and HIV exploit receptors of the Ig superfamily, EBV, through its specific interaction with CR2, is the only virus known to gain intracellular access through a member of the SCR family of proteins. Third, murine CR2 is the only known example of a viral receptor homologue that binds the heterologous natural ligand but not the virus.

This absolute specificity of EBV and OKB7 for human CR2 and of 7G6 for murine CR2, yet comparable affinities of human iC3b and C3dg for both receptors, was fundamental to the strategy used in this study. Our major concern when designing these experiments was that failure of a particular mutant to bind EBV might not be due to substitution of residues critical for direct ligand contact or even disruption of a critical epitope but rather the result of a more general disruption of protein conformation. We have demonstrated previously that both SCR-1 and SCR-2 of human CR2 are required and sufficient to bind EBV and C3dg (28). This observation, together with the known capacity of murine B lymphocytes to bind human C3dg, suggested that it would be possible to construct and characterize a panel of human-murine CR2 chimeras where only some would bind EBV but where all would bind C3dg, thus ensuring that the SCR conformation was maintained. This strategy was also regarded as preferable to an approach based upon peptides because of our anticipation that discontinuous portions of the linear sequence might contribute to the ligand binding site(s), as well as our concern that a proper pattern of disulfide bonds would be critical to the native SCR conformation. Therefore, 24 human-murine CR2 chimeras were created and characterized for their capacity to bind EBV, human C3dg and iC3b, OKB7, 7G6, and HB5 in an attempt to determine if the requirements for binding natural and viral ligands to human CR2 are separable, and to identify the structural basis for preferential binding of EBV to the human receptor.

The findings reported here demonstrate that species-specific epitopes within human CR2 and murine CR2 are based upon critical linear as well as nonlinear determinants, and the capacities of these receptors to bind EBV, OKB7, and 7G6 are all influenced by substitutions within the same NH_2-terminal peptide. Specifically, replacement of the sequence EVKNARKP between cys-1 and cys-2 of the first SCR of murine CR2 with the corresponding peptide PILNGRIS from human CR2 eliminates the 7G6 epitope. In addition, when this NH_2-terminal sequence is combined with elimination of a potential N-linked glycosylation site (NKT) between SCR-1 and SCR-2 of murine CR2, epitopes for both EBV and OKB7 are created. The epitopes for OKB7 and 7G6 are, in fact, mutually exclusive, and the structural requirements for binding C3dg, EBV, and OKB7 to human CR2 are distinct and separable. Finally, any region of murine CR2, including the entire first or second SCR, can substitute for the corresponding region of human CR2 with retention of the capacity to bind EBV. These observations indicate that this NH_2-terminal peptide may well bind OKB7 and 7G6 directly, but it cannot represent a site of unique contact for EBV on the human receptor since it can be replaced with murine sequence without disturbing this interaction. It is more likely that when human sequence in this region is incorporated into the murine receptor, it results in a conformational change that allows the virus access to critical residues involved in direct ligand contact. Presumably, the human receptor is able to accommodate murine sequence in this region and still bind EBV because of a nonlinear portion of the receptor that differs in the murine homologue.

Substitution of eight residues at the NH_2 terminus of murine CR2 with the corresponding amino acids from human CR2 (chimera 16-127) rendered COS cells bearing this receptor capable of binding EBV. In fact, a single replacement of proline with serine at position 15 was sufficient to accomplish this. However, only some of the K562 cells bearing receptor 16-127 were capable of binding EBV. An additional substitution at position 68 was required, as demonstrated by the capacity of all K562 cells bearing receptor 16-127/Y to bind the virus. There are at least two possible explanations for this discrepancy. First, the level of receptor expressed transiently on COS cells was consistently several-fold greater than that observed on stable K562 transfectants as determined by indirect immunofluorescence. Therefore, the COS cells bearing receptor 16-127 could correspond to those observed in the shoulder of the 16-127 histogram of K562 cells (Fig. 3). A second possibility is that the NKT consensus sequence between SCR-1 and SCR-2 of murine CR2 is glycosylated in K562 cells but not in COS cells. In fact, the finding that only some of the K562 cells bearing receptor 16-127 can bind EBV could reflect differential glycosylation of this site among this population of K562 cells. A definitive explanation for this discrepancy will require complementary biosynthetic studies.

