JOURNAL OF VIROLOGY, Aug. 1994, p. 4716–4726
0022-538X/94/$04.00+0

Vol. 68, No. 8

Determination of the Role for CD21 during Epstein-Barr Virus Infection of B-Lymphoblastoid Cells

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Received 25 February 1994/Accepted 21 April 1994

Epstein-Barr virus (EBV), a herpesvirus with oncogenic potential, is camouflaged with glycoprotein 350/220, which mimics the human ligand C3dg and thereby binds to and exploits complement receptor type 2 (CR2; CD21), the EBV receptor. It has not been possible to determine the role of CR2 during postbinding events of viral infection because all B lymphocytes express endogenous CR2, precluding an informative study of receptor mutants. We have overcome this obstacle through creation of a novel experimental system based on molecular dissection of the ligand-binding domains of human CR2 and murine CR2. Our results demonstrate first, that two discontinuous amino acid substitutions within the ligand-binding domain of murine CR2 render it capable of mediating EBV infection of human B-lymphoblastoid cells, and second, that the specific role of CR2 during EBV infection is to capture virions at the cell surface, after which cofactors not associated with CR2 mediate postbinding events. These are the first studies to be described in which a cell that is normally susceptible to viral infection can be manipulated so as to direct entry of virions via recombinant or endogenous receptors.

Epstein-Barr virus (EBV) is an oncogenic herpesvirus that characteristically infects B lymphocytes and certain epithelia of nearly all humans by adulthood. This *Lymphocryptovirus* then escapes immune surveillance and is harbored in a benign, latent state. However, during conditions of immune suppression such as AIDS and posttransplant chemotherapy, as well as within regions of endemicity in Africa for unclear reasons, the oncogenic potential of EBV is unleashed in the form of lymphomas and other lymphoproliferative conditions. Similarly, EBV infection of oral epithelia is intimately associated with the development of nasopharyngeal carcinoma. It is not entirely clear why the human host tolerates viral latency, nor is it fully understood how a state of benign subterfuge is transformed into malignant invasion. However, it has become apparent that the path to immortality for EBV is paved with viral homologs of host factors that ordinarily regulate cellular activation and survival, such as the products of EBV open reading frames BCRF1 and BHRF1, which encode homologs of human interleukin 10 (40) and the proto-oncogene *bcl-2* product (9), respectively. This strategy of molecular mimicry also underlies the initial event during EBV infection, in which the viral envelope is camouflaged with glycoprotein 350/220, which simulates the human ligand C3dg and thereby exploits complement receptor type 2 (CR2; CD21), the EBV receptor (reviewed in references 1 and 10).

CR2 is one of relatively few viral receptors that have been identified. Three of the others are intercellular adhesion molecule-1 (ICAM-1; CD54), the major group rhinovirus receptor (20, 52), CD4, the human immunodeficiency virus type 1 receptor (11, 26), and the poliovirus receptor (33), which are all members of the immunoglobulin (Ig) superfamily. In contrast, CR2 belongs to the family of proteins comprising short consensus repeats (SCRs), which also includes membrane cofactor protein (CD46), which serves as a receptor for measles virus (12, 44). The SCR is a structural module found in at least 30 proteins involved in acute inflammation, tissue repair, and the immune response, including the selectins, coagulation factors, and both host and viral regulators of complement activation. Each SCR consists of hypervariable domains as well as highly conserved sequences including four invariant cysteines that are disulfide bonded in a Cys-1 to Cys-3 and Cys-2 to Cys-4 pattern (23). Electron microscopic studies of CR2 and other members of this family have demonstrated that the multiple SCRs within an individual protein are arranged end-to-end in linear fashion and each SCR is a discrete structural unit (43, 58). High-field two-dimensional nuclear magnetic resonance spectroscopy has been used to determine that each SCR is a β sandwich with two β strands on one face, three β strands on the other face, and a globular compact hydrophobic core. The regions joining the β strands consist of well-defined turns and less well-defined loops (4, 46). Structurally, CR2 consists of a 34-amino-acid cytoplasmic domain, a 24-residue transmembrane region, and an extracellular domain composed entirely of 15 or 16 SCRs (42, 57).

Elucidation of the function of CR2 during viral infection not only is the first step in dissection of the molecular basis for EBV tropism but should also enhance our understanding of the normal function of this receptor during B-cell activation through events triggered by its endogenous ligand C3dg as well as during signal transduction through other membrane constituents with which the viral receptor is associated. CR2 has been recently shown to reside in three forms on the B-cell membrane. First, CR2 coimmunoprecipitates with CD19, a pan-B-cell surface marker and member of the Ig superfamily, from digitonin lysates of the human B-lymphoblastoid cell line Raji (31). Second, CR2 forms a detergent-sensitive complex with CR1 on the surface of human tonsillar B lymphocytes, but not B-lymphoblastoid cells, which is distinct from the CR2-CD19 complex (56). Third, CR2 is present on both cell types in a noncomplexed form.

One role for CR2 in mediating EBV infection is clearly to bind specifically virions at the cell surface. Studies of recombinant deletion and chimeric mutants of human CR2 have determined that SCR-1 and SCR-2 are both necessary and together sufficient to mediate binding of gp350/220, C3dg, and

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monoclonal antibody (MAb) OKB7, which blocks binding of both ligands to the receptor, with an affinity equal to that of wild-type CR2 (7, 28). Furthermore, soluble forms of this pair of SCRs fused to either IgG1 or protein A block cellular infection by EBV (22, 41). Identification of the ligand-binding domain of CR2 was accomplished through expression of recombinant receptors on mouse L cells, COS cells, and K562 erythroleukemia cells; however, postbinding events such as penetration, infection, and replication are absent or inefficient in these and other non-B cells bearing recombinant CR2, demonstrating that although human CR2 is a major determinant of the narrow tropism of EBV, other factors are involved (2, 7). Although exceptional cell lines have been described, B lymphocytes are the only primary cells routinely capable of supporting EBV infection in vivo and in vitro. Yet structure-function studies of the role of human CR2 in mediating postbinding events during EBV infection of B cells have not been possible, because all mature B lymphocytes and EBV-negative B-lymphoblastoid cell lines express endogenous CR2, precluding an informative study of recombinant CR2 mutants. Thus, although it is clear that SCR-1 and SCR-2 are essential for binding virions, it has not been possible to determine whether non-ligand-binding domains of the receptor or the CR2-CD19 and/or CR2-CR1 complexes are specifically required for viral infection, and postbinding events during EBV infection of B cells have remained obscure.

We have developed a novel experimental system that allows, for the first time, characterization of the role played by CR2 during EBV infection of cell types, such as B lymphocytes, that are normally and efficiently infected in vivo and also express endogenous wild-type CR2. The approach is based on separation of residues required for binding EBV from those amino acids required to bind OKB7. Whereas human CR2 serves as a receptor for both C3dg and EBV and binds OKB7, which blocks binding of both ligands to the receptor, murine CR2 (which also has an extracellular domain consisting of 15 SCRs [15, 39]) is capable of binding human C3dg, and this interaction can be blocked with the MAb 7G6 (25), but it does not bind OKB7 or EBV (16). Through creation and characterization of a panel of human-murine chimeric receptors, we have shown previously 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 two discontinuous amino acid substitutions: replacement of proline 15 within SCR-1 and threonine 68 in the linker between SCR-1 and SCR-2 with the corresponding serine 15 and tyrosine 68 from human CR2 (30). This results in a ligand-binding domain, MCR2.SY, that consists of SCR-1 and SCR-2 of murine CR2 except for human residues S-15 and Y-68. This pair of SCRs is unique in its capacity to bind EBV but not OKB7.

