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Antibodies to a Synthetic Oligopeptide That React with Herpes Simplex Virus Type 1 and 2 Glycoprotein C

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Nucleotide sequence and mRNA localization studies have allowed the prediction of the amino acid sequence of herpes simplex virus type 1 (HSV-1) glycoprotein C (gC). We immunized a rabbit with a conjugate of bovine serum albumin and a synthetic peptide having the same sequence as that deduced for amino acids 128 through 139 of HSV-1 gC. A very similar amino acid sequence has been predicted to exist in the related product, herpes simplex virus type 2 (HSV-2) gC, which was formerly designated gF. Preparations of crude antiserum and immunoaffinity-purified antibodies were obtained and shown to react in enzyme-linked immunosorbent assays with purified HSV-1 gC and HSV-2 gC. Although these antibodies did not detectably immunoprecipitate proteins from radiolabeled infected cell extracts, they reacted with HSV-1 gC and HSV-2 gC that were electrophoretically transferred to nitrocellulose membranes from polyacrylamide gels. These results confirm that HSV-1 gC and HSV-2 gC are immunologically related and also define a specific portion of HSV-1 gC that is conserved.

Herpes simplex virus (HSV) type 1 (HSV-1) and type 2 (HSV-2) are highly related viruses. Many of their counterpart gene products have similar molecular weights (9, 12, 18) and common antigenic determinants (19, 20, 25). The genes specifying these products are usually located at equivalent positions on the viral genomes (13, 14).

Recent findings have shown that the 130,000-molecular-weight HSV-1 glycoprotein C (gC) and a 75,000-molecular-weight HSV-2 glycoprotein are antigenically related (26, 29), and their genes are positioned in equivalent regions (16, 27). Since these results show that these glycoproteins are probably counterpart gene products, the name of the HSV-2 glycoprotein has been changed from gF (1) to gC (27). Although HSV-1 gC and HSV-2 gC have considerably different electrophoretic mobilities, their immunological relationship suggests that portions of their amino acid sequences are very similar. Frink et al. (8) have recently deduced the amino acid sequence of HSV-1 gC from DNA sequence and mRNA localization studies. To explore the immunological properties and conservation of a specific region of the HSV-1 gC polypeptide, we prepared antibodies to a synthetic peptide of 12 amino acids, corresponding to that predicted for its amino acids 128 through 139. This amino acid sequence was selected because its hydrophilicity suggests that it may be on the surface of the protein and therefore accessible to antibody binding (10). In this report we show that antibodies to the 128 through 139 peptide react with both HSV-1 gC and HSV-2 gC, thus defining a conserved cross-reactive region of HSV-1 gC. After this report was submitted, we learned of DNA sequencing studies (D. Dowbenko and R. A. Lasky, submitted for publication) predicting that HSV-2 gC possesses an amino acid sequence very similar to that of the 128 through 139 peptide. This finding provides additional support for the results presented in this report.

MATERIALS AND METHODS

Cells and virus. Vero cells were grown in Eagle minimal essential medium with 10% heat-inactivated fetal calf serum. These cells were infected with either HSV-1 strain 14012 or HSV-2 strain 333 at a multiplicity of 10 to 20 as described previously (21, 29).

Antibodies to viral proteins. The properties of the 104-S anti-HSV-2 gC (29) and 19-S anti-HSV-1 gC (21) monoclonal antibodies have been reported earlier. Hyperimmune rabbit antisera to HSV-1 strain MAL and HSV-2 strain MS were produced as previously described (28). Rabbit antibodies to HSV-1 strain McIntrye (lot no. 110A) and HSV-2 strain MS (lot no. 070B), prepared by DAKO Immunoglobulins, Copenhagen, Denmark, were also tested.

