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Identification of gp350 as the Viral Glycoprotein Mediating Attachment of Epstein-Barr Virus (EBV) to the EBV/C3d Receptor of B Cells: Sequence Homology of gp350 and C3 Complement Fragment C3d†

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The major Epstein-Barr virus (EBV) envelope glycoprotein, gp350, was purified from the B95-8 cell line and analyzed for its ability to mediate virus attachment to the isolated EBV/C3d receptor (CR2) of human B lymphocytes. Purified gp350 and EBV, but not cytomegalovirus, exhibited dose-dependent binding to purified CR2 in dot blot immunoassays. Binding was inhibited by certain monoclonal antibodies to CR2 and to gp350. Liposomes bearing incorporated gp350 bound to CR2-positive B-cell lines but not to CR2-negative lines. Liposome binding was also inhibited by the OKB7 anti-CR2 monoclonal antibody. A computer-generated comparison of the deduced gp350 amino acid sequence with that of the human C3d complement fragment revealed two regions of significant primary sequence homology, a finding which suggests that a common region on these two unrelated proteins may be involved in CR2 binding.

Epstein-Barr virus (EBV) binds selectively to a 145-kilodalton B-lymphocyte membrane glycoprotein which also serves as the receptor for the complement C3d fragment (7, 8, 12, 17, 18). Binding of EBV to this receptor on normal peripheral blood B cells is followed by viral endocytosis, while binding to the same receptor on B lymphoblastoid cells leads to viral fusion with the cell membrane (15). Wells et al. found that the dual presence of a major envelope protein of EBV, designated gp350/300 (gp350), and a related protein, gp220/200 (gp220), is required for artificial lipid vesicles containing EBV antigens to bind to B cells (23). There have been no other direct studies of EBV attachment protein(s). It has been found, however, that some monoclonal antibodies (MAbs) to gp350 block EBV infection in vitro (21), and immunization of nonhuman primates with liposomes containing gp350 protects against the development of fatal lymphoma (5). In the studies presented here, we employed purified gp350, EBV, and the EBV/C3d receptor (CR2) to identify gp350 as the EBV attachment protein and to characterize certain aspects of the binding of gp350 to CR2. In addition, two regions of amino acid sequence homology were found in the gp350 and C3d coding sequences and may represent the CR2 binding areas of these two proteins.

MATERIALS AND METHODS

EBV and CMV isolation and cell lines. EBV and the AD169 strain of cytomegalovirus (CMV) were isolated as previously described from phorbol ester-induced B95-8 cells (14) and foreskin fibroblasts (3), respectively. The Ramos cell line was obtained from D. A. Thorley-Lawson, Tufts University School of Medicine, Boston, Mass. As determined by the presence of Epstein-Barr nuclear antigen, this line was not infected with the B95-8 strain of EBV, nor did it possess EBV receptors, a fact ascertained by fluorescence analyses with anti-CR2 MAbs. Another Ramos cell line, designated Ramos-EF, was obtained from Elizabeth Fowler, University of Florida, Gainesville. The cell line was infected with EBV and stained with anti-CR2 MAbs. All cell lines were maintained at 37°C in an atmosphere of 5% CO_2 in RPMI 1640 containing 10% heat-inactivated fetal calf serum (Flow Laboratories, Inc., McLean, Va.), 50 μg of gentamicin per ml, 200 mM glutamine, and 0.2 μg of amphotericin B (Fungizone; GIBCO Laboratories, Grand Island, N.Y.) per ml.

Purification of gp350 and CR2. gp350 was isolated from B95-8 cells as previously described (19), except that either high-pressure liquid chromatography (HPLC) gel filtration or immunoaffinity chromatography was also used to remove low-molecular-weight contaminants. For HPLC purification, gp350 obtained from chromatography on ricin-agarose columns (19) was chromatographed on an HPLC TSK-250 column (Bio-Rad Laboratories, Richmond, Calif.) which was equilibrated with 10 mM phosphate-buffered saline (PBS; pH 7.4; 0.15 M NaCl) containing 0.01% sodium deoxycholate (Calbiochem-Behring, San Diego, Calif.) (Fig. 1). The column was run at a flow rate of 0.5 ml/h. Fractions containing gp350 were identified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (10) on 5% resolving gels which were silver stained (13) and by dot blot assays with anti-gp350 MAbs. The column was precalibrated with gel filtration standards (Bio-Rad).

