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Monoclonal Antibodies Define a Domain on Herpes Simplex Virus Glycoprotein B Involved in Virus Penetration

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In an earlier report (S. D. Marlin, S. L. Highlander, T. C. Holland, M. Levine, and J. C. Glorioso, J. Virol. 59:142–153), we described the production and use of complement-dependent virus-neutralizing monoclonal antibodies (MAbs) and MAb-resistant (*mar*) mutants to identify five antigenic sites (I to V) on herpes simplex virus type 1 glycoprotein B (gB). In the present study, the mechanism of virus neutralization was determined for a MAb specific for site III (B4), the only site recognized by MAbs which exhibited complement-independent virus-neutralizing ability. This antibody had no detectable effect on virus attachment but neutralized viruses after adsorption to cell monolayers. These findings implied that the mechanism of B4 neutralization involved blocking of virus penetration. The remaining antibodies, which recognized sites I, II, and IV, required active complement for effective neutralization. These were further studied for their ability to impede virus infectivity in the absence of complement. Antibodies to sites I (B1 and B3) and IV (B6) slowed the rate at which viruses penetrated cell surfaces, supporting the conclusion that antibody binding to gB can inhibit penetration by a virus. The data suggest that MAbs can interfere with penetration by a virus by binding to a domain within gB which is involved in this process. In another assay of virus infection, MAb B6 significantly reduced plaque development, indicating that antibody binding to gB expressed on infected-cell surfaces can also interfere with the ability of a virus to spread from cell to cell. In contrast to these results, antibodies to site II (B2 and B5) had no effect on virus infectivity; this suggests that they recognized structures which do not play a direct role in the infectious process. To localize regions of gB involved in these phenomena, antibody-binding sites were operationally mapped by radioimmunoprecipitation of a panel of truncated gB molecules produced in transient-expression assays. Residues critical to recognition by antibodies which affect penetration by a virus (sites I, III, and IV) mapped to a region of the molecule (amino acid residues 241 to 441) which is centrally located within the external domain. Antibodies which had no effect on penetration (site II) recognized sequences distal to this region (residues 596 to 737) near the transmembrane domain. The data suggest that these gB-specific MAbs recognize two major antigenic sites which reside in physically distinct components of the external domain of gB. Only the region containing antigenic sites I, III, and IV appeared to colocalize with a molecular structure that directly contributes to the process of virus infectivity.

Herpes simplex virus (HSV) enters the host cell by fusion of the virion envelope with the cell surface plasma membrane and release of the nucleocapsid into the cytoplasm (8, 9, 12, 18, 24, 27). At least four HSV gene products are required for this process (1, 14, 18a, 19, 29). Three of these are glycoproteins designated gB, gD, and gH, while the fourth is a nonenvelope virion component. Temperature sensitivity and/or deletion mutations in these essential genes result in production of enveloped virions which can attach to host cells but subsequently fail to carry out the fusion-penetration process and thus do not enter the cytoplasm (1, 14, 18a, 19, 29).

gB was the first virus component implicated in virus penetration (29). Temperature sensitivity mutations mapping to the gB gene are characterized by failure to fully glycosylate the major precursor of gB to the mature form, as well as by production of noninfectious viruses (19, 20, 29). Virions produced at high temperature attach to cells but penetrate cell surfaces very poorly. However, treatment of the cell-adsorbed mutant virus with the chemical fusogen polyethylene glycol significantly enhances virus infectivity (19, 29). Mutations in the external domain of gB have also been shown to alter the rate of virus penetration (6, 9; S. Highlaner, D. Dorney, P. Gage, T. Holland, M. Levine, and J. Glorioso, manuscript in preparation), and a single mutation in the cytoplasmic domain can induce membrane fusion between infected cells (6, 9). A report that cells that express gB can be induced to form polykaryocytes after brief treatment with low-pH buffer (2) provides additional evidence linking gB with membrane fusion. These observations make it clear that gB is essential to virus infectivity and that it may mediate the fusion event leading to virus penetration.

Previously, we generated a panel of gB-specific monoclonal antibodies (MAbs) which neutralize viruses in the presence of complement (21). Individual antibodies were used to select neutralization-resistant (*mar*) mutants in gB for analysis of its antigenic structure. By comparing the neutralization resistance patterns of these mutants with a panel of 16 gB-specific antibodies, five distinct antigenic sites (I to V) were identified (21). Antigenic variation in at least two of these sites could be correlated with alterations in gB processing and function in virus infection (21). Specifically,

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single *mar* mutations in sites II and III were associated with temperature-dependent defects in Golgi-related glycosylation and transport to the cell surface. Double mutants carrying *mar* mutations in both sites II and III produced a nonfunctional gB at high temperature which failed to support virus growth. Furthermore, other strains of HSV type 1 (HSV-1) that carry temperature sensitivity mutations in the gB gene produced a gB precursor product at high temperature that failed to be recognized by sites II and III antibodies (21). These data link antigenic sites II and III with gB structures that are both antigenic and critical for gB processing and function in virus infectivity.

In this report, we tested gB-specific MAbs representative of antigenic sites I to IV for their ability to impede virus infectivity in the absence of complement. Antibodies specific for sites I, III, and IV were found to interfere with virus infectivity, while a second group of antibodies that recognize site II showed no effect. The physical binding sites for each of these antibodies were operationally defined by immunoprecipitation of a series of truncated gB molecules produced in transient-expression assays. Antibodies that affected penetration were all predicted to react with residues in the middle of the external domain of the molecule. Antibodies which did not affect penetration were predicted to recognize an external-domain sequence near the site of membrane insertion. These observations suggest the presence of two major antibody recognition sites within the external domain of gB, one of which appears to play a key role in virus penetration.

MATERIALS AND METHODS

Cells and virus strains. Vero cells were maintained in Eagle minimum essential medium (MEM; GIBCO Laboratories, Grand Island, N.Y.) supplemented with nonessential amino acids, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), and 5% fetal calf serum (FCS; GIBCO). D6 cells that express HSV-1 (KOS) gB were derived from Vero cells after transformation with pKBXX (see below) and pSV2-neo (7). This cell line was maintained in MEM with 10% FCS and 200 μg of the antibiotic G418 (Sigma Chemical Co., St. Louis, Mo.) per ml. Wild-type HSV-1 (KOS-321) was grown and titered on Vero cells at 37°C as described previously (16). K082 is a KOS strain mutant which contains a unique *Hpa*I linker insertion (GT TAAC) in the coding region of the gene that encodes gB. This mutation causes termination of translation because of generation of a nonsense codon after amino acid 43 (7). This gB^- mutant virus was grown and titered on a gB-producing cell line designated D6 as reported earlier (7).

