Purification and Structural Characterization of Herpes Simplex Virus Glycoprotein C†

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ABSTRACT: Purification of herpes simplex virus glycoprotein C (gC) in microgram amounts yielded sufficient material for an analysis of its secondary structure. Purification was facilitated by using the mutant virus gC⁻3, which bears a point mutation that interrupts the putative hydrophobic membrane anchor sequence, causing the secretion of gC-3 protein into the cell culture medium. gC-3 protein was purified by size fractionation of concentrated culture medium from infected cells on a gel filtration column of Sephacryl S-200, followed by immunoaffinity chromatography on a column constructed of gC-specific monoclonal antibodies cross-linked to a protein A–Sepharose CL-4B matrix. Purified gC-3 had a molecular weight of 130 000 as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the size expected for gC, was reactive with gC-specific monoclonal antibodies in protein immunoblots, and contained amino acid sequences characteristic of gC as determined by radiochemical amino acid microsequence analyses. Polyclonal antisera obtained from a rabbit immunized with gC-3 reacted with wild-type gC in immunoprecipitation, enzyme immunoassay, and immunoelectroblot (western blot) assays. Deglycosylation by treatment with trifluoromethanesulfonic acid reduced the molecular weight of gC-3 by approximately 35%. Analyses of both native and deglycosylated gC-3 by Raman spectroscopy showed that the native molecule consists of about 17% α-helix, 24% β-sheet, and 60% disordered secondary structures, whereas deglycosylated gC-3 consists of about 8% α-helix, 10% β-sheet, and 81% disordered structures. These data were in good agreement with the 11% α-helix, 18% β-sheet, 61% β-turn, and 9% disordered structures calculated from Chou–Fasman analysis of the primary sequence of gC-3.

The envelope glycoproteins of herpes simplex virus (HSV)[1] play a prominent role in virus infectivity and in the immune response of the host against the virus (Spear, 1985). Several envelope glycoproteins, designated B, C, D, E, G, and H, have been identified in both HSV-1 and HSV-2 (Baucke & Spear, 1979; Spear, 1985; Buckmaster et al., 1984). Glycoprotein C (gC) is of particular interest because it is the dominant antigen in both the humoral and the cellular immune response of mice infected with HSV (Glorioso et al., 1985). Although gC is not essential for growth of HSV in tissue culture (Holland et al., 1984; Ruyechan et al., 1979), the HSV-1 deletion mutant gC⁻39, which is missing the entire coding sequence of the gC gene, shows reduced pathogenicity in vivo (Kumel et al., 1985). gC is also a receptor for the C3b component of the complement system of plasma proteins (Friedman et al., 1984; Smiley & Friedman, 1985).

The nucleotide sequence of the structural gene for gC has been determined in both HSV-1 (Frink et al., 1983; Homa et al., 1986) and HSV-2 (Dowbenko & Lasky, 1984). The sequences of HSV-1 and HSV-2 gC are approximately 68% homologous. The molecular weight of HSV-1 gC measured by SDS–PAGE analysis is approximately 130 000, but the molecular weight calculated from the amino acid sequence predicted from the DNA sequence is only approximately 55 000. This is evidence that HSV-1 gC is extensively modified posttranslationally, presumably by glycosylation. Posttranslational modification of gC by attachment of N-linked oligosaccharides (Serafini-Cessi et al., 1984) and O-linked oligosaccharides (Johnson & Spear, 1983; Olofsson et al., 1983; Wenske & Courtney, 1983) and by sulfation (Hope & Marsden, 1983) has been demonstrated. HSV-1 gC has nine possible sites for attachment of N-linked oligosaccharides, and analyses of cyanogen bromide fragments of gC radiolabeled with glucosamine suggest that many of these sites are indeed glycosylated (Kikuchi et al., 1984).