For preparation of liposomes containing gp350 (see below) as well as for dot blot assays, immunoaffinity chromatography with MAbs specific for gp350 was used as the final purification step. Anti-gp350 MAbs were cross-linked to protein A-agarose (17).

CR2, which was isolated from Raji lymphoblastoid cells by HB5 MAb immunoaffinity chromatography, was more

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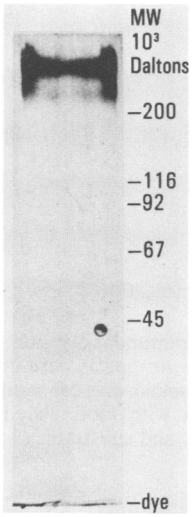

FIG. 1. Affinity purification of gp350. Partially purified gp350 was chromatographed on an anti-gp350 column. Forty microliters (approximately 100 ng) of gp350 was electrophoresed on a 5 to 15% gradient SDS gel. The gel was silver stained. MW, Molecular weight.

than 90% homogeneous as judged by SDS-PAGE, as published previously (12, 17).

MAbs to gp350 and CR2. MAbs to gp350 were raised in the following manner. B95-8 cells were treated with 20 ng of phorbol myristate acetate (tumor-promoting agent [TPA]) per ml for 4 to 6 days, and the intact cells were injected intravenously into 4- to 6-week-old BALB/c mice. The mice were reinjected every 7 to 10 days and at 3 days before the spleens were removed for fusion with the Fox NY myeloma cell line (Hyclone Laboratories, Inc., Ogden, Utah). Wells containing hybridomas were screened by an EBV enzyme-linked immunosorbent assay (ELISA) as previously described (16). Briefly, approximately 10^5 EBV particles per well were coated onto the plastic wells of ELISA plates (Immulon II; Dynatech Industries, Inc., Alexandria, Va.), reacted with culture supernatants, and incubated with biotinylated anti-murine immunoglobulin, streptavidin-horseradish peroxidase (Amersham Corp., Arlington Heights, Ill.), and substrate. A secondary screen for the identification of clones recognizing EBV envelope antigens was carried out by membrane fluorescence assay of TPA-treated B95-8 cells as previously described (18). Hybridomas which were positive in the EBV ELISA and in the TPA–B95-8 fluorescence assay were subcloned at least three times and then grown in ascites. Four of these antibodies, designated BOS-1, 18A7, 5B11, and 11B5, were of the immunoglobulin G2a(κ) isotype and were purified on protein A-agarose (6). The specificity of the MAbs was confirmed by immunoprecipitation of ^{125}I-labeled B95-8 cells followed by SDS-PAGE performed as previously described (18).

MAbs to CR2 used in this study were OKB7, generously provided by Ortho Diagnostics, Inc., Raritan, N.J.; AB-1, kindly donated by Barry Wilson, Hybritech, San Diego, Calif.; and HB5, purchased from the American Type Culture Collection, Rockville, Md. These antibodies were purified by protein A-agarose chromatography as previously described (6).

Dot blot immunoassays. Detection of EBV and gp350 binding to isolated CR2 was carried out by dot blot assays as previously described (17). The specificity of ligand binding was assessed by the ability of the CR2 MAb OKB7 to block receptor binding to EBV and to gp350. The OKB7 antibody has previously been shown to directly inhibit both C3d and EBV binding to intact B cells (18). Another anti-CR2 MAb, designated AB-1, which recognizes a different epitope on the receptor (20), was used as a control in the inhibition studies. Purified receptor (40 ng) was preincubated with 6 μg of each MAb for 60 min at 4°C. This mixture was then reacted with immobilized EBV, CMV, or gp350 for an additional 60 min at 22°C. Detection of receptor binding was determined by incubation with biotinylated HB5 MAb, streptavidin-horseradish peroxidase, and chromogenic substrate as previously described (17).

In parallel studies, MAbs specific for gp350 were used to inhibit receptor binding to immobilized gp350. In these experiments, 100 ng of gp350 was coated onto nitrocellulose, and after nonspecific binding sites were blocked with 2% bovine serum albumin (BSA), the gp350 blots were reacted with various amounts of purified MAbs to gp350 or irrelevant MAbs. After further incubation for 60 min at 22°C, the blots were reacted with purified CR2 and developed as described above.