MAbs. Production of HSV-1 glycoprotein-specific antibodies has been described in detail elsewhere (16). The nine gB-reactive MAbs used in this study have been extensively characterized (21). Antibodies D1 and D8 are specific for HSV-1 gD and were used as positive controls for interference with virus adsorption, postadsorption neutralization, and inhibition of plaque formation assays (15). MAb CC406 does not recognize any HSV determinants (3) and was included as a negative control in these assays. The anti-gB pool consisted of equal amounts of MAbs B2, B3, B4, and B6, while the anti-gC pool consisted of MAbs C1, C3, C7, C8, C11, C13, C15, C16, and C17 (22). Antibodies were produced as mouse ascites fluid, clarified by ammonium sulfate precipitation, and suspended in phosphate-buffered saline (PBS; pH 7.4). Antibody concentrations were determined by using a radial immunodiffusion assay kit (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.).

Inhibition of virus adsorption. HSV-1 (KOS-321) was radiolabeled with [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.), purified by T10 dextran (Sigma) gradient centrifugation, and inhibition of adsorption by antibody binding, assayed as reported earlier (15). A sample of 2.5×10^2 PFU was diluted in 1% bovine serum albumin and treated with antibody B4, D8, or CC406 at concentrations of 0.04, 0.10, 0.25, 0.50, and 1.00 mg/ml for 2 h at 37°C. The antibody-treated virus was plated on Vero cells in 24-well trays (Costar, Cambridge, Mass.) at 250 μl per well for 2 h at 4°C. The inoculum was recovered, the monolayers were lysed, and the counts per minute associated with bound and unbound viruses were determined by liquid scintillation spectrometry. Each antibody dilution was assayed in triplicate, and the values were averaged.

Postadsorption neutralization. Approximately 5×10^2 PFU of wild-type virus in 0.5 ml of MEM was adsorbed to Vero cell monolayers in six-well trays (Costar) for 2 h at 4°C. Monolayers were washed with PBS-$MgCl_2$, overlaid with MEM and antibody B4 or D1 at 10 μg/ml, and incubated for 2 h at 4°C. Monolayers were washed again with PBS-$MgCl_2$, overlaid with methylcellulose, and incubated for 3 days at 37°C. Similar samples were treated with antibody for 2 h at 4°C before plating. Monolayers were stained with crystal violet, and surviving PFU were compared between samples treated with antibody before and after virus adsorption (15).

Antibody inhibition of virus penetration. The rate at which viruses penetrated cell surfaces was measured by inactivation of extracellular viruses with a low-pH citrate buffer with a modification of a protocol described by Huang and Wagner (17) as reported previously (15). Wild-type virus was diluted in MEM and incubated with 20 μg of each antibody per ml for 3 h at 37°C. Approximately 5×10^5 Vero cells in each well of six-well trays (Costar) were infected with the antibody-treated virus at 400 PFU per well for 2 h at 4°C. Monolayers were washed, overlaid with MEM–5% FCS, and incubated at 37°C. At various times after temperature shift, the monolayers were treated with citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) for 1 min, washed promptly with PBS-$MgCl_2$, and overlaid with methylcellulose, and plaques were scored after 3 days at 37°C. The percentage of intracellular virus at a given time after the temperature shift was calculated as the number of plaques formed on citrate-treated monolayers, divided by the number of plaques produced on untreated monolayers, multiplied by 100.

Plaque size reduction assay. Antibodies were tested at low (10 μg/ml), medium (50 μg/ml), and high (500 μg/ml) concentrations for ability to reduce the size of plaques. Vero cells in six-well trays (Costar) were infected with 200 PFU of wild-type virus per well at 37°C in MEM–5% FCS. Monolayers were washed after 4 h and overlaid with antibody diluted in methylcellulose. After 3 days at 37°C, cells were stained with crystal violet and plaque size was scored by comparing average plaque diameters with a dissecting microscope (15).

Construction of truncated gB-encoding genes. The construction of a series of *Hpa*I linker insertion mutations in the gB-coding region will be presented in detail elsewhere (W. Cai, S. Person, C. DebRoy, and B. Gu, J. Mol. Biol., in press). Briefly, an *Xho*I-to-*Bam*HI fragment containing the regulatory and coding sequences of the HSV-1 (KOS) gB-encoding gene was cloned into pUC9 and designated pKBXX. This plasmid was partially digested with four frequent- and blunt-cutting enzymes (*Alu*I, *Fnu*DII, *Hae*III, and *Rsa*I) such that the molecule was cut once. The linear-

ized plasmids were isolated from agarose gels, *Hpa*I linkers were ligated to the blunt ends, digested with *Hpa*I (which does not cut within pKBXX), and religated. The locations of linker insertions were mapped by restriction enzyme digestion and confirmed by DNA sequencing. Seventeen different insertions were identified which generated stop codons in the gB-coding region. Nine of these were used in this study and expressed polypeptides ranging in length from 240 to 737 amino acid residues, in comparison with the 904 residues present in the full-length wild-type molecule.