We now demonstrate that when the ligand-binding domain of wild-type human CR2 is replaced with the amino-terminal pair of SCRs from MCR2.SY, this chimeric receptor faithfully mediates EBV infection of human B-lymphoblastoid cells in the presence of OKB7, which completely blocks infection through wild-type human CR2. This is the molecular basis for a novel experimental system in which we are now able to characterize the role for non-ligand-binding domains of human CR2 during EBV infection of cells that express endogenous CR2 as well.

MATERIALS AND METHODS

Cell lines. Ψ-2 (29), an ecotropic murine packaging cell line, and PA317 (American Type Culture Collection [ATCC], Rockville, Md.), an amphotropic murine packaging cell line, were maintained in Dulbecco modified Eagle medium with D-glucose (4,500 mg/liter) supplemented with 10% bovine calf serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). The EBV-negative B-lymphoblastoid cell line BJA-B (34) was obtained from Richard Ambinder (Johns Hopkins University, Baltimore, Md.), and EBV-negative B-lymphoblastoid cell lines CA46 and Ramos were purchased from ATCC. They were maintained in RPMI 1640 supplemented with 10% bovine calf serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml).

TABLE 1. Oligonucleotides used in this study

Name	5′-3′ sequence
JA.5	TTCTGATCACACTTTACAAGCTGT
JA.6	TGCAGATCTCGTTCACTTGCTCCTACCATCCCCATCGTGGGC
JA.9	CGTGATATCAAGGAGGAAGAGCTCAGAT
JA.19	TTTGTCGACCTGCGGCCCCAAGGG
JA.20	CGGGATCCGCGGCCGCCCTCAAGATCTCGAGTG
JA.43	GTTCGCGTTGCTAGGCCACC
JA.44	CTTCTACGGACTCGTCTGGG
JA.47	TGCAGATCTCGTTCACTTGCTCCTGTC
JA.48	TGAATCGATAGCAGTTTCTTTCTAATC
JA.49	GTTCACGTGTGGAGCTAG

Antibodies. YZ-1 (IgG1) is a murine MAb specific for human CD35 (CR1) (8), HD37 (IgG1) (Dako Corp., Santa Barbara, Calif.) is a murine MAb specific for human CD19, 7G6 (IgG2b) (25) is a rat MAb that recognizes murine CR2, HB-5 (IgG2a) (ATCC) and OKB7 (IgG2b) (Ortho Diagnostic Systems, Inc., Raritan, N.J.) are murine MAbs specific for human CR2, and R6.5 (IgG2a) and CBRIC 1/13.3.2 (IgG2a) (provided by Mike Diamond and Tim Springer, Center for Blood Research, Boston, Mass.) are murine MAbs that recognize Ig domains 2 and 3, respectively, of ICAM-1. 7D2-1.4.1.5 (IgG2b) (ATCC) is a nonspecific rat MAb. RPC5.4 (IgG2a) (ATCC) is a nonspecific murine MAb. Nasopharyngeal carcinoma patient serum 00448 was obtained from S. Spring (National Cancer Institute, Bethesda, Md.). Fluorescein (DTAF)-conjugated goat anti-rat IgG, DTAF-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, Pa.), and fluorescein-conjugated goat anti-guinea pig C3 (Organon Teknika Corp., Durham, N.C.) were purchased.

Creation of plasmids encoding CR2 chimeric receptors. A 3.9-kbp *Sma*I-*Sal*I restriction fragment from pBS.CR2.1 (28) containing the full-length human CR2 cDNA was cloned into the *Hpa*I and *Xho*I sites of the retroviral eukaryotic expression vector LXSN (37) to generate LXSN.CR2. The human CR2 insert from LXSN.CR2 was replaced with the 3.2-kbp cDNA insert from piPS/Y (30), using a partial *Eco*RI restriction digestion, to create plasmid LXSN.MCR2.SY.

Oligonucleotide-directed mutagenesis and a two-step PCR strategy (30) were used to introduce a silent C-to-T transition in the third position of a serine codon within SCR-15, six residues amino-terminal to the extracellular-transmembrane junction of MCR2.SY (Tables 1 and 2). This created a unique *Bgl*II restriction site in plasmid LXSN.MCR2.SY.Bgl II.

The 1.2-kbp *Bam*HI-*Cla*I restriction fragment from plasmid LXSN.MCR2.SY.Bgl II was cloned into pBluescript KS+ (Stratagene) to create the intermediate pBS.MCR2.SY.BC. This construct has a unique *Bgl*II site 21 bp 5′ to the extracellular-transmembrane junction, a unique *Eco*RV site 7 bp 5′ to the transmembrane-cytoplasmic junction, and a unique *Bcl*I site at the carboxyl terminus of the cytoplasmic domain encoded by the MCR2.SY cDNA.

TABLE 2. PCR strategies for construction of CR2 mutants

Mutant	Oligonucleotide		cDNA template
	5′	3′	
MCR2.SY.Bgl II	JA.47	JA.48	CR2
	JA.49	PCR 1 product	CR2
MCR2.SY.C	JA.9	JA.5	HLA-A2
MCR2.SY.TMC	JA.6	JA.5	HLA-A2
MCR2.SY.ICAM	JA.19	JA.20	ICAM-1

Plasmid LXSN.MCR2.SY.CAM, encoding a chimeric construct comprised of SCR-1 and -2 of MCR2.SY fused to the amino terminus of Ig domain 3 of ICAM-1, was created in two steps as follows. An 800-bp ICAM-1 cDNA fragment, extending from the junction of Ig domains 2 and 3 to its extracellular-transmembrane junction, was amplified by PCR using plasmid CD1.8 (52) as a template (Tables 1 and 2). The 5′ end of this fragment contained a novel *Sal*I restriction site encoded by the primer. The 3′ end of the fragment contained a *Xho*I restriction site present within the ICAM-1 cDNA, as well as novel *Not*I and *Bam*HI sites encoded by the primer and located 3′ to the *Xho*I site. This PCR product was restricted with *Sal*I and *Bam*HI and cloned into *Xho*I-*Bam*HI-restricted LXSN.MCR2.SY to generate LXSN.MCR2.SY.CAM.X, and the nucleotide sequence of the PCR-generated insert was confirmed. A 500-bp *Xho*I-*Not*I ICAM-1 cDNA fragment, extending from its extracellular-transmembrane junction through its transmembrane, cytoplasmic, and 3′ untranslated regions, was removed from plasmid CD1.8 and cloned into the 5′ *Xho*I and 3′ *Not*I sites of LXSN.MCR2.SY.CAM.X to generate LXSN.MCR2.SY.CAM.

Cassettes composed of cDNA encoding the carboxyl terminus of human CR2 in which the cytoplasmic (MCR2.SY.C) or the combined transmembrane and cytoplasmic (MCR2.SY.TMC) domains from the human class I major histocompatibility complex (MHC) antigen HLA-A2 were substituted for the corresponding regions of human CR2 were created through PCRs (Tables 1 and 2). Primers used to create the chimeras spanned the extracellular-transmembrane, transmembrane-cytoplasmic, and cytoplasmic-3′ untranslated region junctions, with their 3′ ends homologous to HLA-A2 cDNA and their 5′ ends homologous to CR2 cDNA and encoding unique *Bgl*II, *Eco*RV, or *Bcl*I restriction sites. The PCR template for the MCR2.SY.C and MCR2.SY.TMC mutants was pTR4.9, which contains the HLA-A2 cDNA. The PCR products were restricted at the appropriate flanking unique sites and subcloned into plasmid pBS.MCR2.SY.BC, and the nucleotide sequences were confirmed. The *Bam*HI-*Cla*I cassettes were removed and cloned into the expression vector LXSN.MCR2.SY.Bgl II.