Preparation of antibodies to synthetic peptide. The synthetic peptide NH_2-Asp-Arg-Arg-Asp-Pro-Leu-Ala-Arg-Tyr-Gly-Ser-Arg-COOH which corresponds to amino acids 128 through 139 of HSV-1 gC as deduced by Frink et al. (8) was purchased from Peninsula Laboratories, Inc. Analysis by reverse-phase high-pressure liquid chromatography indicated that the preparation predominantly contained a single species of peptide. The peptide was coupled to bovine serum albumin (Miles Laboratories, Inc.) with glutaraldehyde as described by Baron and Baltimore (2) and extensively dialyzed against phosphate-buffered saline (pH 7.2). Rabbits were immunized three times at 2-week intervals with bovine serum albumin-conjugated peptide (500 μg of protein per injection) by intramuscular and intradermal injections. The conjugated peptide was emulsified in complete Freund adjuvant for the first injection and in incomplete Freund adjuvant for subsequent injections. Antisera were collected at weekly intervals starting 7 days after the final immunization and stored in samples at −20°C. Antibodies to the peptide were immunoaffinity purified on a peptide-containing column by a procedure adopted from Tamura et al. (22). The column was prepared by coupling the N terminus of the peptide (5 mg) to 1 g of CH-Sepharose 4B (Pharmacia Fine Chemicals) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Bio-Rad Laboratories) according to instruction provided by Pharmacia Fine Chemicals. The peptide-coupled Sepharose beads were mixed end over end on a rotator with 6 ml of antiserum for 18 h at 4°C. The beads were sedimented by centrifugation (1,200 × *g* for 5 min), and the supernatant representing the nonabsorbing serum fraction was removed.

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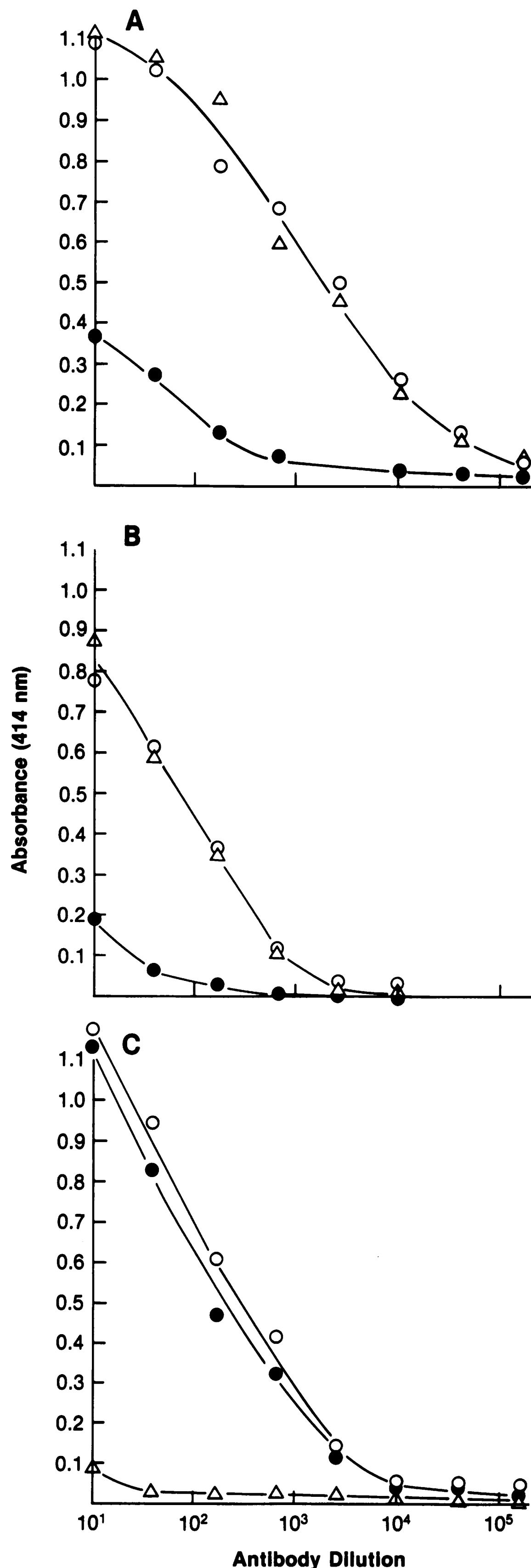

The beads were resuspended in phosphate-buffered saline (pH 7.2) and then loaded into a polypropylene column (Bio-Rad Laboratories). The column was first washed with phosphate-buffered saline and then with phosphate-buffered saline containing 0.5 M NaCl. After equilibration in 0.1 M sodium phosphate buffer (pH 7.2), the bound antibodies were eluted with 0.1 M glycine-hydrochloride buffer (pH 2.5), neutralized with 1 M Tris base and dialyzed against phosphate-buffered saline (pH 7.2).