Transient expression and radioimmunoprecipitation of truncated gB molecules. Transfections were performed by using a modification of the method of Sompayrac and Danna (31). Vero cells were plated at 10^6/60-mm (diameter) dish (Costar) 24 h before transfection. Two micrograms of plasmid DNA, 1 ml of serum-free MEM, and 10 μl of a 5-mg/ml stock of DEAE-dextran (M_r, 500,000; Pharmacia, Uppsala, Sweden) in sterile H_2O were added, in that order, to polystyrene culture tubes (17 by 100 mm; Becton Dickinson Labware, Oxnard, Calif.). Samples were allowed to equilibrate for 30 min at room temperature. The culture medium was aspirated from cells, and the inocula were pipetted gently onto the monolayers. Plates were incubated for 4 h at 37°C. At that time, the inocula were removed and 5 ml of fresh MEM–5% FCS was added gently to each monolayer. Plates were incubated for an additional 42 h at 37°C before infection with K082 at a high multiplicity of infection of 5 to induce expression of the plasmid-borne gB-encoding gene. After infection with K082 for 7 h, transfected monolayers were washed with PBS-$MgCl_2$ and overlaid with 0.5 ml of methionine-free MEM supplemented with 50 μCi of [^{35}S] methionine (New England Nuclear) per ml. After 1 h at 37°C, monolayers were washed again with PBS and solubilized with NP-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 10 mM Tris hydrochloride, pH 8.8) as described previously (16). To inhibit proteolytic degradation of gB in cell lysates, 1 mM phenylmethylsulfonyl fluoride (Sigma)–50 μg of *N*α-*p*-tosyl-*l*-lysine chloromethyl ketone (Sigma)–5% dry milk (Carnation, Los Angeles, Calif.) was added to detergent cell extracts. gB was precipitated from these extracts by overnight incubation with 5 μl of individual MAbs or MAb pools at 4°C. Immunoprecipitates were recovered with protein A-conjugated Sepharose beads (Sigma). Electrophoresis on *N*,*N*′-diallyltartardiamide cross-linked 10% polyacrylamide gels and fluorography were performed as previously described (16).

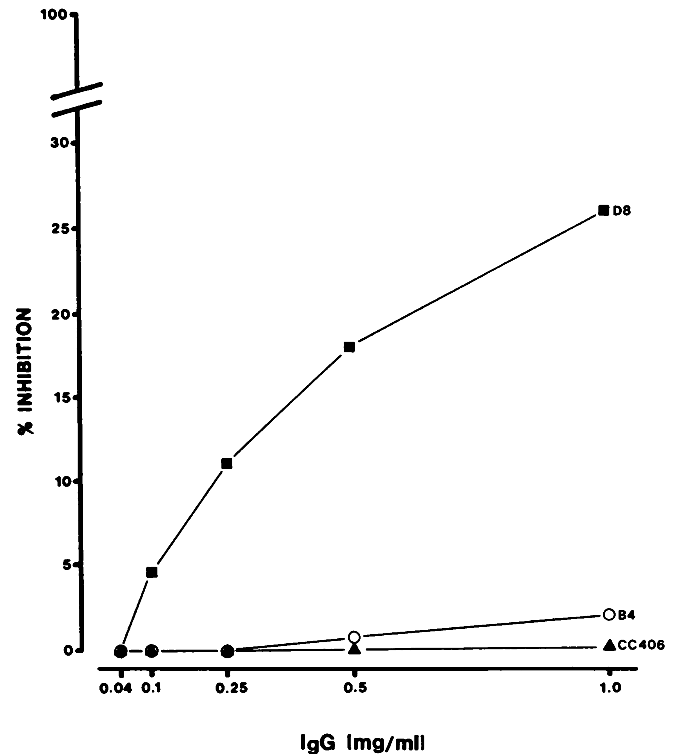

FIG. 1. Effect of antibody B4 on HSV-1 adsorption to Vero cells. [^{35}S]methionine-labeled KOS-321 was treated with different concentrations of each antibody at 37°C for 2 h. The virus was then allowed to adsorb to the cell surfaces for 2 h at 4°C. Bound and unbound viruses were determined by liquid scintillation spectrometry. The results are reported as percent inhibition of adsorption compared with an untreated KOS-321 control. D8 recognizes HSV-1 strain KOS-321 gD and has been previously shown to inhibit virus attachment (15). CC406 does not recognize any HSV determinant and has been previously shown to have no effect on virus attachment (15). IgG, Immunoglobulin G.

RESULTS

Occurrence of complement-independent virus neutralization after virus adsorption. Antibody B4 (site III) was previously shown to neutralize HSV-1 in the absence of complement (21). Because the HSV-1 envelope is shed during virus entry (10, 27), neutralization by glycoprotein-specific antibodies is unlikely to involve inhibition of nucleocapsid uncoating. Rather, binding of neutralizing antibody should either prevent the virus attachment or penetration step in the infectious process. The following experiments were performed to distinguish between these two possibilities.

Antibody B4 was tested for the ability to prevent attachment of a purified, radiolabeled virus to cell surfaces. The virus was treated with antibody and plated on cells at 4°C, which allows attachment but prevents penetration (15). The percent inhibition of virus attachment after antibody treatment with B4 was compared with that of an untreated virus. Antibodies D8 and CC406 were used as positive and negative controls, respectively, for antibody interference with virus attachment. At concentrations far in excess of that needed for neutralization (1 mg/ml), antibodies B4 and CC406 had little or no effect on virus adsorption (Fig. 1). In agreement with earlier findings (15), D8 inhibited virus adsorption by about 27% at the highest concentration tested. These data indicated that the neutralizing activity exhibited by B4 does not involve inhibition of virus adsorption.

To confirm that neutralization inhibits a step subsequent to virus attachment, the ability of B4 to neutralize a virus adsorbed to cell surfaces was tested. An inoculum of 5×10^2 PFU of the virus was adsorbed to cell monolayers at 4°C to prevent virus penetration. These monolayers were treated with antibody B4 for 2 h at 4°C and overlaid with methylcellulose, and plaques were scored after 3 days of incubation at 37°C. Antibody D1 was included as a positive control for postadsorption neutralization (15). Duplicate virus samples were treated with each antibody for 2 h at 4°C before adsorption and plated on monolayers for comparison. At 10 μg/ml, B4 neutralized 42% of the adsorbed virus compared with 83% for the virus treated with this antibody before adsorption. D1 neutralized 67% of the input virus at this concentration regardless of whether antibody treatment was given before or after adsorption. Although less efficient, antibody B4 was still effective at neutralizing the surface-

bound virus, indicating that neutralization occurred at a step subsequent to virus attachment.