The retroviral expression system. The plasmids LXSN.MCR2.SY.CAM, LXSN.MCR2.SY.TMC, and LXSN.MCR2.SY.C were transfected into ecotropic Ψ-2 cells, and culture supernatant from each respective transfection was used to transduce amphotropic PA317 cells, which were selected with 750 μg of G418 (Gibco BRL, Gaithersburg, Md.) per ml. PA317 clones producing high viral titers were isolated, cell supernatant from each PA317 clone was harvested at 18 to 24 h, and DEAE-dextran was added to a final concentration of 50 μg/ml. The supernatants were passed through a 0.8-μm-pore-size filter, and 5 ml was used to resuspend 10^7 B-lymphoblastoid cells which had been grown to a concentration of 5×10^5/ml. The cells were transferred to a 50-ml flask, incubated for 18 to 24 h, pelleted, washed with RPMI, resuspended in 10 ml of RPMI with 20% fetal bovine serum, cultured for an additional 18 to 24 h, and placed into selection with G418 (2 mg/ml).

Immunofluorescence and flow cytometric analyses. Cells were incubated with a primary MAb in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 0.02% sodium azide for 30 min on ice, washed, incubated with DTAF-conjugated goat anti-rat IgG or DTAF-conjugated goat anti-mouse IgG, and washed. Either 5×10^3 cells were assayed with a FACScan (Becton Dickinson & Co., Mountain View, Calif.), or 10^7 cells were sorted with a FACStar (Becton Dickinson) to obtain the brightest 2 to 5%.

EBV infection and detection. EBV was obtained by concentrating (500-fold) and filtering supernatant from induced Akata cell cultures as described previously (53). Cellular infection with EBV was performed by preincubating 2.5×10^6 B-lymphoblastoid cells for 30 min on ice with 50 μl of PBS containing 0.1% BSA (PBSA) with or without MAb OKB7 (10 μg/ml). Purified, 500-fold-concentrated Akata cell EBV in 50 μl of RPMI was added, and the cells were incubated in a 37°C water bath for 60 min. Cells were then washed with PBS, resuspended in 0.25% trypsin or RPMI, incubated at 37°C for 5 min, washed twice, resuspended in 10 ml of medium, cultured for 48 h, and assayed for expression of Epstein-Barr nuclear antigen (EBNA) as described previously (47), using nasopharyngeal carcinoma serum 00448. Alternatively, 10^6 cells were harvested 48 h after addition of EBV and assayed for the presence of EBV nucleic acid as follows. Cells were pelleted, washed, and resuspended in 100 μl of buffer consisting of 50 mM KCl, 10 mM Tris (pH 8.3), 2.5 $MgCl_2$, 5% Tween 20, and 100 μg of proteinase K per ml. A 100-μl PCR was performed in buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 200 μM (each) deoxynucleoside triphosphates, and 2.5 U of Amplitaq (Perkin-Elmer Cetus), using 10 μl of cell lysate as the template and 0.25 μM each primers JA.43 and JA.44, resulting in amplification of bp 14256 to 14783 of the EBV genome, a segment which is within the *Bam*HI W repeat (3). The reaction mixtures were covered with mineral oil and subjected to 20 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. A 20-μl sample of each reaction was analyzed by electrophoresis through 2% SeaKem agarose and stained with ethidium bromide.

Immunoprecipitation. A total of 2×10^7 B-lymphoblastoid cells were radiolabeled with ^{125}I (New England Nuclear, Boston, Mass.). Cells were lysed in 0.8 ml of buffer containing 1% digitonin (Sigma Chemical Co., St. Louis, Mo.), 10 mM triethanolamine, 150 mM NaCl, 1 mM EDTA, 5 mM diisopropylfluorophosphate, 1 μg of antipain per ml, 0.5 μg of chymostatin per ml, 1 μg of pepstatin A per ml, and 1 μg of leupeptin per ml. Insoluble material was removed by centrifugation of the lysates. Lysates were precleared twice by rocking at 4°C with 80 μl of immobilized protein A on trisacryl (Pierce, Rockford, Ill.) and were divided into 0.4-ml replicate samples to which 10 μg of MAb RPC5.4 or 10 μg of MAb R6.5 was added. The lysates were rocked at 4°C for 15 min, 40 μl of protein A-trisacryl beads was added, the lysates were rocked for an additional 45 min at 4°C, and the beads were pelleted. The lysate from the R6.5 tube was reprecipitated with R6.5 and protein A-trisacryl beads, the beads were pelleted, and 10 μg of MAb CBRIC 1/13.3.2 was added to the lysate. This mixture was rocked at 4°C for 15 min, 40 μl of protein A-trisacryl beads was added, the lysates were rocked for an additional 45 min at 4°C, and the beads were pelleted. The RPC5.4, R6.5, and CBRIC 1/13.3.2 beads were washed five times with 600 μl of lysis buffer, boiled in 100 μl of 2× sample buffer containing 6% sodium dodecyl sulfate (SDS) and 200 mM dithiothreitol, and

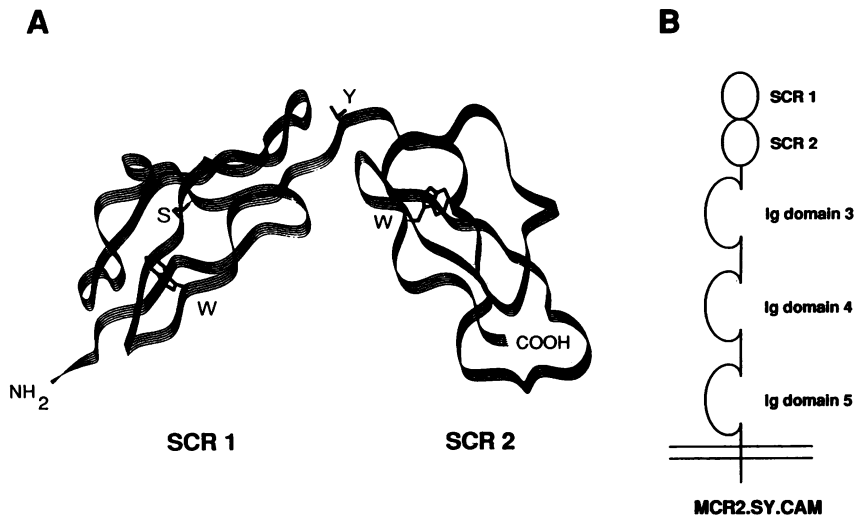

FIG. 1. (A) Schematic representation of the pair of SCRs that comprise the MCR2.SY ligand-binding domain, based on studies of factor H by Barlow et al. (5). Each SCR consists of a β sandwich with three β strands on one face, two on the other, and a compact hydrophobic core. The five extended β strands are separated by four turns, with turns 1 and 3 located near the carboxyl terminus and turns 2 and 4 near the amino terminus. Locations of the serine 15 and tyrosine 68 substitutions are indicated by S and Y, respectively, and the invariant tryptophan residues are indicated by W. (B) Schematic representation of the MCR2.SY.CAM chimeric receptor which was created by replacing the first two Ig domains of ICAM-1 with SCR-1 and SCR-2 of MCR2.SY.

analyzed by SDS–5% polyacrylamide gel electrophoresis (PAGE).