Preparation of liposomes containing gp350. Liposomes containing gp350 were prepared by detergent dialysis as previously described (12), except that cholesterol was added to the phosphatidylcholine in preparation of the membranes. The composition of the liposome mixtures was 50 μg of gp350 per ml in 10 mM Tris–150 mM NaCl (pH 8.0) containing 0.5% deoxycholate, 6 mM egg yolk phosphatidylcholine (Supelco Inc., Bellefonte, Pa.), 0.75 mM cholesterol (Supelco), and 0.5 μCi of [^{14}C]dipalmitoyl phosphatidylcholine (New England Nuclear Corp., Boston, Mass.). Control liposomes were prepared with 10 mM Tris–150 mM NaCl (pH 8.0)–0.5% deoxycholate lacking gp350. Liposomes were separated from unincorporated protein by ultracentrifugation on discontinuous sucrose gradients (12). gp350 was detected in liposomes by dot blot assays with purified MAbs to gp350 as described above. Control liposomes prepared in the absence of gp350 did not react in this assay.

gp350 liposome-binding studies. For cell-binding assays, 10 μl of liposomes containing 2,500 cpm of ^{14}C-lipid was incubated for 60 min at 4°C with 5×10^6 B lymphoblastoid cells in 250 μl of PBS containing 0.1% BSA. Cells were pelleted for 30 s at 2,000 × *g* in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) and washed three times in PBS-BSA. The supernatants and pellets were counted for ^{14}C. To assess the specificity of the liposome binding, 5×10^6 lymphoblastoid cells were incubated with 5 μg of HB5 or OKB7 anti-CR2 MAb for 30 min on ice. Cells were pelleted, and supernatants were removed before the liposomes were added for binding.

Quantitation of CR2 sites on B lymphoblastoid cells. ^{125}I-HB5, which was generously donated by John Bohnsack, Scripps Clinic and Research Foundation, La Jolla, Calif., was trace labeled with ^{125}I and Iodo-Beads (Pierce Chemical Co., Rockford, Ill.) according to the instructions of the manufacturer to a specific activity of 250,000 cpm/μg. B lymphoblastoid cells (5×10^6) were incubated with 2 μg of ^{125}I-HB5 in 500 μl of PBS–0.1% BSA for 30 min at 4°C. The cells were then washed three times, and pellets were counted. Background binding was determined by incubating cells with 200 μg of unlabeled HB5 for 15 min before the labeled antibody was added and represented less than 0.5% of the total counts per minute.

gp350 and C3d sequence comparison. The deduced amino acid sequences of gp350 (1, 2) and human C3d (4) were

TABLE 1. Antigenic properties of MAbs

MAb[a]	Membrane fluorescence (% positive)[b]	EBV ELISA reactivity (A_{405})[c]	gp350 dot blot reactivity
BOS-1	13.2	1.690	+
18A7	19.7	2.031	+
11B5	17.2	1.915	+
5B11	20.2	1.299	+
AB-1	2.3	0.158	−
Medium (control)	1.0	0.029	−

[a] BOS-1, 18A7, 11B5, and 5B11 are MAbs generated by immunization with B95-8 cells. AB-1 is MAb to CR2 included as a control.

[b] B95-8 cells were treated for 4 days with 20 ng of TPA per ml before fluorescence analysis with each MAb and fluorescein isothiocyanate anti-mouse immunoglobulin.

[c] Purified EBV, approximately 10^5 particles per well, was coated onto plastic wells of ELISA plates and reacted with undiluted culture supernatants containing antibody and then with biotinylated anti-murine immunoglobulin, streptavidin-horseradish peroxidase, and substrate.

compared by an alignment program, ALIGN, of the National Biomedical Research Foundation, Georgetown University Medical Center, Washington, D.C.

RESULTS

Isolation of gp350. Affinity-purified gp350 was devoid of low-molecular-weight contaminants (Fig. 1). The purified protein also contained detectable amounts of the related gp220, only small amounts of which are present in the B95-8 cell line from which the gp350 was purified. gp350 isolated by HPLC gel filtration lacked the gp220 but contained minor amounts (≤5%) of a host cell 160- to 170-kilodalton protein (data not shown).