Effect of antibody on rate of virus penetration. Previously it was reported that antibodies specific for HSV-1 gD greatly slowed the rate at which viruses penetrated cell surfaces in the absence of complement (15). Although complement-independent neutralizing antibodies were more effective, a reduction in the rate of virus entry could also be demonstrated for complement-dependent virus-neutralizing antibodies. To determine whether gB-specific antibodies could impede the rate of virus penetration in a manner similar to that observed for antibodies reactive with gD, antibodies that recognize the different antigenic sites on gB were tested for their effects on the kinetics of virus penetration. Samples of wild-type virus were treated with the individual antibodies, and the infection was synchronized by adsorption at 4°C. After 2 h, cultures were shifted to 37°C to allow penetration to proceed. At various times after the temperature shift, replicate monolayers were washed with low-pH citrate buffer to inactivate the surface-bound, nonpenetrated virus, and plaque formation was scored after 3 days. The percent survival represents that fraction of the input virus that had penetrated cells at each time point. Since antibody B4 neutralized the virus in the absence of complement, most of the input PFU were neutralized by complete blocking of penetration. In this case, only the rate of penetration for the virus which survived neutralization can be determined. Although antibodies B1 and B3 (site I) and B6 (site IV) showed little or no complement-independent neutralizing capability (21), they nevertheless slowed the rate of virus penetration (Fig. 2). In agreement with the finding that B4 neutralized attached viruses, this antibody also severely reduced the rate at which antibody-treated viruses penetrated cell membranes, confirming that B4 neutralized viruses by interfering with virus penetration (Fig. 2). Antibody B4-treated virions did not begin to enter cells until 5 to 6 h postinfection, 3 h after untreated viruses had completely penetrated cell membranes (data not shown). In contrast, antibodies B2 and B5 (site II) had no effect on penetration (Fig. 2), similar to other site II-reactive antibodies (B7, B8, and B9) and CC406 (data not shown). Taken together, these data demonstrated that the ability of an antibody to interfere with virus penetration depends on epitope specificity and is not strictly related to virus neutralization in the absence of complement.

Antibody-mediated inhibition of plaque development. Another measure of virus infectivity is the ability of the virus to spread from an infected cell to an adjacent uninfected cell. The effect that antibodies specific for gB had on plaque size under methylcellulose was examined to measure the ability to inhibit cell-to-cell spread of viruses in the absence of complement. Only one of the nine antibodies, B6 (site IV), had any effect on plaque development. This antibody reduced the diameters of plaques measurably at concentrations as low as 10 μg/ml and by 50% at 50 μg/ml. Figure 3 shows the effects on plaque size at a concentration of 500 μg/ml for antibodies representing four antigenic sites defined on gB (21). Antibodies D1 and CC406 were included as positive and negative controls, respectively, for inhibition of plaque development (15). Because B6 does not neutralize viruses in the absence of complement, inhibition of plaque development was not the result of neutralization of viruses released into the medium. This conclusion is supported by the finding that B4 had no such effect. Rather, a mechanism by which neighboring cells may become infected without virus release appears to be inhibited by MAb B6.

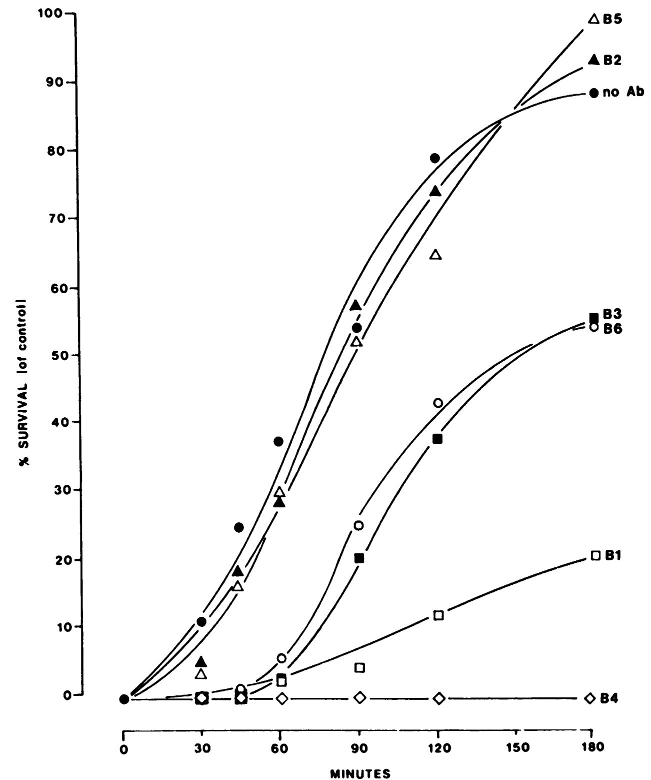

FIG. 2. Effect of gB-specific antibodies on kinetics of HSV-1 entry into Vero cells. Virus samples containing 5×10^2 PFU of KOS-321 were treated with 20 μg of each antibody per ml for 3 h at 37°C and added to Vero cell monolayers. After adsorption for 2 h at 4°C, cultures were shifted to 37°C and washed with citrate buffer (pH 3.0) at various times after the shift. The time shown is the time after the shift to 37°C. Survivor virus plaques were scored after 3 days of incubation at 37°C.

Mapping of antibody-binding sites by radioimmunoprecipitation of truncated gB molecules. Since the antibodies showed distinct differences in their abilities to affect virus penetration, they might be expected to recognize different regions of the gB protein. To examine this possibility, MAb-binding sites were identified by using a panel of chain-terminating mutant gB-encoding genes. These mutants were engineered to contain a nonsense codon within the gB-coding region (Fig. 4A). Nine different truncations that express polypeptides ranging from residues 240 to 737 were tested. These mutant plasmids were individually transfected into Vero cells with DEAE-dextran (31). At 48 h posttransfection, cells were infected with K082, a virus which encodes a gB protein that is truncated after 43 amino acids and produces no detectable gB product (7). This virus provides the immediate-early regulatory functions necessary for gB expression from the transfected plasmids (Cai et al., in press). At 7 h postinfection, cells were labeled with [^{35}S] methionine for 1 h, lysed, and immunoprecipitated with glycoprotein-specific antibody pools. Figure 4B shows the migration pattern for each of the truncated molecules after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (lanes 5 to 13). Two cellular polypeptides were nonspecifically immunoprecipitated from mock-treated cell lysates with the gB-specific antibody pool (lane 1) and were seen in each of the experimental precipitates. No gB-related product was detected after immunoprecipitation of K082-infected cell extracts with a gB-specific antibody pool (lane 3),