RESULTS

Creation of a novel experimental system based on MCR2.SY. We have previously created and characterized a human-murine CR2 chimera designated MCR2.SY that has a ligand-binding domain consisting of SCR-1 and SCR-2 of murine CR2 except for P-15 and T-68, which have been replaced by residues S-15 and Y-68 from human CR2 (Fig. 1), followed by SCR-3 through SCR-15 and the transmembrane and cytoplasmic domains of human CR2. This chimeric recombinant receptor is uniquely capable of binding human C3dg, EBV, and MAb 7G6, which ordinarily blocks binding of C3dg to murine CR2, but it does not bind MAb OKB7, which completely blocks binding of both C3dg and EBV to human CR2. These binding studies were performed with K562 human erythroleukemia cells which we were unable to infect with EBV through either mutant or wild-type recombinant human CR2 despite numerous attempts and a wide range of conditions. However, these findings suggested that it would be possible to infect B lymphocytes or B-lymphoblastoid cells through MCR2.SY in the presence of OKB7. Therefore, retrovirus-mediated gene transfer was used to express MCR2.SY on Ramos B-lymphoblastoid cells, which are EBV negative yet express endogenous CR2 and can be infected efficiently with EBV. Wild-type Ramos cells or Ramos cells bearing MCR2.SY (Ramos.MCR2.SY cells) were preincubated with either PBSA or OKB7, incubated with EBV for 60 min, washed, cultured for 48 h, and stained by indirect immunofluorescence for EBNA. As anticipated, wild-type Ramos cells were infected with EBV in the presence of buffer alone, yet no EBNA-positive cells were detected when the infection was performed in the presence of MAb OKB7. In contrast, Ramos cells bearing MCR2.SY as well as endogenous CR2 were infected both in the absence and in the presence of OKB7 (not shown).

EBV infection is mediated by a CR2–ICAM-1 chimeric receptor. These observations demonstrated that two discontinuous amino acid substitutions could transform the ligand-binding domain of murine CR2 into a viral receptor and suggested that it would now be possible to manipulate the non-ligand-binding domains of receptor MCR2.SY to assess their potential roles in mediating EBV infection. These components of CR2 could modulate postbinding events critical to viral infection in several ways such as by determining that viral attachment occurs at a discrete distance from the host membrane, by promoting fusion of viral and B-cell membranes, by triggering endocytosis, or by otherwise influencing the viral life cycle through postbinding signal transduction events. Furthermore, the non-ligand-binding domains of CR2 could mediate these processes either directly or indirectly through association with other membrane constituents such as those within the CR2-CR1 and CR2-CD19 complexes. It is also possible that the role for CR2 and CR2-containing membrane complexes differs during EBV infection of peripheral B lymphocytes which express CR2, CD19, and CR1 compared with EBV infection of B-lymphoblastoid cells that express CR2 and CD19 but not CR1. This possibility is supported by previous observations that EBV enters peripheral B lymphocytes via endocytosis, whereas penetration of B-lymphoblastoid cells is achieved through direct membrane fusion (45, 54).

To determine absolutely whether non-ligand-binding domains of CR2 are specifically required for EBV infection of B-lymphoblastoid cells, we first determined whether the ligand-binding domain of CR2 could mediate EBV infection if substituted for the ligand-binding domain of another viral receptor. For this purpose we selected ICAM-1, the rhinovirus receptor, which consists of a 453-amino-acid extracellular domain composed entirely of five Ig domains, the first two of which form the viral binding domain, a 24-residue transmembrane region, and a 28-amino-acid cytoplasmic tail (49, 50). It has been determined that Ig domain 1 is the primary site for rhinovirus contact, although the amino-terminal two domains appear to interact conformationally (51). Domains 3 to 5

TABLE 3. Expression of selected surface molecules by B-lymphoblastoid cells[a]

Cell line	Mean channel fluorescence				
	Nonspecific	CD35	CD21	CD19	CD54
BJA-B	12	12	26	276	107
CA46	7	7	14	274	58
Ramos	6	6	16	180	67

[a] Expression of CR1 (CD35), CR2 (CD21), CD19, and ICAM-1 (CD54) on three wild-type B-lymphoblastoid cell lines was determined through indirect immunofluorescence and flow cytometry using MAbs YZ-1, HB-5, HD37, and CBRIC 1/13.3.2, respectively, compared with the two nonspecific MAbs MOPC21 and RPC5.4 (which produced identical results).

influence accessibility of the viral binding domain either by ensuring its protrusion from the glycocalyx or by providing the receptor with required flexibility. Therefore, we created a CR2–ICAM-1 chimeric receptor, MCR2.SY.CAM, in which the two Ig domains that comprise the rhinovirus binding site were replaced with the first two SCRs of receptor MCR2.SY (Fig. 1). Retrovirus-mediated gene transfer was used to express this chimeric receptor on three different EBV-negative B-lymphoblastoid cell lines, BJA-B, CA46, and Ramos, all of which also express endogenous CR2, ICAM-1, and CD19 but are CR1 negative (Table 3). The recombinant receptors were detected through indirect immunofluorescence and flow cytometric analyses using anti-murine CR2 MAb 7G6, which should recognize MCR2.SY.CAM but neither wild-type human CR2 nor human ICAM-1, and those cells bearing the highest levels of chimeric receptor were obtained with two rounds of fluorescence-activated cell sorting (Fig. 2).

We anticipated that fusion of a pair of SCRs with three Ig domains would result in a chimeric molecule in which the proper intrachain disulfide bonds would be formed and result in five discrete structural domains because of our previous success in creating a functional $(CR2)_2$-IgG1 chimera (22). To ensure that the CR2–ICAM-1 chimera expressed on each of the three cell lines not only bound MAb 7G6 but was otherwise intact structurally, we immunoprecipitated MCR2.SY.CAM from each of the three transfected cell lines. The first two Ig domains of ICAM-1 are 88 and 97 residues, respectively, whereas the two SCRs that comprise the MCR2.SY ligand-binding domain are 59 and 57 amino acids, respectively, indicating that the primary structure of MCR2.SY.CAM should be approximately 7 kDa smaller than wild-type ICAM-1. However, SCR-2 of MCR2.SY contains one potential N-linked glycosylation site, compared with four in Ig domain 2 of ICAM-1 (neither SCR-1 nor Ig domain 1 of these receptors is glycosylated). Differential glycosylation of MCR2.SY.CAM compared with ICAM-1 would reduce its posttranslational size an additional 6 kDa; therefore, the recombinant receptor should be 7 to 13 kDa smaller than endogenous ICAM-1 on these same cells.

To demonstrate that the MCR2.SY.CAM chimeric receptors were of the correct anticipated size, we prepared digitonin lysates from ^{125}I-labeled wild-type and MCR2.SY.CAM-bearing BJA-B, CA46, and Ramos B-lymphoblastoid cells. Each lysate was sequentially immunoprecipitated twice with MAb R6.5, which recognizes Ig domain 2 of ICAM-1, which is not present on MCR2.SY.CAM, followed by immunoprecipitation with MAb CBRIC 1/13.3.2, which should recognize Ig domain 3 within MCR2.SY.CAM and any residual wild-type ICAM-1 remaining in the lysates. Analysis of the radiolabeled immunoprecipitates by SDS-PAGE under reducing conditions revealed that the first R6.5 immunoprecipitation from wild-type