Preparation of monoclonal antibody-Sepharose beads. 19-S antibody was purified as described previously (28, 29) by affinity chromatography, using protein A-Sepharose CL-4B (Pharmacia Fine Chemicals). Since the 104-S antibody binds poorly to protein A-Sepharose CL-4B (29), it was purified by DEAE Affi-gel blue chromatography (Bio-Rad Laboratories) by the stepwise elution procedure described by Bruck et al. (3). The purified antibodies were linked to CNBr-Sepharose 4B (Pharmacia Fine Chemicals) at 5 mg of protein per ml of gel according to the instructions of the manufacturer.

Purification of HSV glycoproteins. HSV-1 and HSV-2 gC were purified from extracts of infected cells by immunoaffinity chromatography with monoclonal antibody-Sepharose beads as reported earlier (28, 29). The viral glycoproteins were eluted from the beads with 3 M NaSCN–0.1% Nonidet P-40–0.01 M Tris-hydrochloride (pH 8.0) and extensively dialyzed against phosphate-buffered saline (pH 7.2). The purity of the preparations was verified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis (see below).

Enzyme-linked immunosorbent assay. The enzyme-linked immunosorbent assay was performed as described previously (28, 29). Briefly, after antigen was adsorbed to wells (0.5 μg of protein per well) of Immulon 2 plates (Dynatech Laboratories, Inc.), the wells were washed with phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20, and the remaining protein-binding sites were saturated by incubation in either 5% bovine serum albumin or 10% goat serum in phosphate-buffered saline. Antibody was added to the wells, and the plates were incubated for 1 h at 37°C. After the wells were washed, peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G (heavy and light chains; Kirkegaard and Perry Laboratories, Inc.) was added. The color-producing substrate used was 2,2'azino-di-(3-ethylbenzylthiazoline-sulfonate) (Sigma Chemical Co.). Absorbance was measured at 414 nm by a Titertek Multiskan spectrophotometer (Flow Laboratories, Inc.).

Protein blot analysis. At 20 h after HSV infection, the cell sheet was washed twice with Tris-buffered saline (pH 7.4), and the cells were scraped and pelleted (1,500 × *g* for 5 min). The pellet was resuspended in 2 volumes of 0.5% Nonidet P-40–0.01 M Tris-hydrochloride (pH 8.0)–0.2 mM phenyl-

FIG. 1. Enzyme-linked immunosorbent assay for the immunoaffinity purification of antibodies to the 128 through 139 peptide. The wells of microtiter plates were coated with 128 through 139 peptide (1 μg per well [A]), purified HSV-1 gC (0.5 μg per well [B]), or bovine serum albumin (0.5 μg per well [C]) and incubated with various dilutions of crude antiserum to the 128 through 139 peptide (○), the antiserum fraction adhering to the 128 through 139 peptide column (△), or the antiserum fraction not adhering to the 128 through 139 peptide column (●). The wells were washed and incubated with peroxidase-conjugated goat antibodies to rabbit immunoglobulin, followed by the addition of the color-producing substrate, 2,2'azino-di-(3-ethylbenzylthiazoline-sulfonate). The absorbance was then measured.