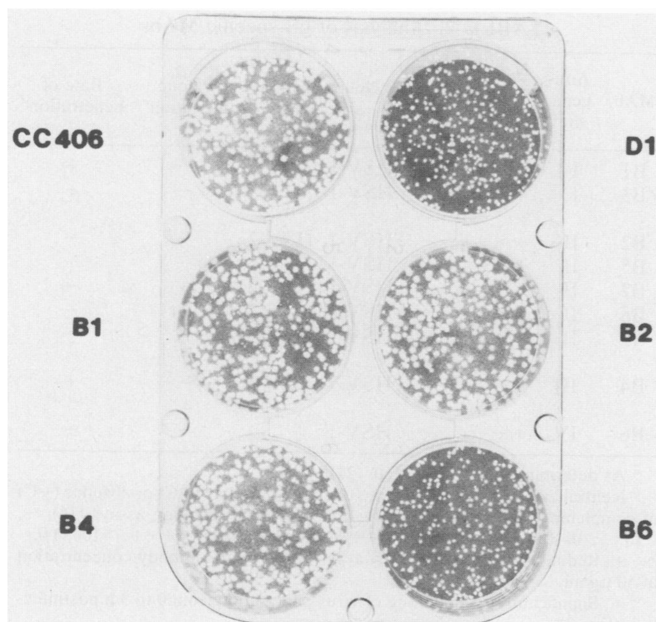

FIG. 3. Effect of gB-specific antibodies on HSV-1 plaque size. Cell monolayers were infected with approximately 200 PFU of KOS-321 for 4 h, at which time the medium was replaced with 0.5% methylcellulose and 500 μg of each antibody per ml. After 3 days of incubation at 37°C, the monolayers were stained with crystal violet and plaque diameters were estimated with a dissecting microscope.

although precursor gC and mature gC were detected by immunoprecipitation with a gC-specific antibody pool (lane 2), indicating that infection had occurred. Lane 4 shows the normal-size gB molecule expressed from the plasmid pKBXX after immunoprecipitation by the gB-specific antibody pool. The truncated polypeptides are arranged from left to right in order of decreasing molecular ratios (lanes 5 to 13). In each case, the predicted size of the truncated product correlated with the relative mobility of the polypeptide in the gel. All truncations larger than 240 amino acids could be detected by immunoprecipitation, indicating that at least one of the antigenic determinants was intact in each of these molecules. No gB-related polypeptide was detected in cells transfected with mutant plasmid 343. Since gB expressed from this plasmid would represent only about one-quarter of the normal gB molecule, it is possible that either this polypeptide is unstable and cannot be detected by this method or the remaining antigenic structure is disrupted. However, it is only 42 amino acids shorter than a detectable polypeptide (230) and has the same three methionine residues available for radiolabeling, as predicted by DNA sequencing of the mutant plasmid. Therefore, it may be argued that the truncated molecule 343 lacked all of the antigenic determinants recognized by these antibodies.

The results of using individual MAbs against each polypeptide are shown in Table 1. All antibodies precipitated polypeptide 159 (residues 1 to 737), placing binding sites I to IV in the external domain of gB (Fig. 4A). Site I antibodies also precipitated polypeptide 366, indicating that their binding sites could be further resolved to include only residues 1 to 441. The site III antibody precipitated polypeptides as short as 147 (residues 1 to 380), and the site IV antibody precipitated polypeptides as short as 230 (residues 1 to 282). The most direct explanation for the loss of reactivity of an antibody with a given polypeptide would be that a truncation removed residues recognized by that antibody. Therefore,

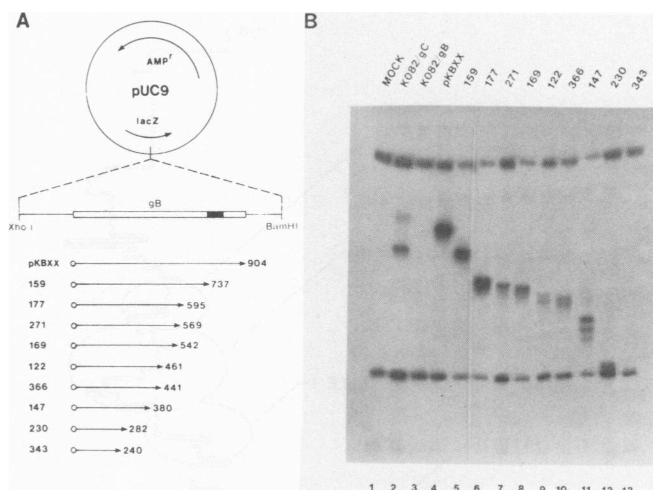

FIG. 4. Radioimmunoprecipitation of truncated gB polypeptides. (A) An *Xho*I-to-*Bam*HI clone containing gB regulatory and coding sequences was inserted into pUC9 and designated pKBXX. The gB protein is shown by the open rectangle, and the location of the transmembrane domain is represented by the black rectangle. Partial digestion of pKBXX with blunt-cutting enzymes and *Hpa*I linker ligation resulted in generation of stop codons and polypeptide termination at the indicated residues (see Materials and Methods). (B) Two micrograms of each truncation plasmid was transfected into Vero cells by DEAE-dextran, and expression was induced by infection with K082 (gB$^-$) 48 h later. At 7 h postinfection, cells were labeled for 1 h with 50 μCi of [^{35}S]methionine per ml and immunoprecipitated with a pool of gB-specific MAbs. Samples were electrophoresed on 10% polyacrylamide gels and fluorographed. Lanes: 1, mock-treated Vero cells precipitated with anti-gB pool; 2 and 3, K082-infected cells precipitated with anti-gC pool or anti-gB pool, respectively; 4 to 13, cells transfected with the indicated plasmids, infected with K082, and precipitated with anti-gB pool.

binding sites may be operationally defined to include residues that are lost between a truncated polypeptide and the next smaller one, the larger of which is precipitated by an antibody while the smaller is not. On this basis, residues 241 to 282 may be operationally defined as the binding site for site IV antibodies, residues 283 to 380 would represent the