The antigenic properties of HSV-1 gC have been investigated by using a panel of gC-specific virus-neutralizing monoclonal antibodies (Marlin et al., 1985). Two classes of mutant viruses that were resistant to neutralization with antibodies in this panel were selected. In one class, termed

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[1] Abbreviations: HSV, herpes simplex virus; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; gC, glycoprotein C; *mar*, monoclonal antibody resistant; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; NP-40, Nonidet P-40; TFMS, trifluoromethanesulfonic acid; TBS, Tris-buffered saline; BSA, bovine serum albumin; PTH, phenylthiohydantoin; HEL, human embryonic lung.

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monoclonal antibody resistant (*mar*) mutants, a change in reactivity with a monoclonal antibody occurs at a single epitope. Two major antigenic sites composed of nine epitopes were operationally defined in gC (Marlin et al., 1985) by using these *mar* mutants. The other class of mutants, termed gC^- mutant viruses, lacks an immunoreactive envelope form of gC. Some gC^- mutant viruses have the unusual phenotype in which gC is secreted from infected cells (Holland et al., 1984; Homa et al., 1986) instead of being expressed as an integral membrane protein as is wild-type gC (Spear, 1985). Radiochemical amino acid microsequence analyses of some of these mutants demonstrated that secretion of gC is due to a truncation that removes the hydrophobic anchor sequence at the carboxy terminus (Kikuchi et al., 1984). Nucleotide sequence analyses of mutants that secrete gC showed that in each case a nonsense or frameshift mutation caused premature termination of translation (Homa et al., 1986). One mutant virus, gC^-3, secretes a soluble gC-3 protein having approximately the same molecular weight as wild-type gC as determined by SDS–PAGE analyses (Holland et al., 1984). A single nucleotide deletion in the gC^-3 mutant virus causes a frameshift and premature termination of translation, disrupting the putative membrane anchor sequence (Homa et al., 1986).

In this report, we describe the purification of HSV-1 gC protein to homogeneity from cells infected with gC^-3 virus. Homogeneity was established by SDS–PAGE analyses, which showed a single band of M_r 130000, and by direct protein sequence analyses. The purified gC-3 protein reacts with gC-specific monoclonal antibodies and was used to elicit polyclonal rabbit antisera that react with wild-type gC. Raman spectroscopic analysis was used to determine the relative percentages of α-helix, β-sheet, and disordered secondary structure of both native gC-3 and gC-3 deglycosylated by treatment with trifluoromethanesulfonic acid.

Materials and Methods

Cells, Viruses, and Monoclonal Antibodies. Human embryonic lung (HEL) cells, a plaque-purified isolate of wild-type (KOS) HSV-1 designated KOS 321, and the gC^- mutant virus gC^-3 were grown by methods described previously (Holland et al., 1983, 1984). Monoclonal antibodies recognizing gC were prepared as described previously by Marlin et al. (1985).

Preparation of Material Enriched with gC-3 by Gel Filtration. A total of 3×10^8 HEL cells grown in monolayers in ten 490 cm^2 roller bottles were infected with gC^-3 virus at a multiplicity of 10 in minimal Eagle medium (Gibco, Grand Island, NY) as described previously (Holland et al., 1983), except that no serum was added. At 20-h postinfection, the cell culture medium (750 mL) was removed and concentrated to 20 mL in an Amicon ultrafiltration cell (Model 850) using a Diaflo YM30 membrane (Amicon, Danvers, MA). The concentrate was then separated by chromatography on a 2.5 × 180 cm column of Sephacryl S-200 (Pharmacia, Piscataway, NJ) equilibrated in 0.15 M NaCl–0.05 M Tris-HCl, pH 7.4, containing 0.02% NaN_3.