methylsulfonyl fluoride and incubated for 1 h at 0°C. Cellular debris was removed by centrifugation (12,000 × g for 5 min), and 4 volumes of a modified electrophoresis sample buffer (2% SDS–2% β-mercaptoethanol–0.0625 M Tris-hydrochloride [pH 6.8]–20% glycerol–0.01% Pyronine Y) was added to the supernatant. Samples of extracts of 10^5 infected cells, purified viral glycoprotein, bovine serum albumin, and prestained protein molecular weight standards (catalog no. 6041SA; Bethesda Research Laboratories, Inc.) in the modified electrophoresis sample buffer were heated at 100°C for 5 min and electrophoresed in a 10% polyacrylamide gel containing SDS (11, 29). Protein from the polyacrylamide gel was electrophoretically transferred to a nitrocellulose sheet (BA85; Schleicher & Schuell, Inc.) in a TE-42 apparatus (Hoefer Scientific Instruments) at 250 mA for 4 h as described by Towbin et al. (23). A portion of the nitrocellulose blot was stained with amido black to detect transferred protein (23). The protein-binding sites on the remaining portion was saturated by incubation in 10% horse serum in Tris-buffered saline (pH 7.4)–0.05% NaN_3 for 2 h at 37°C. The blot was reacted with rabbit antibodies in 2% horse serum in Tris-buffered saline–0.05% NaN_3 for 18 h at 4°C. After the blot was extensively washed in Tris-buffered saline (7.4), it was incubated in a 1:400 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G (heavy and light chains; Kirkegaard and Perry Laboratories, Inc.) in Tris-buffered saline for 2 h at 37°C. After several washes with Tris-buffered saline, the blot was reacted with 4-chloro-1-naphthol–H_2O_2 as described by Nielsen et al. (15). The staining was stopped by rinsing the membrane with water.

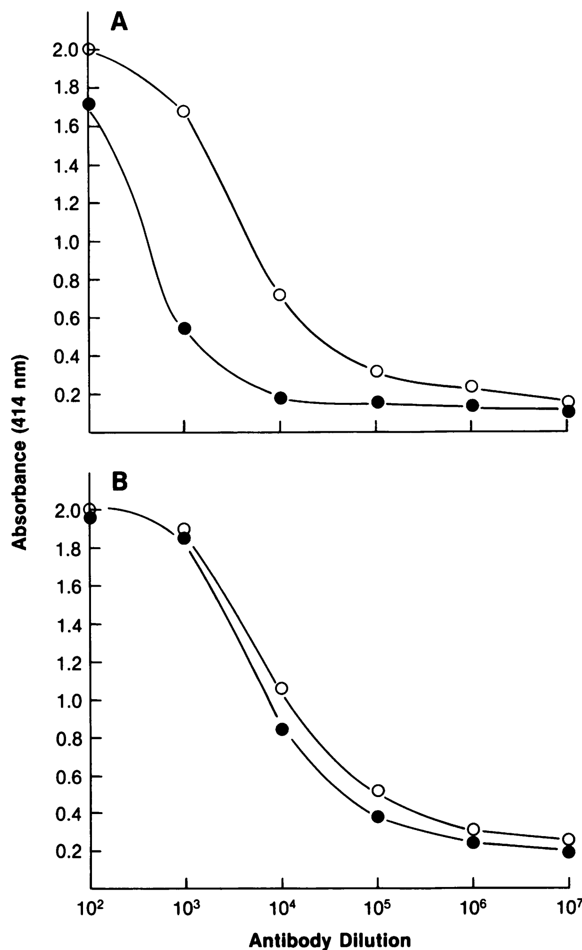

FIG. 2. Titration curves of rabbit antibodies reacting with HSV-2 gC by enzyme-linked immunosorbent assay. HSV-2 gC-coated wells of a microtiter plate were incubated with various dilutions of rabbit antibodies to HSV-1 (DAKO) (A) or rabbit antibodies to HSV-2 (no. 661) (B) that were either absorbed with 0.1 μg of purified HSV-1 gC (●) or unabsorbed (○). The wells were then treated as described in the legend of Fig. 1, and the absorbance was measured.