TABLE 1. Radioimmunoprecipitation of truncated gB polypeptides

Plasmid	No. of NH_2-terminal residues	Precipitation by the following MAb[a]:								
		2	5	7	8	9	1	3	4	6
pKBXX	904	+	+	+	+	+	+	+	+	+
159	737	+	+	+	+	+	+	+	+	+
177	595	–	–	–	–	–	+	+	+	+
271	569	–	–	–	–	–	+	+	+	+
169	542	–	–	–	–	–	+	+	+	+
122	461	–	–	–	–	–	+	+	+	+
366	441	–	–	–	–	–	+	+	+	+
147	380	–	–	–	–	–	–	–	+	+
230	282	–	–	–	–	–	–	–	–	+
343	240	–	–	–	–	–	–	–	–	–

[a] The antigenic sites and MAb designations were previously defined by Marlin et al. (21). MAbs 1 and 3 are antigenic site I MAbs; 2, 5, 7, 8, and 9 are site II MAbs; 4 is a site III MAb; and 6 is a site IV MAb.

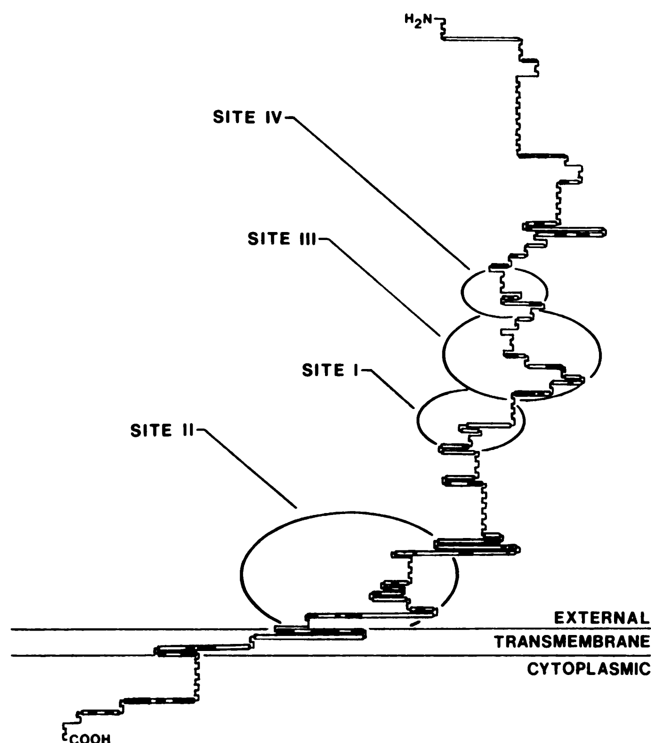

FIG. 5. Location of antigenic sites on HSV-1 gB. The putative secondary structure of KOS-321 gB displayed with the graphics routine of the protein analysis program MSEQ (4). The predicted alpha helix (■), beta sheet (□), random coil (——), and beta turn (⊐) are depicted, and the predicted location of each antigenic site (I to IV) is indicated. Scale, 0.0450 cm/A; MSEQ v., 1.19; hydrophobicity set, 1.

binding site for site III antibodies, and residues 381 to 441 would include the binding site for site I antibodies. All of these sites reside in the middle of the external domain of gB. In contrast, site II would map to residues 596 to 737, more proximal to the transmembrane domain (Fig. 5). Within each site, the antibodies showed identical neutralization patterns, indicating that their binding sites are in close proximity to one another.

An alternative cause for loss of antibody reactivity would involve disruption of the conformation of a truncated molecule such that a polypeptide is unstable and rapidly broken down. However, all polypeptides of 282 or more residues can be detected by the gB-specific antibody pool, discounting this possibility for all but polypeptide 343 (Fig. 4B). It is also possible that truncation may destabilize antigenic structures and prevent their recognition by an antibody without removing the binding site. If this is the case, however, only one antigenic site at a time is affected by the progressive truncations (Table 1). In view of these alternative explanations, the regions of gB involved in binding (Fig. 5) can only be said to contain some structure critical for antibody recognition.

DISCUSSION

Table 2 summarizes the properties of nine gB-specific antibodies generated in our laboratory. Antigenic sites I through IV were defined by complement-dependent virus neutralization resistance patterns of *mar* B mutants of KOS-321 (21). Only one of these antibodies, B4, neutralized the virus in the absence of complement and severely inhibited the rate at which the antibody-treated virus entered cells. Another antibody, B6, also slowed the rate of virus penetration and demonstrated the unique ability to inhibit cell-to-cell virus spread. Two other antibodies, B1 and B3, slowed the rate of virus penetration but did not neutralize the virus or inhibit its spread to adjacent cells. Although these four antibodies all affected virus infectivity, they could be distinguished on the basis of different antiviral activities (Table 2). Moreover, these differences correlated with antigenic recognition sites defined by these antibodies (21). The data indicate that the various antiviral activities of these antibodies depend on the location of the corresponding epitopes within gB. In contrast, site II antibodies (B2, B5, B7, B8, and B9) failed to affect virus infectivity in the absence of complement, operationally distinguishing this antigenic site from the other three (Table 2). The precipitation of the truncated gB molecules presented here (Table 1) is consistent with the above-described analysis. Antibodies reactive with the same antigenic site precipitated the same truncated polypeptides and therefore react with sequences in a similar region of the molecule. Furthermore, antibodies which affected virus infectivity in the absence of complement all appeared to recognize the same general region of gB, whereas antibodies which did not exhibit these effects recognized a separate region of the molecule.

TABLE 2. Analysis of gB-specific MAbs

MAb	Antigenic site[a]	Neutralization titer[b]		Neutralization specificity	Plaque inhibition[c]	Rate of penetration[d]
		+C	−C			
B1	I	+	−	HSV-1	−	+
B3	I	+	−	HSV-1	−	+
B2	II	++++	−	HSV-1, HSV-2	−	−
B5	II	+++	−	HSV-1, HSV-2	−	−
B7	II	+++	−	HSV-1, HSV-2	−	−
B8	II	+++	−	HSV-1, HSV-2	−	−
B9	II	+++	−	HSV-1, HSV-2	−	−
B4	III	++	+	HSV-1, HSV-2	−	+
B6	IV	++	−	HSV-1	+	+

[a] As determined by Marlin et al. (21).
[b] Neutralization titer was determined in the presence (+C) or absence (−C) of complement in 50% plaque reduction microneutralization assays (16). −, <10; +, 10–100; ++, 100–1,000; +++, 1,000–100,000; ++++, >100,000.
[c] +, Reduction of plaque size by at least 50% at an antibody concentration of 50 μg/ml.
[d] +, Significantly reduced rate of virus penetration from 0 to 3 h postinfection (Fig. 2).