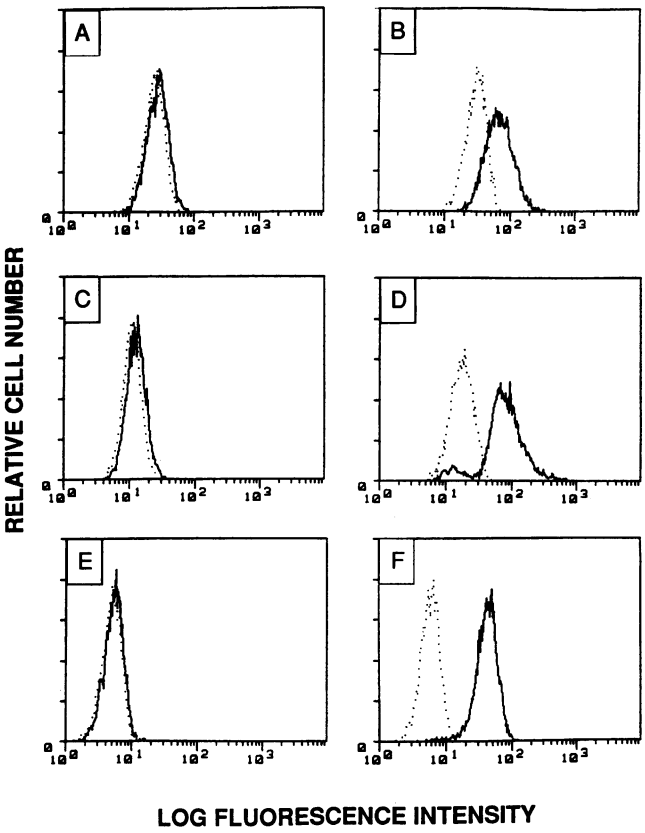

FIG. 2. Flow cytometric analyses of human B-lymphoblastoid cell lines expressing MCR2.SY.CAM. Shown are histograms of wild-type BJA-B (A), CA46 (C), and Ramos (E) cells and MCR2.SY.CAM-bearing BJA-B (B), CA46 (D), and Ramos (F) cells, indirectly labeled with the anti-murine CR2 MAb 7G6 (solid lines) or with the nonspecific control antibody 7D2-1.4.1.5 (dotted lines).

BJA-B, CA46, and Ramos as well as from the respectively paired transfectants cleared the majority of endogenous ICAM-1 with an apparent molecular mass that ranged from 80 to 90 kDa among the three cell types (Fig. 3). This result is consistent with the previous observation that although the polypeptide core of ICAM-1 has a predicted and observed size of 55 kDa, differential glycosylation among cell types results in a mature product that varies between 76 to 114 kDa (13). The second sequential immunoprecipitation with R6.5 recovered specifically a small amount of residual ICAM-1 from wild-type Ramos cells and Ramos bearing MCR2.SY.CAM which was less apparent in the other four lysates. The absence of detectable ICAM-1 within the six lysates after these two sequential immunoprecipitations was confirmed with a third immunoprecipitation with CBRIC 1/13.3.2, which specifically precipitated a protein from BJA-B.MCR2.SY.CAM, CA46.MCR2.SY.CAM, and Ramos.MCR2.SY.CAM cells but not from the respectively paired wild-type cells. The protein immunoprecipitated from the BJA-B.MCR2.SY.CAM lysate migrated with an apparent molecular mass that was 5 to 10 kDa smaller than that of endogenous ICAM-1 recovered from the same sample, whereas a smaller but distinct difference in electrophoretic mobility was observed between the chimeric proteins recovered from CA46.MCR2.SY.CAM and Ramos.MCR2.SY.CAM cells and wild-type ICAM-1 from the same cell lysates.

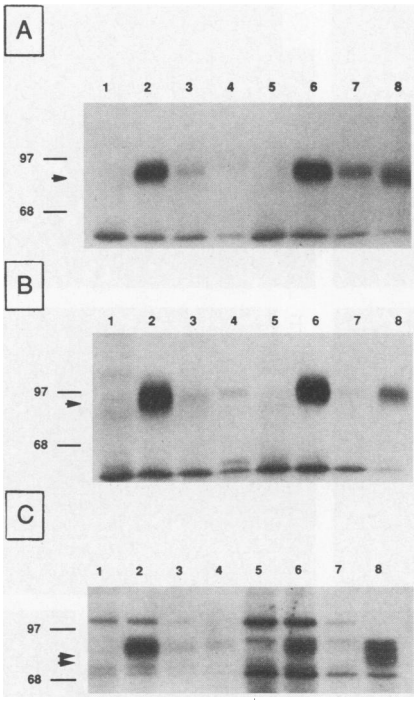

FIG. 3. Expression of human ICAM-1 and MCR2.SY.CAM on human B-lymphoblastoid cell lines. Digitonin lysates were prepared from ^{125}I-labeled wild-type (lanes 1 to 4) and MCR2.SY.CAM-bearing (lanes 5 to 8) Ramos cells (A), CA46 cells (B), and BJA-B cells (C). Each cell lysate was subjected to two sequential immunoprecipitations with MAb R6.5, which recognizes Ig domain 2 of ICAM-1 (lanes 2, 3, 6, and 7), followed by a third sequential immunoprecipitation with MAb CBRIC 1/13.3.2, which recognizes Ig domain 3 of ICAM-1 (lanes 4 and 8). Nonspecific immunoprecipitates were also obtained (lanes 1 and 5). Proteins were resolved on SDS-polyacrylamide gels and detected by autoradiography. Sizes are indicated in kilodaltons.

The differential capacity of the protein specifically recovered from the three cell lines bearing chimeric receptors to bind CBRIC 1/13.3.2 but not R6.5, the absence of this protein in lysates from the three wild-type cell lines, its smaller size compared with that of wild-type ICAM-1 in the same lysates, and the capacity of the transfected cells to bind specifically MAb 7G6 demonstrate the presence of functional epitopes represented by each of the two chimeric portions of the receptor and suggest that the chimeric polypeptide was appropriately folded, processed, and expressed in each of the three cell lines. Furthermore, the absence of specifically coprecipitating proteins such as CD19, which is abundantly present on all three cell lines, suggests that MCR2.SY.CAM is not capable of maintaining the multimolecular associations observed in digitonin lysates of wild-type CR2 from B-lymphoblastoid cells. This observation is consistent with our recent determination that the transmembrane domain of human CR2 is required for its interaction with CD19 (32).

We then assayed the capacity of these cell lines to support infection with EBV in the presence of OKB7. BJA-B, CA46, and Ramos wild-type cells and those bearing MCR2.SY.CAM were preincubated in PBSA with or without OKB7 for 30 min, exposed to EBV for 60 min, washed twice with RPMI, cultured for 48 h, and assayed for EBNA expression as well as for the presence of EBV nucleic acid. As shown in Fig. 4, the three wild-type cell lines were infected by EBV in the absence, but not in the presence, of OKB7. In contrast, BJA-B.MCR2.SY.CAM, CA46.MCR2.SY.CAM, and Ramos.MCR2.SY.CAM cells were infected with EBV both in the absence and in the presence of OKB7. As a more sensitive and absolute determination of cellular infection with EBV, a PCR assay was used to detect a 527-bp fragment of the *Bam*HI W segment of the EBV genome. These studies confirmed that when cells were exposed to EBV after preincubation with OKB7, the BJA-B, CA46, and Ramos cells bearing MCR2.SY.CAM were infected, but the viral genome could not be detected in the three respective wild-type cell lines (Fig. 5). To eliminate the possibility that virions might be captured on MCR2.SY.CAM and later released to infect the cells via endogenous CR2 that is expressed during subsequent incubation, similar experiments were performed in which cells were treated with trypsin following the incubation with EBV to remove any residual virions and receptors from the cell surface. Indirect immunofluorescence analysis demonstrated that no virus or receptor was detected on the cell surface following trypsinization (not shown). The results of three similar experiments (Table 4) demonstrate definitively that the virus entered these cells through the MCR2.SY.CAM chimeric receptor.

Cellular infection by EBV in the absence of OKB7 was also studied to address the relative efficiencies of wild-type CR2 versus MCR2.SY.CAM in mediating this process. Shown in Table 5 are representative data from several experiments in which we consistently observed that cells bearing recombinant receptors in addition to endogenous wild-type receptors were more frequently infected in the absence of antibody blockade, either suggesting that infection was more efficient through the recombinant receptors or simply demonstrating an effect of more total binding sites per cell.

