Soluble Recombinant CR2 (CD21) Inhibits Epstein-Barr Virus Infection†

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Epstein-Barr virus (EBV), an oncogenic herpesvirus of humans, displays selective tropism for B lymphocytes and epithelial cells. EBV tropism is thought to be determined in part by a unique host cell receptor termed CR2 (CD21). Although previous studies have demonstrated that CR2 mediates EBV binding to B cells, its role in initiating EBV infection and B-cell transformation is less certain. In the studies reported here, soluble recombinant CR2 was shown to cause substantial inhibition of EBV infection of B cells in vitro, indicating that CR2 binding initiates EBV infection. Soluble CR2 may represent a therapeutic agent for acute and chronic EBV infections in humans.

Epstein-Barr virus (EBV), a human herpesvirus, causes infectious mononucleosis (9), a benign lymphoproliferative syndrome, and is an important cofactor in several human malignancies (5, 10). EBV-induced B-cell lymphomas also arise in immunocompromised patients receiving allografts and in patients infected with the human immunodeficiency virus (27).

A 145-kilodalton (kDa) B-lymphocyte membrane glycoprotein, CR2 (CD21), serves as a receptor for EBV and the C3dg complement fragment (6, 8, 19). EBV attachment to CR2 is mediated by a viral envelope glycoprotein (gp350/220) (17, 22, 23), specifically by a 9-amino-acid sequence epitope located near the N-terminus of the molecule (16). Purified CR2 (17, 18) and cells transfected with CR2 cDNA (1) bind EBV, and gp350/220 selectively absorbs CR2 from B-lymphocyte membrane extracts (6, 22). However, EBV infection of CR2-transfected cells is inefficient (1 of 200) (1), and CR2 expression does not always correlate with EBV infection (3).

In order to examine the role of CR2 in initiating EBV infection of B cells, a baculovirus expression system (21) was used to generate milligram amounts of the complete extracellular domain of CR2 (CD21). To produce a soluble receptor, plasmid Bluescript containing the 16-repeat form of the receptor (13) (pBS/CR2) was truncated proximal to the predicted membrane-spanning region (Fig. 1) and was ligated to a *Bam*HI linker immediately 5' to the transcription initiation codon. The internal *Bam*HI site present in repeat element 10 of CR2 was also deleted by changing a single nucleotide at position 2014 in CR2 cDNA by site-directed mutagenesis (12) without changing the original amino acid sequence. Next, a transcription termination codon and *Bam*HI site were inserted immediately following the last repeat (repeat 16) of the CR2 extracellular domain. The altered CR2 construct was then inserted downstream of the polyhedrin promoter in the pAc373 transfer vector and integrated into the *Autographa californica* nuclear polyhedrosis virus genome by homologous recombination (21). Pure recombinant viruses were identified by microscopic

examination and used to infect *Spodoptera frugiperda* clone 9 cells (Sf9). For large-scale production of CR2, 1-liter Spinner cultures of Sf9 were infected. Cells were infected with recombinant virus, and soluble CR2 was isolated on day 3 from culture supernatant by immunoaffinity chromatography (20).

Characterization of secreted recombinant CR2 protein was carried out by metabolic labeling of cells infected with recombinant baculovirus with [³⁵S]methionine followed by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. A 125-kDa protein was immunoprecipitated from the supernatants of cells infected with recombinant virus with two CR2-specific monoclonal antibodies, HB5 and OKB7, but not with an irrelevant control antibody (Fig. 2A). The size of the truncated recombinant molecule is smaller than that of the nontruncated 145-kDa, fully glycosylated, cell-associated CR2 but is larger than the predicted size of the 110-kDa, nonglycosylated, truncated recombinant receptor. Cells infected with the wild-type baculovirus did not produce the 125-kDa protein (Fig. 2A, lanes 4 to 6). The HB5 monoclonal antibody was subsequently used to isolate large amounts of recombinant CR2 for functional analysis. Supernatants derived from cells infected with recombinant virus were reacted with an HB5 monoclonal antibody immunoabsorbent and after the resin was washed, the receptor was eluted with 3 M MgCl₂. A highly purified receptor (>90% homogeneous) was obtained by this method (Fig. 2B).