Sites I (residues 381 to 441), III (residues 283 to 380), and IV (residues 241 to 282) all mapped to the amino-terminal half of the molecule (Fig. 5) and all reduced the rate of virus penetration (Fig. 2). The single antibody representing the site III neutralized virus in the absence of complement (Table 2). Showalter et al. (30) showed that another gB-specific MAb, designated 3S, also neutralized the virus in the absence of complement. Both B4 and 3S were previously shown to recognize KOS-321 gB site III (21). Site IV-specific antibody B6 inhibited cell-to-cell virus spread as measured by reduction in plaque size under methylcellulose (Fig. 3). The region of gB recognized by these antibodies has also been shown to be a target for conditional-lethal mutations that cause defects in virus penetration at elevated temperature. These mutations result in amino acid substitutions in

residues 273 and 377 (5) and amino acid insertions in residues 313 and 437 (Cai et al., in press). The data suggest that these sites are contained within a major antigenic domain of gB whose integrity is critical to the function of gB in virus penetration.

Site II appears to represent a second antigenic domain which contained residues more proximal to the transmembrane domain (residues 596 to 737; Fig. 5). One of its distinguishing characteristics was that it was the only site for which representative antibodies did not decrease the rate of virus penetration. Yet, this site lies near a region where insertion mutations cause lethal defects in gB function (Cai et al., in press). Furthermore, *mar* mutations in site II cause a decrease in the rate of virus penetration (Highlander et al., in preparation), as well as temperature-dependent effects on gB processing and cell surface transport (21). Thus, it appears that this domain may not be directly involved in virus penetration but may contain a structure which influences the ability of gB to participate efficiently in this process. Another distinguishing characteristic of site II antibodies is that they possess very high complement-dependent neutralization titers (Table 2). This method of virus neutralization results from disruption of the virion envelope by antibody activation of the complement cascade (25). The proximity of site II and the viral envelope bilayer may contribute to the efficiency with which site II antibodies initiate this process.

It is clear that gB and gD are both essential to virus penetration (18a, 19, 29) and may both participate in membrane fusion (2, 6, 12, 15, 23, 26). Both gB^- and gD^- mutants exhibit the same phenotype: a lethal defect in penetration of cell membranes (7; Ligas and Johnson, submitted). Consistent with this finding, antibodies which recognize a number of distinct antigenic sites on gD (11, 15, 23, 28) and one on gB neutralize viruses in the absence of complement (21). Furthermore, both gB-specific (Fig. 2) and gD-specific antibodies (15) slow the rate of virus penetration and inhibit plaque formation (15, 23; Fig. 3). Cells that express gB or gD can also be induced to form polykaryocytes, the former with low pH (2) and the latter with low concentrations of the membrane fusogen polyethylene glycol (O. Fuller, R. Johnson, and P. Spear, 12th Int. Herpesvirus Workshop, 1987, abstr. no. 139, p. 139). There are, however, significant differences in behavior between these two molecules, suggesting that, while they are both essential to virus entry into cells, they may perform distinctly different activities. (i) gD-expressing cells, but not gB-expressing cells, have been shown to be resistant to infection by HSV (8; R. Johnson, O. Fuller, and P. Spear, 12th Int. Herpesvirus Workshop, 1987, abstr. no. 137, p. 137), implying that gD may directly interact with a cellular component during virus infection at a stage subsequent to virus attachment. (ii) Antibodies to gD, but not gB, will inhibit syncytium formation (26; S. Highlander, unpublished data), even though both glycoproteins have been shown to be necessary for polykaryocyte formation (13, 18a, 20; W. Cai, B. Gu, and S. Person, submitted for publication). However, only gB has been shown to be susceptible to mutations which induce syncytia (6, 9). It remains to be determined whether a *syn* mutant in the gD gene can be isolated.

In summary, the results shown here indicate that two distinct regions of gB exist: one that is susceptible to defects in penetration caused by antibody binding and one that is resistant to such effects. These data suggest regions of the external domain of gB which may contribute to its ability to function in virus infection. Further mutagenesis experiments are in progress for genetic fine mapping of structures of gB which contribute to virus penetration and cell membrane fusion. These studies should lead to a better understanding of the structural requirements for these activities, as well as provide insight into the mechanism by which gB participates in the penetration process.