EBV infection is mediated by CR2-MHC class I chimeric receptors. The capacity of chimeric receptor MCR2.SY.CAM to mediate efficiently EBV infection demonstrated that the non-ligand-binding domains of human CR2 are not specifically required for EBV infection of B-lymphoblastoid cells. Although these regions of CR2 are not specifically needed for virus uptake, it is still possible that MCR2.SY.CAM is capable of mediating EBV infection because functions served by these regions of CR2 are also served by the corresponding regions of ICAM-1 through its role as the rhinovirus receptor. Thus, although unlikely, it is possible that EBV enters these B-lymphoblastoid cells bearing MCR2.SY. CAM through an ICAM-mediated rhinovirus pathway. Therefore, to determine further the role of the transmembrane and cytoplasmic domains of human CR2 in mediating EBV infection of B-lymphoblastoid cells, we created two additional chimeric receptors, MCR2.SY.C and MCR2.SY.TMC, in which the cytoplasmic domain alone or the transmembrane and cytoplasmic domains of receptor MCR2.SY were replaced with the corresponding regions of class I HLA (Fig. 6). Each of these two chimeric receptors was expressed on BJA-B lymphoblastoid cells by using retrovirus-mediated gene transfer to create cell lines BJA-B.MCR2.SY.C and BJA-B.MCR2.SY.TMC, and expression of the appropriate chimeric receptors was confirmed by indirect immunofluorescence with MAb 7G6, which recognizes the ligand-binding domains of receptors MCR2.SY.C and MCR2.SY.TMC but not that of endogenous CR2 on these cell lines. BJA-B cells bearing each of these chimeric receptors were obtained through fluorescence-activated cell sorting and assayed for the ability to mediate cellular infection with EBV. Indirect immunofluorescence detection of EBNA demonstrated that 7% of BJA-B cells bearing MCR2.SY.C and 11% of BJA-B cells bearing MCR2.SY.TMC were susceptible to infection with EBV in the presence of OKB7, whereas no BJA-B wild-type cells were infected in the

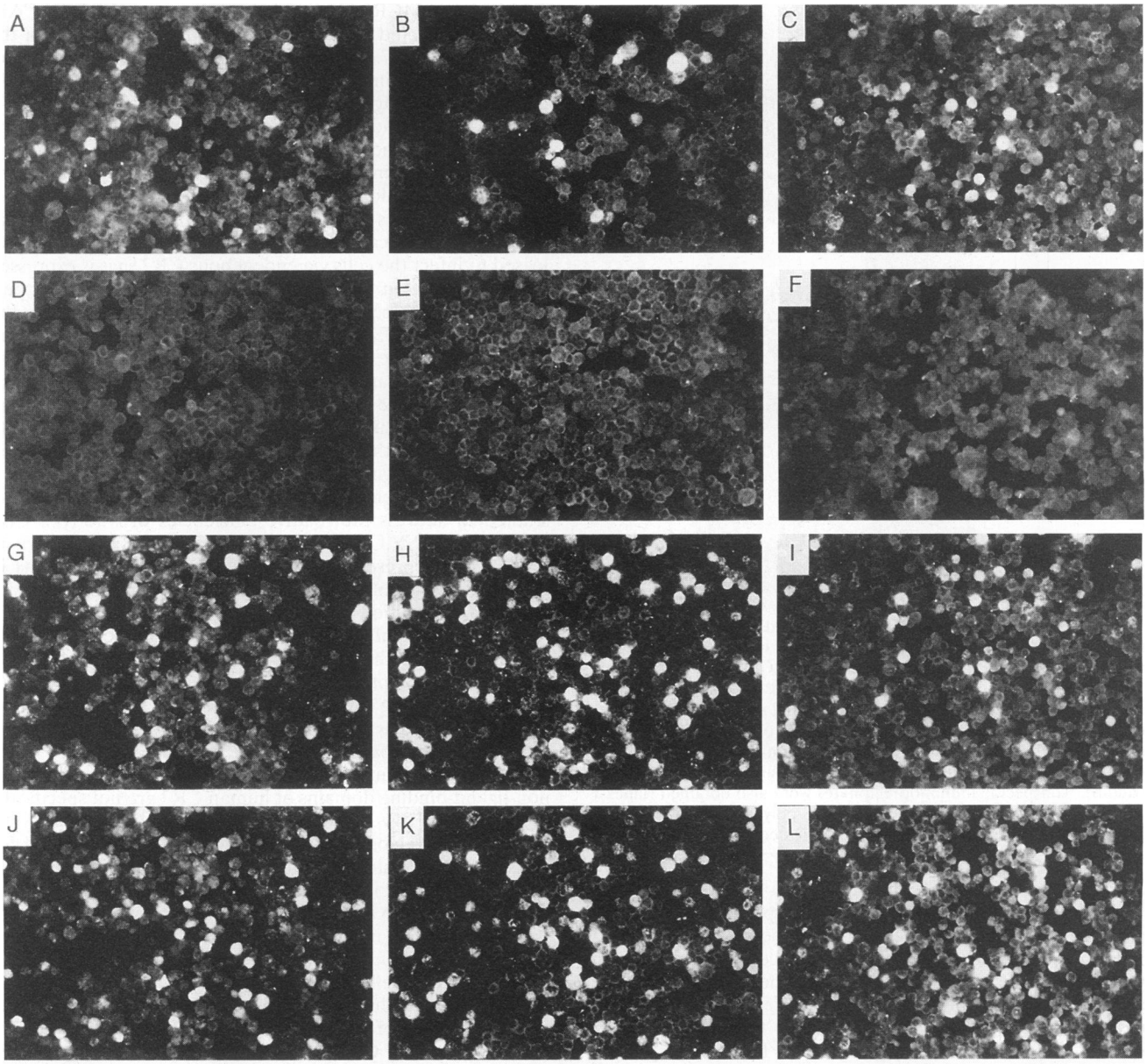

FIG. 4. MCR2.SY.CAM mediates infection of B-lymphoblastoid cells with EBV. Wild-type BJA-B (A and D), CA46 (B and E), and Ramos (C and F) cells and MCR2.SY.CAM-bearing BJA-B (G and J), CA46 (H and K), and Ramos (I and L) cells were preincubated with PBSA (A, B, C, G, H, and I) or MAb OKB7 (D, E, F, J, K, and L), exposed to EBV, washed, and cultured for 48 h prior to detection of EBNA. Fluorescence photomicrographs were taken and are shown at a magnification of ×600.

presence of OKB7. PCR detection of the EBV *Bam*HI W genomic fragment confirmed that only cells bearing the chimeric receptors were infected (Fig. 7).

DISCUSSION

We have developed an experimental system, unique among studies of viral receptors to date, in which we are able to infect the same cell through either endogenous wild-type or recombinant mutant receptors. We have used this strategy to demonstrate that the specificity provided by CR2 in mediating EBV tropism for B-lymphoblastoid cells is determined entirely by the ligand-binding domain. Previous observations have indicated that SCR-1 and SCR-2 of CR2 are both necessary and together sufficient to bind the virus, MAbs specific for this domain block viral infection, and soluble forms of this domain compete effectively for virions and thereby block cellular infection by EBV (7, 22, 28, 41). However, the ubiquitous presence of CR2 on cells normally infected by EBV and the inability to render CR2-negative cells permissive of efficient viral infection through expression of recombinant receptor required development of an experimental system to overcome this obstacle to characterizing the role of CR2 in mediating postbinding events during EBV infection.