Characterization of anti-gp350 MAbs. Four MAbs were produced which reacted with the membranes of a subpopulation of TPA-treated B95-8 cells, indicating that they probably reacted with a structural EBV glycoprotein (Table 1). These MAbs also reacted strongly with purified EBV in an ELISA and with purified gp350 in a dot blot assay. AB-1, an immunoglobulin G2a CR2 MAb included as a control, did not react with B95-8 cells, EBV, or gp350. The BOS-1 antibody-stained TPA induced B95-8 cells but did not react with untreated or phosphonoformate-treated B95-8 cells as determined by fluorescence-activated cell sorter analyses (data not shown). To confirm the specificity of the MAbs for gp350, immunoprecipitation analyses were carried out with radiolabeled, TPA-treated B95-8 cells. BOS-1, 18A7, 11B5, and 5B11 all reacted strongly with a 350-kilodalton protein and reacted weakly with a 200-kilodalton protein, as shown for BOS-1 in Fig. 2. A control MAb of the same isotype but directed against CR2 (HB5) did not immunoprecipitate any proteins from B95-8 cells.

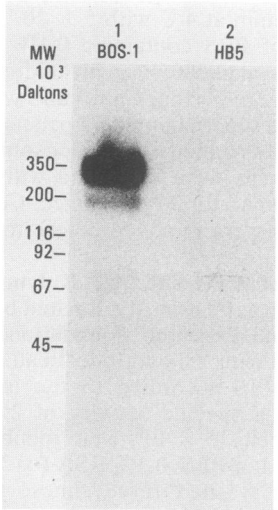

FIG. 2. SDS-PAGE analysis of immunoprecipitates of ^{125}I-labeled TPA-treated B95-8 cells. ^{125}I-labeled B95-8 cell membrane lysates were immunoprecipitated with 5 μg of protein A-linked BOS-1 or with HB5, an anti-CR2 MAb, as a control. The precipitates were eluted with 2% SDS (and 5% BME) and electrophoresed on 5 to 15% SDS-PAGE gradient gels. MW, Molecular weight.

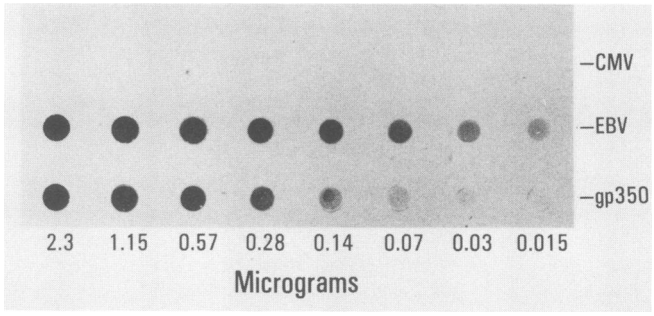

FIG. 3. Dot blot immunoassay for CR2 binding. Various amounts of CMV, EBV, or gp350 were immobilized on nitrocellulose. The blots were blocked with 5% nonfat milk and reacted with purified CR2 and then with biotinylated anti-CR2, streptavidin-horseradish peroxidase, and substrate.

Dot blot assays for gp350 binding to CR2. Dot blot immunoassays were used to assess the ability of purified gp350 to bind to isolated CR2. EBV, as well as purified gp350 (Fig. 3), showed dose-dependent binding to CR2. Binding of as little as 70 ng of gp350 or 15 ng of EBV to CR2 was detectable. No binding of CMV to CR2 in doses as high as 2,300 ng was detectable (Fig. 2).

Effect of anti-CR2 MAbs on gp350 and EBV binding to CR2. Further experiments were done to document the specificity of gp350 binding to CR2 in the dot blot assay. The substitution of BSA for CR2 abrogated reactivity (Fig. 4, upper right), indicating that gp350 and EBV were not di-

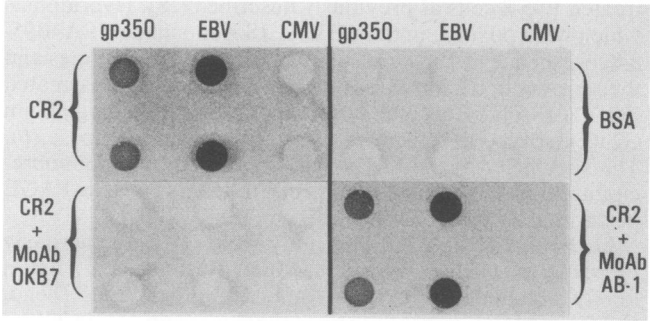

FIG. 4. Specificity of CR2 binding to gp350. Purified CR2 was reacted directly with EBV, CMV, or gp350 (upper left); with 6 μg of MAb OKB7 (lower left); or with 6 μg of MAb Ab-1 (lower right) in solution before incubation with immobilized gp350, EBV, or CMV. BSA was substituted for CR2 (upper right) in the blotting experiments. The reactions were developed as described in the legend to Fig. 3.