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LITERATURE CITED

1. **Addison, C., F. Rixon, J. Palfreyman, M. O'Hara, and V. Preston.** 1984. Characterization of a herpes simplex virus type 1 mutant which has a temperature-sensitive defect in penetration of cells and assembly of capsids. Virology **138**:246–259.
2. **Ali, M., M. Bucher, and H. Ghosh.** 1987. Expression and nuclear envelope localization of biologically active fusion glycoprotein B of herpes simplex virus in mammalian cells using cloned DNA. Proc. Natl. Acad. Sci. USA **84**:5675–5679.
3. **Bishop, G. A., G. Kümel, S. Schwartz, and J. C. Glorioso.** 1986. Clonal specificity of herpes simplex virus type 1 reactive human natural killer cells for determinants of viral glycoproteins. J. Virol. **57**:294–300.
4. **Black, S., and J. Glorioso.** 1986. MSEQ: a microcomputer-based approach to the analysis, display, and prediction of protein structure. Biotechniques **4**:448–460.
5. **Bzik, D., B. Fox, N. DeLuca, and S. Person.** 1984. Nucleotide sequence specifying the glycoprotein gene, gB, of herpes simplex virus type 1. Virology **133**:301–313.
6. **Bzik, D., B. Fox, N. DeLuca, and S. Person.** 1984. Nucleotide sequence of a region of the herpes simplex virus type 1 gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion. Virology **137**:185–190.
7. **Cai, W., S. Person, S. C. Warner, J. Zhou, and N. A. DeLuca.** 1987. Linker-insertion nonsense and restriction-site deletion mutations of the gB glycoprotein gene of herpes simplex virus type 1. J. Virol. **61**:714–721.
8. **Campadelli-Fiume, G., M. Arsenakis, F. Farabegoli, and B. Roizman.** 1988. Entry of herpes simplex virus 1 in BJ cells that constitutively express viral glycoprotein D is by endocytosis and results in the degradation of the virus. J. Virol. **62**:159–167.
9. **DeLuca, N., D. Bzik, V. Bond, S. Person, and W. Snipes.** 1982. Nucleotide sequences of herpes simplex virus type 1 (HSV-1) affecting virus entry, cell fusion, and production of glycoprotein B (VP7). Virology **122**:411–423.
10. **DeLuca, N., D. Bzik, S. Person, and W. Snipes.** 1981. Early events in herpes simplex virus type 1 infection: photosensitivity of fluorescein isothiocyanate-treated virions. Proc. Natl. Acad. Sci. USA **78**:912–916.
11. **Eisenberg, R. J., D. Long, L. Pereira, B. Hampar, M. Zweig, and G. H. Cohen.** 1982. Effect of monoclonal antibodies on limited proteolysis of native glycoprotein gD of herpes simplex virus type 1. J. Virol. **41**:478–488.
12. **Fuller, O., and P. Spear.** 1987. Anti-glycoprotein D antibodies that permit adsorption but block infection by herpes simplex virus 1 prevent virion-cell fusion at the cell surface. Proc. Natl. Acad. Sci. USA **84**:5454–5458.
13. **Glorioso, J., M. Szczesiul, S. Marlin, and M. Levine.** 1983. Inhibition of glycosylation of herpes simplex virus glycoproteins: identification of antigenic and immunogenic partially glycosylated glycopeptides on the cell surface membrane. Virology **126**:1–18.

14. **Gompels, U., and A. Minson.** 1986. The properties and sequence of glycoprotein H of herpes simplex virus type 1. Virology **153**:230–247.
15. **Highlander, S. H., S. L. Sutherland, P. J. Gage, D. C. Johnson, M. Levine, and J. C. Glorioso.** 1987. Neutralizing monoclonal antibodies specific for herpes simplex virus glycoprotein D inhibit virus penetration. J. Virol. **61**:3356–3364.
16. **Holland, T. C., S. D. Marlin, M. Levine, and J. Glorioso.** 1983. Antigenic variants of herpes simplex virus selected with glycoprotein-specific monoclonal antibodies. J. Virol. **45**:672–682.
17. **Huang, A., and R. Wagner.** 1964. Penetration of herpes simplex virus into human epidermoid cells. Proc. Soc. Exp. Biol. Med. **116**:863–869.
18. **Johnson, D. C., M. Wittels, and P. G. Spear.** 1984. Binding to cells of virosomes containing herpes simplex virus type 1 glycoproteins and evidence for fusion. J. Virol. **52**:238–247.

18a. **Ligas, M. W., and D. C. Johnson.** 1988. A Herpes Simplex Virus Mutant in Which Glycoprotein D Sequences Are Replaced by β-Galactosidase Sequences Binds to but Is Unable To Penetrate into Cells. J. Virol. **62**:1486–1494.

19. **Little, S., J. Jofre, R. Courtney, and P. Schaffer.** 1981. A virion-associated glycoprotein essential for infectivity of herpes simplex virus type 1. Virology **115**:149–160.
20. **Manservigi, R., P. Spear, and A. Buchan.** 1977. Cell fusion induced by herpes simplex virus is promoted and suppressed by different viral glycoproteins. Proc. Natl. Acad. Sci. USA **74**:3913–3917.
21. **Marlin, S. D., S. L. Highlander, T. C. Holland, M. Levine, and J. C. Glorioso.** 1986. Antigenic variation (*mar* mutations) in herpes simplex virus glycoprotein B can induce temperature-dependent alterations in gB processing and virus production. J. Virol. **59**:142–153.
22. **Marlin, S. D., T. C. Holland, M. Levine, and J. C. Glorioso.** 1985. Epitopes of herpes simplex virus type 1 glycoprotein gC are clustered in two distinct antigenic sites. J. Virol. **53**:128–136.
23. **Minson, A., T. Hodgman, D. Digard, D. Hancock, S. Bell, and F. Buckmaster.** 1986. An analysis of the biological properties of monoclonal antibodies against glycoprotein D of herpes simplex virus and identification of amino acid substitutions that confer resistance to neutralization. J. Gen. Virol. **67**:1001–1013.
24. **Morgan, C., H. M. Rose, and B. Mednis.** 1968. Electron microscopy of herpes simplex virus. I. Entry. J. Virol. **2**:507–516.
25. **Müller-Eberhard, H.** 1986. The membrane attack complex of complement. Annu. Rev. Immunol. **4**:503–528.
26. **Noble, G., G. Lee, R. Sprague, M. Parish, and P. Spear.** 1983. Anti-gD monoclonal antibodies inhibit cell fusion induced by herpes simplex type 1. Virology **129**:218–224.
27. **Para, M. F., R. B. Baucke, and P. G. Spear.** 1980. Immunoglobulin G(FC)-binding receptors on virions of herpes simplex virus type 1 and transfer of these receptors to the cell surface by infection. J. Virol. **34**:512–520.
28. **Para, M. F., M. L. Parish, A. G. Noble, and P. G. Spear.** 1985. Potent neutralizing activity associated with anti-glycoprotein D specificity among monoclonal antibodies selected for binding to herpes simplex virions. J. Virol. **55**:483–488.
29. **Sarmiento, M., M. Haffey, and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7(B_2) in virion infectivity. J. Virol. **29**:1149–1158.
30. **Showalter, S. D., M. Zweig, and B. Hampar.** 1981. Monoclonal antibodies to herpes simplex virus type 1 proteins, including the immediate-early protein ICP 4. Infect. Immun. **34**:684–692.
31. **Sompayrac, L., and K. Danna.** 1981. Efficient infection of monkey cells with DNA of simian virus 40. Proc. Natl. Acad. Sci. USA **78**:7575–7578.