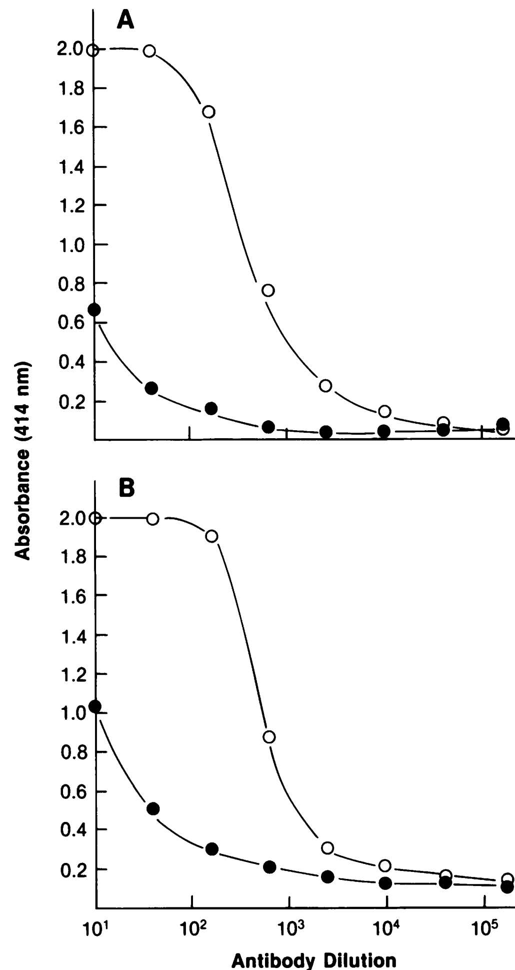

FIG. 3. Reactivity of antibodies to the 128 through 139 peptide with HSV-1 gC and HSV-2 gC. Wells of microtiter plates were coated with HSV-1 gC (A) or HSV-2 gC (B) and incubated with various dilutions of purified antibodies to the 128 through 139 peptide which were either absorbed with 0.05 μg of 128 through 139 peptide (●) or unabsorbed (○). The wells were then treated as described in the legend of Fig. 1, and the absorbance was measured.

RESULTS

Purification of antibodies by immunoaffinity chromatography. A synthetic peptide (the 128 through 139 peptide) having the same sequence as that predicted for amino acids 128 through 139 of HSV-1 gC (8) was conjugated to bovine serum albumin with glutaraldehyde and injected into a rabbit. Antibodies to the 128 through 139 peptide were purified from the resulting antiserum by immunoaffinity chromatography on a column containing Sepharose linked to the 128 through 139 peptide. Antibody activity against the

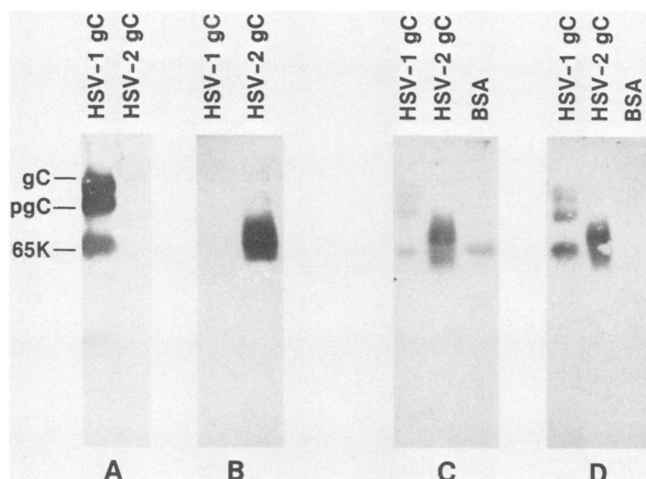

FIG. 4. Antibodies reacting with HSV-1 gC and HSV-2 gC as assayed by protein blot analysis. One microgram of purified HSV-1 gC and HSV-2 gC and five micrograms of bovine serum albumin (BSA) were electrophoresed in a polyacrylamide gel and electrophoretically transferred to nitrocellulose sheets. The sheets were reacted with 1:50 dilutions of (A) antibodies to HSV-1 (DAKO), (B) antibodies to HSV-1 (no. 661), (C) crude antiserum to the 128 through 139 peptide. or (D) purified antibodies to the 128 through 139 peptide. The sheets were washed and incubated with peroxidase-conjugated goat antibodies to rabbit immunoglobulin, followed by the addition of the 4-chloro-1-naphthol substrate.