Although the three-dimensional structure of CR2 has not yet been determined, the tertiary structure of SCR-16 from human complement factor H has been solved through high field two-dimensional nuclear magnetic resonance spectroscopy (21). As shown in Fig. 5, the SCR structure is based upon a β sandwich in which the NH_2 and COOH termini are at opposite ends of the module. One face consists of three β strands, the other face is formed from two β strands, and the regions joining the β strands are comprised of well-defined turns and less well-defined loops. The two faces contribute highly conserved side chains to form a globular compact hydrophobic core. Alignment of the >140 SCR sequences available from >20 proteins indicates that the most highly conserved residues are found in regions with critical structural importance while the more variable positions are located in the less well-defined loops. On this basis, it has been predicted that the general structure determined for SCR-16 of factor H will be shared by other SCR-containing proteins (21). Based upon this assumption, the structural significance of our functional data derived from mutagenesis of the ligand binding domain of CR2 becomes apparent.

First, two residues within SCR-16 of factor H, Phe 37 and His 13, are semi-conserved and highly solvent exposed, yet play no demonstrable role in stabilizing the tertiary structure of the single SCR. These observations, together with the determination that they are located in adjacent positions within the tertiary structure and reside proximate to the COOH terminus, suggest that they might participate in interactions between SCRs, and are consistent with our findings in this study (Fig. 5). Amino acids 8–15, which we find to be structurally critical to converting several functional epitopes between murine and human CR2, correspond to residues 10-17 of SCR 16 of factor H (because of two additional amino acids before cysteine 1 in factor H), which include His-13. In addition, Phe 37 of SCR-16 of factor H corresponds to Phe 35 of SCR-1 of human CR2, which is replaced by tyrosine in mutant 24-36. As shown in Fig. 3, this chimeric receptor appears to have a diminished capacity to bind EBV. Furthermore, our finding, that preferential binding of EBV to human CR2 is based at least in part on critical substitutions among residues 8-15 combined with changes in the inter-SCR sequence, is supported by the fact that these two regions are proximate in the tertiary SCR structure. Finally, high local mobility of residues 17-22 within SCR-16 of factor H, combined with observations that the sequence in this region tends to be nonconserved among SCRs and includes large insertions, suggests a functional domain (21). Position 17 in this sequence corresponds with a human serine versus a murine proline in position 15 of CR2, a substitution that could easily distort this potentially functional loop, and was alone sufficient to render COS cells bearing receptor PS capable of binding EBV. This same loop within SCR-2 of human CR2 is replaced by murine sequence in the chimera 74-90 (Fig. 2), which also appears to have a reduced capacity to bind EBV when expressed on K562 cells as determined by the location of the EBV histogram to the left of that for HB5 (Fig. 3).

In summary, the findings reported here are consistent with functional predictions based upon the SCR consensus structure proposed by Norman et al. (21). Specifically, the peptide PILNGRIS of human CR2, particularly the serine at position 15, might be critical for proper alignment of SCR-1 and SCR-2 with respect to one another and is likely to be responsible for the distinct receptor conformation required to bind EBV. In addition, the inter-SCR region appears to be proximate to and perhaps a part of this functional domain, and glycosylation of this region in murine CR2 may contribute to its lack of affinity for EBV. Further characterization of the significance of these critical amino acid substitutions, such as whether they represent sites of direct ligand contact, lead to local disruption of species-specific epitopes, or alter the three-dimensional alignment of SCR-1 and SCR-2 relative to one another, will require complementary structural analyses of CR2, such as two-dimensional nuclear magnetic resonance and crystallography.

Finally, we have demonstrated that the requirements for binding EBV and C3dg to human CR2 are separable. Thus, although the viral and natural ligand binding sites overlap, the human receptor should be mutable so as to avoid the virus while maintaining normal function. This observation at least suggests the possibility that the capacity to support EBV infection is somehow an advantage shared by humans.

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