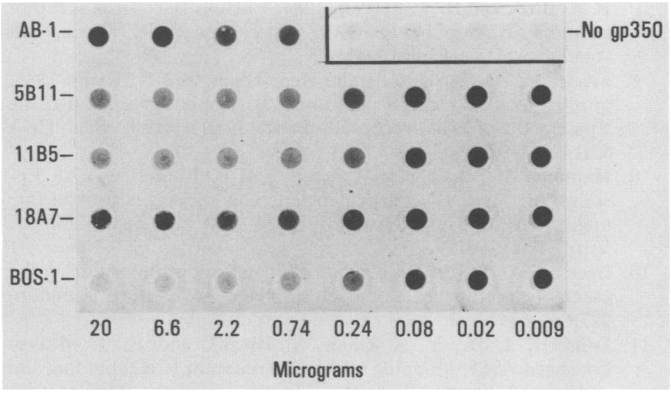

FIG. 5. Effect of anti-gp350 MAbs on CR2 binding to gp350. Various amounts of anti-gp350 MAbs 5B11, 11B5, 18A7, and BOS-1 were reacted for 60 min with immobilized gp350. After further incubation with purified CR2, the blots were developed as described in the legend to Fig. 3. AB-1, an anti-CR2 MAb, was also included as a control. The four wells at the upper right were coated with albumin instead of gp350 and reacted with CR2.

rectly interacting with the immunologic reagents. The preincubation of CR2 with OKB7 MAb, which blocks EBV binding to B cells and to CR2, completely abrogated CR2 binding to both gp350 and EBV (Fig. 4, cf. lower left with upper left). Another anti-CR2 MAb, AB-1, which does not directly block CR2 ligand binding to B cells, did not interfere with the binding of purified CR2 to EBV or gp350 (Fig. 4, lower right). These findings document the specificity of the dot blot receptor assay for CR2-EBV ligand interactions.

Effect of anti-gp350 MAbs on gp350 binding to CR2. The ability of the various anti-gp350 MAbs to alter gp350 binding to CR2 was examined. Pretreatment of immobilized gp350 with BOS-1 MAb strongly inhibited binding of CR2 in a dose-dependent manner (Fig. 5). MAbs 5B11 and 11B5 inhibited modestly, while 18A7 was without inhibitory activity. AB-1, an anti-CR2 MAb included as a control, failed to inhibit binding of CR2 to gp350. CR2 also did not react with uncoated nitrocellulose.

gp350 liposome-binding studies. To confirm by another experimental approach the binding of gp350 to CR2, the ability of liposomes containing gp350 to bind to several B lymphoblastoid cell lines was assessed. gp350-containing liposomes, but not control liposomes, bound to Raji and Ramos-EF cells, both of which had approximately 2×10^4 CR2 sites per cell (Table 2). gp350-containing liposomes failed to bind to B95-8 and Ramos cells, both of which lacked detectable CR2. Furthermore, binding of gp350 liposomes to Raji and Ramos-EF cells was abrogated by pretreatment with OKB7 MAb but not with HB5 MAb, indicating that the liposome binding is mediated through CR2.