128 through 139 peptide, HSV-1 gC, and bovine serum albumin was measured by enzyme-linked immunosorbent assay (Fig. 1). The purified antibody preparation and the crude antiserum had comparable titers to both the 128 through 139 peptide and HSV-1 gC, although the protein concentration of the purified antibody preparation was ca. 100-fold lower. The portion of antiserum not adhering to the column had a sharply lower titer to the 128 through 139 peptide and HSV-1 gC. The crude antiserum and the antiserum fraction that did not stick to the peptide column had about the same titer to bovine serum albumin, whereas the purified antibody preparation retained negligible activity. These findings demonstrate copurification by immunoaffinity chromatography of antibodies reacting with the 128 through 139 peptide and the HSV-1 gC antigens and their separation from antibodies reacting with bovine serum albumin.

Antibodies reacting with HSV-2 gC. We developed an enzyme-linked immunosorbent assay for antibodies to HSV-2 gC to further investigate the immunological relationship between HSV-1 gC and to test the specificity of our anti-peptide antibodies. Although the rabbit antibodies to HSV-1 and HSV-2 each reacted with HSV-2 gC in this assay (Fig. 2), only the titer of the antibodies to HSV-1 was significantly reduced by preabsorption with purified HSV-1 gC. These results are consistent with the proposal that HSV-2 gC possesses both type-specific determinants and determinants which are shared with HSV-1 gC.

Enzyme-linked immunosorbent assay of antibodies to the 128 through 139 peptide. The immunological relationship between HSV-1 gC and HSV-2 gC suggests that they have common amino acid sequences. Recent DNA sequencing studies have indicated that a major portion of the 128 through 139 peptide of HSV-1 gC is present in HSV-2 gC (Dowbenko and Lasky, submitted for publication). Indeed, the antibodies to this peptide reacted with both glycoproteins in the enzyme-linked immunosorbent assays shown in Fig. 3, and their binding was blocked by absorbing the antibodies with the 128 through 139 peptide. These findings confirm that the amino acid 128 through 139 region of HSV-1 gC is at least partially conserved in HSV-2 gC.

Protein blot analysis of antibodies to the 128 through 139 peptide. Although the antibodies to the 128 through 139 peptide reacted in immunofluorescence tests with antigen in Vero cells infected with HSV-1 and HSV-2, they neither neutralized HSV-1 or HSV-2 nor immunoprecipitated protein from [^{35}S]methionine-labeled infected cell extracts (data not shown). Therefore, protein blot analyses (23) were performed to further establish their specificity. In these analyses, purified HSV-1 gC and HSV-2 gC were electrophoresed in SDS-polyacrylamide gels and electrophoretically transferred to nitrocelluose sheets. In our initial studies, transfer of immunologically active gC proteins was monitored by reacting the blots with rabbit antibodies to HSV-1 and HSV-2, since our monoclonal antibodies (21, 29) did not react in this assay. The rabbit antibodies only reacted with homologous gC antigen (Fig. 4), even though they reacted with both types of gC in enzyme-linked immunosorbent and immunoprecipitation assays (Fig. 2; 28; unpublished data). The purified antibodies to the 128 through 139 peptide reacted with both HSV-1 gC and HSV-2 gC. In comparison to the rabbit antibodies to HSV-1, the antibodies to the peptide appeared to preferentially react with the 65,000-molecular-weight component of the HSV-1 gC preparation. We previously obtained results (29) suggesting that at least a portion of this 65,000-molecular-weight protein is a partial degradation product that is formed during extraction. The crude antiserum to the peptide reacted less strongly with the gCs, although it also reacted with transferred bovine serum albumin, since this protein was present in the immunogen. The reactivity of the antibodies was blocked by preabsorption with the 128 through 139 peptide (Fig. 5). Endoglycosidase F (7) digestion of HSV-1 gC before electrophoresis caused a more intense reaction with the anti-peptide antibodies (Fig. 6). Presumably, sugar residues attached to the HSV-1 gC polypeptide partially blocked the antibody-antigen reaction.