A CR2-specific enzyme-linked immunosorbent assay was used to determine whether the purified receptor retained ligand-binding activity. Samples of purified gp350/220 (100 ng per well) or C3dg or ovalbumin (300 ng per well) were dried on 96-well plates (Immulon II, Dynatech Industries, Inc., McLean, Va.) at 37°C for 18 h. Nonspecific binding sites were blocked by the addition of 100 µl of 5% bovine serum albumin in phosphate-buffered saline for 1 h at 4°C. After the blocking reagent was removed, purified CR2 diluted in 100 µl of 0.5% bovine serum albumin in phosphate-buffered saline was added with further incubation for 2 h at 22°C. CR2 binding was then detected by sequential incubations with biotinylated HB5 monoclonal antibody, streptavidin-horseradish peroxidase (Amersham Corp.), and substrate as previously described (16). Purified recombinant

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FIG. 1. Construction of a CR2-baculovirus expression vector and production of soluble CR2. The PstI-BglI-derived CR2 insert was modified by the addition of a synthetic oligonucleotide *Bam*HI linker (5'-dCGGATCCG-3'; Pharmacia LKB Inc., Piscataway, N.J.) ligated to the 5' end of the blunt-ended CR2 insert. The construct was further modified by two successive oligonucleotide-based, site-directed mutagenesis (12) reactions in order to abolish the *Bam*HI site at nucleotide position 2017 by the substitution of a single nucleotide (GGATCC to GGA $\overline{A}$ CCC) which preserved the amino acid coding sequence of the receptor. A second mutagenesis step incorporated a termination codon and *Bam*HI site (TGAGGATCC) following CR2 nucleotide 3137 and immediately preceding the transmembrane domain of the receptor. The modified CR2 fragment was then inserted into the *Bam*HI cloning site of pAc373.

CR2 bound to both immobilized recombinant gp350/220 (26) and C3dg in a dose-dependent manner, while only background binding was observed with an irrelevant protein (Fig. 3a). In parallel studies, recombinant CR2 was assessed for its ability to compete with gp350/220 for binding to B cells. Approximately 20 to 40 ng of purified gp350/220 was coated onto fluorescent microspheres (Baxter, Chicago, Ill.) as previously described. Nonspecific binding sites were blocked by the addition of 5% bovine serum albumin for 1 h at 4°C. The microspheres were then pelleted, suspended in phosphate-buffered saline, and incubated with buffer alone or with various amounts of CR2 for 18 h at 4°C. These samples were then directly reacted with 5×10^5 cells for 1 h at 4°C and then prepared for fluorescence-activated cell sorter analysis as previously described (16). CR2 caused dose-dependent inhibition of gp350/220 fluorescent-microsphere binding to Raji B cells (Fig. 3b), while no inhibition was observed with microspheres incubated in buffer alone or in buffer plus an irrelevant protein (results not shown).

Experiments were next carried out to determine whether recombinant CR2 could also alter EBV infection of peripheral blood B cells as measured by the outgrowth of transformed B cells and by virus-induced stimulation of DNA synthesis. In these experiments, EBV was preincubated with various amounts of recombinant CR2 prior to the

FIG. 2. Characterization and large-scale purification of recombinant CR2. (A) [^{35} S]methionine metabolic labeling of CR2. SF9 cells were infected with either CR2 recombinant baculovirus (lanes 1 to 3) or wild-type *A. californica* nuclear polyhedrosis virus (lanes 4 to 6), followed by incubation in [^{35} S]methionine-containing media. Cell supernatants were immunoprecipitated with anti-CR2 monoclonal antibody HB5 (American Type Culture Collection, Rockville, Md.) (lanes 1 and 4) or OKB7 (Ortho Diagnostics, Inc., Raritan, N.J.) (lanes 3 and 6) or a control antibody (BOS-1) (lanes 2 and 5). Immunoprecipitates were analyzed under reducing conditions on a 7% sodium dodecyl sulfate-polyacrylamide gel. MW $\times 10^{-3}$ Daltons, molecular sizes in kilodaltons. (B) Silver-stained (14) sodium dodecyl sulfate-polyacrylamide gel analysis of large-scale purification of soluble CR2. Lanes 1 to 5 represent samples of consecutive fractions of recombinant CR2 eluted with 3 M MgCl_2 from HB5 monoclonal antibody immunoadsorbent.