The basis for the experimental approach undertaken in this report is our previous molecular dissection of the ligand-binding domains of human CR2 and murine CR2 and the observation that the mouse receptor could be rendered capa-

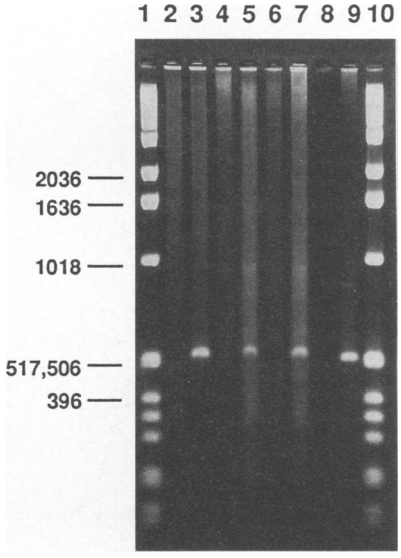

FIG. 5. MCR2.SY.CAM mediates infection of B-lymphoblastoid cells with EBV. Wild-type BJA-B (lane 2), CA46 (lane 4), and Ramos (lane 6) cells and MCR2.SY.CAM-bearing BJA-B (lane 3), CA46 (lane 5), and Ramos (lane 7) cells were preincubated with OKB7, exposed to EBV, and cultured for 48 h. Lysates from each culture were prepared and used as templates for PCR amplification of a 527-bp fragment of the *Bam*HI W segment of the EBV genome. PCR amplification products were resolved on a 2% agarose gel and detected by staining with ethidium bromide. A negative control that lacked template DNA (lane 8) and a positive control that used B95-8 cell lysate as a template (lane 9) were included. Sizes are indicated in base pairs.

ble of binding EBV through two discontinuous single amino acid substitutions. The spatial and dynamic consequences of these substitutions can now be considered in light of the recent determination of the structure of an SCR pair through two-dimensional nuclear magnetic resonance (5). Barlow et al. have demonstrated that the tertiary structure of the SCR-15 and SCR-16 pair from factor H consists of an amino-terminal domain composed of SCR-15 and the eight-amino-acid inter-SCR linker plus a second discrete domain comprised of SCR-16 (5). Although there is limited contact between the two

TABLE 4. Infection of B-lymphoblastoid cell lines with EBV[a]

Cell line	Incubation	% EBNA-positive cells
Wild-type BJA-B	RPMI	0, 0, 0
	Trypsin	0, 0, 0
BJA-B.MCR2.SY.CAM	RPMI	4, 4, 9
	Trypsin	5, 4, 10
Wild-type CA46	RPMI	0, 0, 0
	Trypsin	0, 0, 0
CA46.MCR2.SY.CAM	RPMI	4, 5, 6
	Trypsin	5, 5, 7
Wild-type Ramos	RPMI	0, 0, 0
	Trypsin	0, 0, 0
Ramos.MCR2.SY.CAM	RPMI	7, 7, 9
	Trypsin	7, 7, 9

[a] Wild-type cell lines and the three paired cell lines bearing chimeric receptor MCR2.SY.CAM were preincubated with MAb OKB7, exposed to EBV, treated with culture medium or trypsin, cultured for 48 h, and assayed for expression of EBNA. Three separate experiments were performed, and each value represents the percentage of positive cells observed among at least 300 counted.

TABLE 5. Infection of B-lymphoblastoid cell lines with EBV[a]

Cell line	% EBNA-positive cells
Wild-type BJA-B	7
BJA-B.MCR2.SY.CAM	14
Wild-type CA46	7
CA46.MCR2.SY.CAM	12
Wild-type Ramos	6
Ramos.MCR2.SY.CAM	10

[a] Wild-type cell lines and the three paired cell lines bearing chimeric receptor MCR2.SY.CAM were exposed to EBV, cultured for 48 h, and assayed for expression of EBNA. Each value represents the percentage of positive cells observed among at least 400 counted.

domains, there do appear to be constraints that control flexibility at their interface. The hypervariable domains are positioned away from the inter-SCR region, with a lateral exposure consistent with potential ligand-binding function. Although there are no known specific functions for SCR-15 and SCR-16 from factor H, individual SCRs within this family of proteins might function in ligand recognition, in spacing of multiple ligand-binding domains within a single molecule, in scaffolding to extend ligand-binding domains away from the cell surface, or in determining the degree of flexibility within an extended structure. Therefore, the SCR-15/16 pair of factor H may be involved in scaffolding and thus require greater flexibility and minimal contact with one another compared with SCRs that are involved in ligand binding. In the case of CR2, the pair of critical substitutions at positions 15 and 68 that transform murine CR2 into a viral receptor may reside within a single domain which consists of SCR-1 and the inter-SCR linker and may serve to modulate accessibility of contact residues within this domain. Alternatively, the orientation of SCR-1 with respect to SCR-2 within CR2 may be more critical than such a relationship within a non-ligand-binding scaffolding region, and the pair of substitutions in MCR2.SY may serve to create an orientation of SCR-1 with respect to SCR-2 that is suitable for ligand binding. Finally, it is possible that the amino terminus of CR2 not only must accommodate the virus during attachment but also may need to change either its own conformation or that of the viral envelope glycoprotein to mediate subsequent postbinding events. This would require controlled flexibility within the ligand-binding domain, and changes in the primary structure may influence the capacity of the receptor to bind the virus and/or prime it for subsequent internalization. Final determination of the consequences of these functionally critical substitutions must await complementary two-dimensional nuclear magnetic resonance and crystallographic studies of these receptors and their ligands.

Recent identification and characterization of a B-cell membrane signal transduction complex composed of CR2, CD19, TAPA-1, and Leu-13 suggested that this complex may be required for EBV infection of B cells (31). This concept was supported by the fact that although EBV binds to both B- and T-lymphoblastoid cell lines (35), it is internalized only by the B cells, to which this complex is unique. Our results indicate that infection of B-lymphoblastoid cells by EBV does not require the CD21–CD19–TAPA-1–Leu-13 membrane complex because MCR2.SY.CAM efficiently mediates viral infection although it does not assemble into such a complex, as demonstrated by immunoprecipitations of digitonin lysates. This observation is consistent with our recent demonstration that the transmembrane domain of CR2, which is absent in