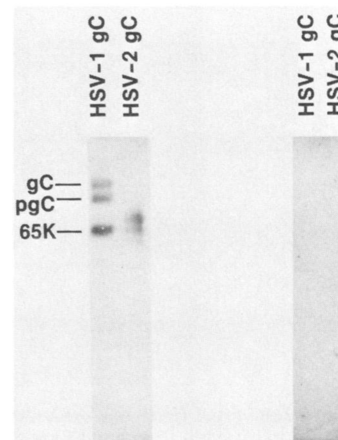

FIG. 5. Effect of absorption of 128 through 139 peptide. Purified HSV-1 gC and HSV-2 gC were electrophoresed in a polyacrylamide gel and electrophoretically transferred to nitrocellulose sheets. The sheets were reacted with a 1:50 dilution of purified antibodies to 128 through 139 peptide that were either unabsorbed (left) or absorbed with 10 μg of the 128 through 139 peptide (right). The sheet was then treated as described in the legend of Fig. 4.

The crude antiserum and the purified anti-peptide antibodies also reacted with HSV-1 gC and HSV-2 gC on blots containing infected cell extracts instead of purified protein (Fig. 7). The slightly lower molecular weight of HSV-2 gC in this extract is probably the result of partial degradation. The purified antibodies did not react with an extract of cells infected with the MP strain of HSV-1, which is deficient in HSV-1 gC production (data not shown). The reaction between the crude antiserum and several extract proteins appears to be nonspecific, since the reaction was not blocked by preabsorption with peptide. This finding illustrates the importance of immunoaffinity purification of antibodies to obtain unambiguous results.

DISCUSSION

We prepared rabbit antibodies against a synthetic peptide having the same sequence as that previously proposed for amino acids 128 through 139 of HSV-1 gC (8) and demonstrated in enzyme-linked immunosorbent assays and protein blot tests that these antibodies react with HSV-1 gC and HSV-2 gC. With the exceptions of amino acids 128 and 130, the same sequence is present in HSV-2 gC (Dowbenko and Lasky, submitted for publication). Our findings indirectly show that at least portions of the predicted amino acid sequence of HSV-1 gC and HSV-2 gC are correct.

In contrast to our anti-peptide antibody preparation, previous preparations of monoclonal and polyclonal antibodies to intact HSV-1 gC produced in several laboratories (4, 6, 17, 21, 24) have been shown not to readily react with HSV-2 antigen. This lack of reactivity has contributed to the proposal that HSV-1 gC is a type-specific antigen (4, 6, 24). The antigenic relationship between HSV-1 gC and HSV-2 gC has been discovered only recently (26, 29), probably because it is somewhat limited.

Antibodies can be divided into a class (class I) which binds a determinant whose conformation is specified by the amino acid sequence in the immediate area and a second class (class II) which binds a determinant whose conformation is affected by secondary or tertiary structure which is influenced by amino acid sequences in distant portions of the polypeptide chain. In contrast to class II antibodies, class I antibodies could theoretically react with denatured protein.

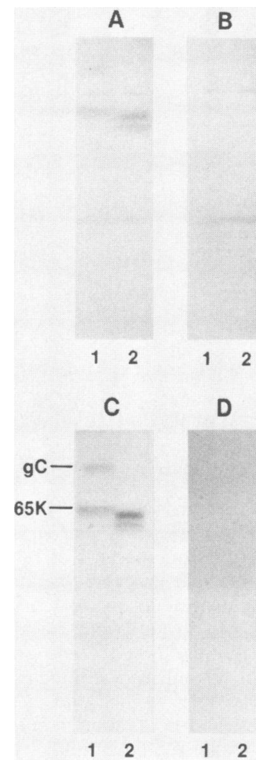

FIG. 7. Protein blot analysis of infected cell extracts. Extracts of 10^5 cells infected with HSV-1 or HSV-2 were electrophoresed in a polyacrylamide gel and electrophoretically transferred to nitrocellulose sheets, which were reacted with 1:50 dilutions of crude antiserum (A and B) or purified antibodies to the 128 through 139 peptide (C and D) that were unabsorbed (A and C) or absorbed with 10 μg of the 128 through 139 peptide (B and D). These sheets were treated as described in the legend to Fig. 4.