FIG. 3. Recombinant CR2 binding to gp350/220 and C3dg and inhibition of gp350/220 binding to B cells. (a) Purified C3dg (●), gp350/220 (▲), or ovalbumin (■) was coated onto 96-well plates and then reacted with various amounts of purified CR2. Receptor binding was determined by an enzyme-linked immunosorbent assay. (b) Fluorescent microspheres coated with purified recombinant gp350/220 (a gift of Elliot Kieff, Boston, Mass.) were reacted with CR2-negative SF9 cells (A) or with CR2-positive Raji cells (B). Gp350/220-coated microspheres were also reacted with 2.0 μg (C) or 10 μg (D) of purified recombinant CR2 prior to incubation with Raji cells. Binding of gp350/220 was determined by fluorescence-activated cell sorter analysis.

addition of unseparated peripheral blood mononuclear cells. After further incubation with EBV and CR2, the cells were washed, cultured for 14 to 21 days, and assessed for transformation as previously described (15). Preincubation of EBV with CR2 resulted in substantial inhibition (65 to 70%) of EBV infection, as measured by the outgrowth of trans-

formed colonies and by [³H]thymidine incorporation (Fig. 4A). The ability of CR2 to inhibit EBV infection was not due to toxic effects of the receptor on the B cells, since the highest dose of CR2 used in this experiment (7.5 μg) did not abolish infection of B cells if EBV was allowed to bind to B cells prior to being exposed to the receptor (Fig. 4A). In

FIG. 4. Inhibition of EBV infectivity by recombinant CR2. (A) EBV-containing B95-8 culture supernatant was incubated with various amounts of purified recombinant CR2 prior to the addition of 6×10^5 human peripheral blood mononuclear cells. The cells were washed and cultured in the presence of 0.1 μg of cyclosporine per ml and assessed for outgrowth of transformed B-cell colonies or stimulation of [³H]thymidine incorporation. In control experiments, EBV was incubated with cells prior to the addition of recombinant CR2 (○, △). (B) Various amounts of EBV supernatant were incubated with media alone (—) or with 10 μg of purified recombinant CR2 (---). Samples were then reacted with peripheral blood mononuclear cells as described above and assessed for B-cell transformation by measuring the colony outgrowth and stimulation of DNA synthesis. Bars represent standard deviation.

parallel experiments (Fig. 4B), various amounts of EBV were preincubated with a constant amount of CR2 (10 μ g) prior to the addition of B cells. EBV-induced B-cell transformation was reduced by 70 to 90% by preincubation with CR2 (Fig. 4B). Since complete inhibition of EBV infection with soluble CR2 was not achieved in these experiments, we cannot rule out the role of other cellular factors in EBV entry; however, CR2 appears to be the primary site of attachment. The inability of soluble CR2 to completely inhibit EBV infection may be due to the failure of the receptor to completely saturate all of the virus-associated gp350/220 molecules which possess high affinity for the receptor (23).

The ability of recombinant soluble CR2 to block EBV infection in vitro clearly indicates that CR2 is the primary EBV receptor on B cells. Similar approaches have been used to show that CD4 is the human immunodeficiency virus type 1 receptor on various cell types (2, 4, 7, 11, 24, 25). These findings suggest that soluble viral receptors may have potential as antiviral agents in vivo. For EBV, soluble CR2 may have value in treating immunosuppressed individuals or patients with severe, chronic infectious mononucleosis who are at risk for the development of fatal EBV-induced B-cell lymphomas. There are numerous advantages in the use of soluble viral receptors to block acute infection, if the approach is found to be useful. First, efficacy would likely not be impaired by antigenic changes and polymorphism of viral envelope proteins or by strain variation, since the viral receptor-binding domain(s) would be unaffected by such changes. Second, the soluble receptor would not likely be antigenic, toxic, or immunosuppressive, and effectiveness would not require the use of adjuvants. Furthermore, efficacy would not be dependent on the presence of an intact immune system. Regardless of the potential of CR2 as an antiviral agent, the availability of relatively large amounts of soluble CR2 should facilitate further analysis of its functional roles in EBV infection and B-cell activation.

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