DISCUSSION

In the studies presented here, purified ligands and receptors have been used to show that gp350 mediates binding of EBV to B-cell CR2. Affinity-purified gp350 employed in these studies was devoid of minor amounts of cellular contaminants and contained a minor amount of gp220. gp220 is related to gp350 but differs in size because of altered mRNA splicing (2, 9). Isolated gp350 bound to the receptor in a dose-dependent manner. This reaction was specifically blocked by OKB7 anti-CR2 MAb, which also blocks EBV and C3d binding to B cells (18), but was unaffected by AB-1 and HB5 anti-CR2 MAbs, which do not directly inhibit EBV and C3d binding to B cells. Certain anti-gp350 MAbs also specifically blocked the interaction of purified gp350 with the receptor. Finally, liposomes containing purified gp350 bound to B cells expressing CR2 but did not bind to CR2-negative cell lines. This binding also was blocked by OKB7 but was not blocked by HB5 anti-CR2 MAb. These studies indicate that gp350 mediates adsorption of EBV to CR2. We are unable to explain the gp220 requirement previously reported by Wells et al. with gp350- and gp220-containing liposomes (23) since the HPLC-purified gp350, which lacked gp220, also mediated CR2 binding.

The nature of the ligand-binding site on CR2 to which EBV and C3d bind is not known, and it is also unclear whether both ligands react with the same site or bind to distinct sites. It is not possible to address this question directly by performing competitive binding studies with C3d,g and gp350 because the binding affinity of monomeric C3d,g for CR2 is very low (22) and because isolated gp350 is insoluble in the absence of detergent. The ability to selectively inhibit EBV or C3d binding to B cells with different anti-CR2 MAbs which was recently reported (R. Frade, M. Barel, L. Krikoria, and C. Charriaut, 6th Int. Congr. Immunol., p. 189, 1986) does not prove that EBV and C3d bind to different sites because the attachment of large ligands such as EBV- and C3d-coated particles to cells may involve not only the primary ligand-binding site but also secondary interactions. In an alternative approach to determining the binding sites, we questioned whether gp350 and C3d, two proteins of diverse origin, might exhibit regions of primary sequence homology. A computer search done by using the ALIGN program identified two regions of homologous sequence in the deduced gp350 coding sequence (1) and the human C3d sequence (4). One of these regions (region 1, Fig. 6) consisted of five identical amino acids. This sequence was not found in any of the other 3,000 proteins contained in the data base. In addition, a second region (region 2, Fig. 6) encompassing the region of C3d which has been reported to be involved in CR2 binding (11) was also identified. Whether either of these two sequences has any role in CR2 binding remains to be determined, but their presence raises the posibility that a common domain on gp350 and C3d is used for CR2 binding.

The identification of gp350 as the EBV protein responsible for receptor attachment represents the first identification of a distinct herpesvirus envelope glycoprotein which is utilized

TABLE 2. Binding of gp350-containing liposome to B lymphoblastoid cells

Cell line	CR2 (molecules/cell)[a]	MAb[b]	Liposome binding (cpm)[c]	
			With gp350	Control
Raji	20,500 ± 200		256 ± 31	48 ± 4
		OKB7	58 ± 6	ND
		HB5	281 ± 7	ND
Ramos-EF	19,000 ± 1,300		141 ± 21	34 ± 11
		OKB7	46 ± 8	
Ramos	0		76 ± 12	67
B95-8	0		62 ± 2	ND

[a] Determined by binding of ^{125}I-labeled HB5. Results are mean ± standard error.

[b] Cells were preincubated with 5 μg of MAb to CR2.

[c] Liposome-binding assays were done in duplicate. Results of three experiments were combined and expressed as mean ± standard error of counts per minute in pellet. ND, Not determined.

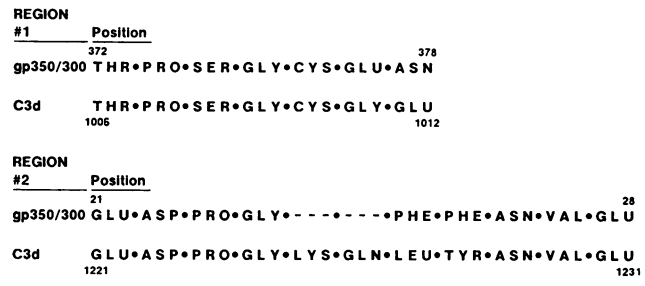

FIG. 6. Primary sequence homology between gp350 and C3d. The deduced gp350 coding sequence and that of C3d were compared for homology by ALIGN.

in virus adsorption to host cells. This system provides a unique model for the analysis of the structure and function of virus receptor-ligand interations. Since CR2, with which both gp350 and C3d interact, mediates viral endocytosis or fusion depending on the cell type, this system also provides the means to address not only the nature of, and epitopes involved in, ligand-receptor interactions but also the functional consequences of these reactions.

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