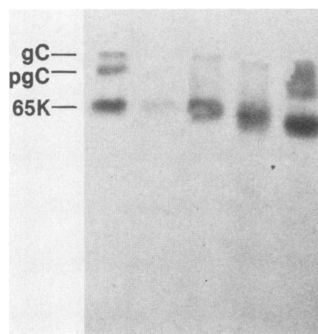

FIG. 6. Effect of treatment of HSV-1 gC with endoglycosidase F. Purified HSV-1 gC (1 μg) from left to right was untreated and treated in phosphate-buffered saline with 1.6×10^{-3}, 8.0×10^{-3}, 40×10^{-3}, or 200×10^{-3} U of endoglycosidase F (New England Nuclear Corp.) for 2 h at 37°C. After 3 volumes of modified electrophoresis buffer was added, the samples were heated at 100°C for 3 min and electrophoresed in a polyacrylamide gel. Electrophoretic transfer to a nitrocellulose sheet was conducted, and the sheet was reacted with a 1:50 dilution of purified antibodies to the 128 through 139 peptide and treated as described in the legend to Fig. 4.

The protein blot procedure is primarily an assay for only class I antibodies, since the secondary and tertiary structures of the antigen are often lost or altered as a result of electrophoresis in SDS. The immunoprecipitation and the enzyme-linked immunosorbent assay techniques measure both class I and II antibodies, since the antigens in these assays usually retain some of their native structure.

Each of the rabbit antibodies to HSV-1 and HSV-2 reacted with both types of gC in the enzyme-linked immunosorbent assay and in the immunoprecipitation test but only with the homologous gC in the protein blot procedure. This could mean that these antibodies contain only class II cross-reacting antibodies to gC. In other words, the common antigenic determinants that were detected with these antibodies are exclusively specified by secondary and tertiary structure. The monoclonal antibodies to HSV-1 gC and HSV-2 gC that we have described (21, 29) do not react in the protein blot assay, suggesting that they belong to the class II type. The antibodies to the 128 through 139 peptide are by their very nature class I antibodies, and therefore, they readily reacted with both types of gC in the protein blot procedure. Their failure to precipitate gC from infected cell extracts may mean that the amino acid 128 through 139 portion of the gCs is inaccessible to antibody binding due to

secondary or tertiary structure. It is also possible that the affinity of these antibodies for the gC antigens is too weak for immunoprecipitation.

The gC, the precursor to gC (pgC), and the 65,000-molecular-weight components of the HSV-1 gC preparation each reacted with the anti-peptide antibodies, indicating that they all share the 128 through 139 amino acid sequence. The relative intensity of these bands by amido black staining resembles that produced by the reaction with the anti-peptide antibodies. Therefore, the relatively enhanced reactivity of the rabbit antibodies to HSV-1 with the gC and pgC components is probably due to the presence of antibodies to additional antigenic determinants on these larger components.

Endoglycosidase F cleaves both high-mannose and complex glycans from glycoproteins (7). The stronger reaction of the antibodies to the 128 through 139 peptide after endoglycosidase F digestion of HSV-1 gC suggests that sugar residues may sterically hinder antibody binding. Eight potential glycosylation sites on the HSV-1 gC polypeptide have been discovered (8). The closest sites to the 128 through 139 peptide are in the regions of amino acids 110 and 150.

The enhanced reactivity and specificity of our antibody preparations resulting from immunoaffinity purification demonstrate the importance of this procedure. In other studies, we have found that unpurified anti-peptide antibodies often bind protein nonspecifically, which could lead to misinterpretation of results.

In the next few years, a considerable amount of sequencing of HSV DNA will be conducted, leading to the discovery of new open reading frames. The preparation of anti-peptide antibodies resulting from such discoveries as demonstrated in this and other (5) reports will be invaluable in identifying HSV gene products and deciphering their physiological roles.

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