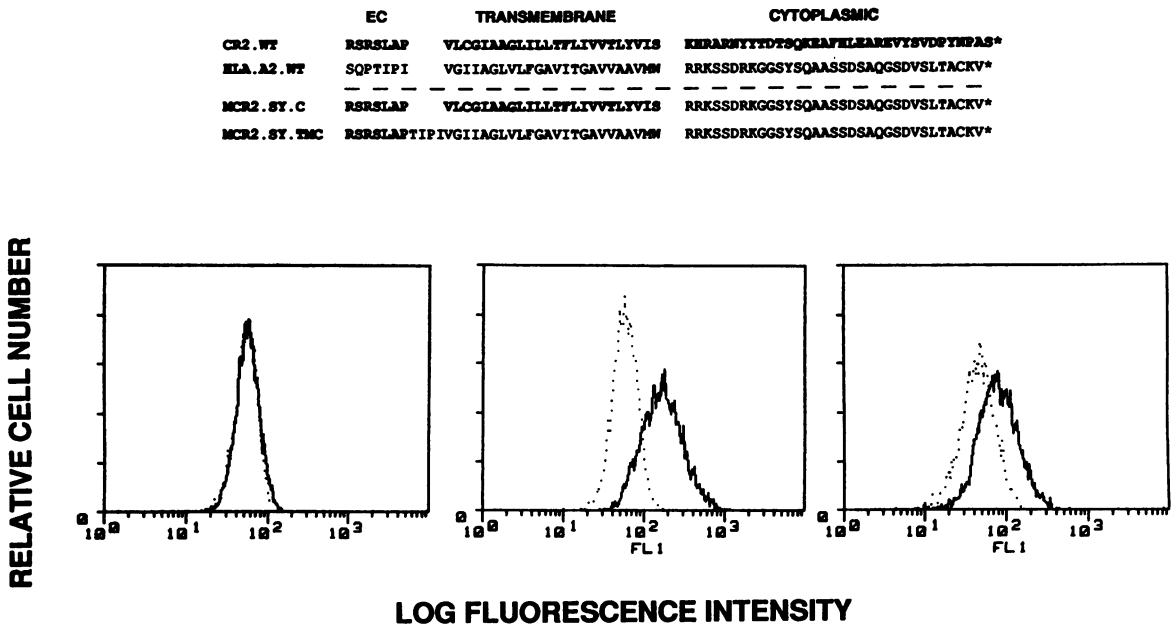

FIG. 6. Expression of CR2-MHC class I chimeric receptors on B-lymphoblastoid cells. Shown at the top are the wild-type amino acid sequences of the carboxyl-terminal extracellular (EC), transmembrane, and cytoplasmic domains of wild-type human CR2 (CR2.WT) and wild-type HLA class I (HLA.A2.WT) and the corresponding sequences of chimeric receptors in which the cytoplasmic (MCR2.SY.C) and transmembrane and cytoplasmic (MCR2.SY.TMC) domains of MCR2.SY have been replaced with those of HLA class I. Shown below are flow cytometric analyses of BJA-B wild-type (left), BJA-B.MCR2.SY.C (center), and BJA-B.MCR2.SY.TMC (right) cells. Cells were indirectly fluorescently labeled with anti-murine CR2 MAb 7G6 (solid lines) or nonspecific MAb 7D2-1.4.1.5 (dotted lines).

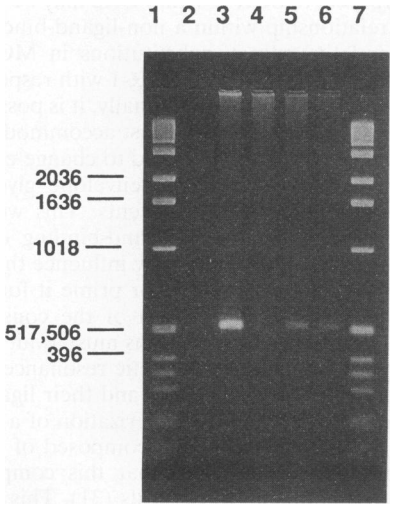

FIG. 7. CR2-MHC class I chimeric receptors mediate infection of B-lymphoblastoid cells with EBV. Wild-type BJA-B (lane 4), BJA-B.MCR2.SY.C (lane 5), and BJA-B.MCR2.SY.TMC (lane 6) cells were preincubated with OKB7, exposed to EBV, and cultured for 48 h. Lysates from each culture were prepared and used as templates for PCR amplification of a 527-bp fragment of the *Bam*HI W segment of the EBV genome. PCR amplification products were resolved on a 2% agarose gel and detected by staining with ethidium bromide. A negative control that lacked template DNA (lane 2) and a positive control that used B95-8 cell lysate as a template (lane 3) were included. Sizes are indicated in base pairs.

MCR2.SY.CAM, is critical for assembling and/or maintaining the noncovalent association between CR2 and CD19 (32). Furthermore, these results demonstrate that EBV is not required to bind its receptor at a critical distance from the B-cell membrane. The lengths of CR2 (43) and ICAM-1 (51) are 38.6 and 18.7 nm, respectively, as demonstrated by electron microscopy, indicating that the lengths of each SCR within CR2 and each Ig domain within ICAM-1 are 2.4 and 3.7 nm, respectively. The length of CR2, together with the observation that EBV binds to B cells at a distance of 50 nm from the surface (45), indicates that CR2 is expressed as an extended structure on the cell surface. The predicted length of MCR2.SY.CAM is approximately 17 nm, which is less than half the length of the normal receptor. Therefore, the capacity of the virus to tolerate a relatively wide range of binding distances from the cell surface and the limited portion of CR2 required for viral infection together suggest that not only is the CR2-CD19 membrane complex not required to mediate EBV infection, but it is also unlikely that CR2 must interact directly with any other cell surface molecules to mediate infection. Postbinding events are more likely to occur directly between the viral envelope and constituents of the B-cell membrane that do not associate with CR2.

Demonstration that the role played by CR2 during EBV infection of B-lymphoblastoid cells is mediated entirely by the ligand-binding domain does not necessarily imply that CR2 simply functions as a passive magnet to capture virions at an arbitrary distance from the cell surface. Observations made in the study of other viruses such as herpes simplex virus (19), poliovirus (18), and Sindbis virus (36) indicate that the structure of the virus primed for cellular entry can be quite different from the structure of that virus prior to cellular attachment. Thus, it is possible that the pair of SCRs at the amino terminus of human CR2 not only captures EBV but also subsequently

induces a conformational change in viral envelope glycoprotein gp350/220 that induces subsequent membrane fusion events and viral penetration. This concept is supported by the observation that antibodies to the EBV envelope glycoprotein gp85 do not block binding of virions to B cells but do block cellular infection (21, 38). Furthermore, although EBV is harbored by several types of T-cell lymphomas (6, 24, 27, 55), it does not routinely infect peripheral T cells that are now known to express CR2 (17). At least one reason for this is that the virus is able to bind but does not fuse with the T-cell membrane, and therefore it is not internalized (35). Thus, EBV may infect B cells through a strategy analogous to human immunodeficiency virus infection of T cells (reviewed in reference 14). Whereas binding of human immunodeficiency virus gp120 to CD4 induces conformational changes in the virion that catalyze interaction of viral gp41 with the T-cell membrane, binding of EBV gp350/220 to CR2 may induce conformational changes in the virion that catalyze interaction of EBV gp85 with the B-cell membrane.

Thus, although the initial event during EBV infection of human B cells is binding of EBV to the amino terminus of CR2, there is at least one other cell surface molecule required for EBV to enter human B lymphocytes, and this other membrane constituent may not be able to recognize EBV prior to modification of the virion by CR2. Evidence that viral attachment and internalization can be separate but cooperative events that are mediated by distinct cell surface molecules has recently been generated through studies of cellular infection by adenovirus (59). Just as EBV is able to bind but not infect all cells bearing CR2, adenovirus also infects only a subset of cells to which it binds (48). It has been determined by Wickham et al. that in addition to binding an attachment receptor, subsequent internalization of adenovirus is mediated by RGD-dependent integrins on the cell surface (59). There is most likely an analogous internalization receptor on human B lymphocytes that mediates cellular entry by EBV.

The experimental system described here can now be extended to studies of other cell types that support EBV infection and express endogenous CR2, such as peripheral B lymphocytes, certain epithelia, and thymocytes. For example, whereas B-lymphoblastoid cells have been shown to internalize EBV via direct membrane fusion without internalization of CR2, infection of peripheral B lymphocytes occurs through endocytosis of virions and receptor, with subsequent fusion of the virus with the endosome (45, 54). It is possible, therefore, that infection of peripheral B lymphocytes by EBV is mediated by the CR2-CR1 membrane complex which is not present on B-lymphoblastoid cells (56). Finally, although CR2 was the first viral receptor composed of SCRs to be identified, there are certain to be others, as demonstrated by the recent identification of the measles virus receptor as membrane cofactor protein (CD46), which is composed of four SCRs (12, 44). Our findings based on molecular dissection of CR2 should help to elucidate the role of other members of the SCR superfamily of proteins in mediating viral infection and also guide the identification of critical residues among those SCRs that mediate binding of specific viral ligands.

ACKNOWLEDGMENTS

We thank Tim Springer for pCD1.8, Mike Diamond and Tim Springer for MAbs CBRIC 1/13.3.2 and R6.5, Mike Krangel for pTR4.9, Taroh Kinoshita for MAb 7G6, Rich Ambinder for BJA-B cells, Paul Barlow for assistance with Fig. 1, and Lloyd Klickstein for helpful suggestions.

This work was supported by National Institutes of Health grant GM-43803, a grant from the Maryland Chapter Arthritis Foundation, and an Arthritis Investigator Award (J.M.A.) and by National Institutes of Health grant 1K11-AR01888-01 (D.R.M.).

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