Immunoslot Blot Analysis. Samples to be analyzed were applied to a Schleicher & Schuell (Keene, NH) Minifold II SCR 0720 slot blotter using nitrocellulose (Schleicher & Schuell BA85) as an adsorbent. The nitrocellulose was washed for 1 h in 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.4, and 3% bovine serum albumin (TBS–BSA), treated for 1 h with a pool of nine gC-specific monoclonal antibodies diluted 1:100 in TBS–BSA, washed for 15 min in TBS–BSA, and treated for 1 h with protein A–horseradish peroxidase conjugate (Bio-Rad) diluted 1:2000 in TBS–BSA. After being washed twice in water, the blot was developed with 4-chloro-1-naphthol (0.01%) and 0.1% hydrogen peroxide (v/v) in water. Densitometric analysis of immunoslot blots was performed by using a Zeineh Model SL-TRFF scanning densitometer (Biomed Instruments, Fullerton, CA) equipped with Zeineh Videophoresis software compatible with the Apple II computer.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). The method of Laemmli (1973) was used, as previously modified (Glorioso et al., 1980).

Immunoaffinity Column Purification. The immunoaffinity matrix was prepared by the method of Schneider et al. (1982). A 250 μL sample of a pool of nine gC-specific monoclonal antibodies was incubated with 500 mg of protein A–Sepharose CL-4B beads (Pharmacia) in 15 mL of 0.1 M borate, pH 8.2, for 45 min. The beads were washed in 0.2 M triethanolamine, pH 8.2, and then cross-linked with 0.05 M dimethyl pimelimidate (Pierce, Rockford, IL) in 0.2 M triethanolamine, pH 8.2, for 45 min. The reaction was stopped with 0.05 M ethanolamine, pH 8.2, and the beads were washed with 0.1 M borate, pH 8.2, and mixed with carrier Sepharose CL-4B at a 1:1 ratio of specific to carrier beads to form a column 1 × 7 cm. Pooled fractions of gC-3-enriched material eluted from the Sephacryl S-200 column were applied to this immunoaffinity column, which was washed with 60 mL of 0.1 M borate, pH 8.2. gC-3 was eluted with 20–30 mL of 0.05 M diethylamine, pH 11.0. Eluted fractions (1.9 mL) were immediately neutralized by the addition of a 1:10 volume of 0.5 M NaH_2PO_4.

Biosynthetic Labeling of Infected Cells. The methods used for radiolabeling murine MHC gene products (Nairn, 1984) were modified for biosynthetic radiolabeling of KOS- or gC^-3-infected HEL cells as described previously (Kikuchi et al., 1984).

Isolation of Radiolabeled gC by Immunoprecipitation. Immunoprecipitation of radiolabeled gC with gC-specific monoclonal antibodies or with polyclonal antiserum was performed as described previously (Kikuchi et al., 1984).

Radiochemical Sequence Analysis of gC-3. gC-3 that was biosynthetically labeled with 5 mCi each of [^{3}H]arginine, [^{3}H]proline, and [^{3}H]leucine was purified by gel filtration and immunoaffinity column chromatography as described above. Cyanogen bromide fragments of immunopurified gC-3 were prepared by the method of Gross (1967) and were separated by chromatography on a Sephacryl S-200 column (1.5 × 190 cm) in 6 M guanidine hydrochloride as previously described (Kikuchi et al., 1984). Fractions 99–103 were combined and desalted on a Sephadex G-15 column (2.5 × 40 cm) in 2 M HCOOH and then lyophilized. The amino acid sequence of this material was determined as described previously (Coligan et al., 1983) in the presence of carrier cytochrome *c* in a Model 890 M automated sequenator (Beckman Instruments), using the 0.1 M Quadrol program 05-22-85 (Q/P). Fractions showing radioactivity above the background level were removed for the preparation of phenylthiohydantoin (PTH) derivatives and then mixed with PTH–amino acid standards. PTH–amino acids were separated by high-pressure liquid chromatography according to the procedure of Gates et al. (1979), and the radioactivity of each was determined. The criteria used for acceptance of assignments made in this way have been described previously (Coligan et al., 1983).

Immunizations. Two New Zealand White rabbits were used for the immunizations. The first, rabbit A, was immunized intradermally with 5 μg of immunopurified gC-3 emulsified with Freund's complete adjuvant (Difco, Detroit, MI) followed by boosting 5 weeks later with 5 μg of immunopurified gC-3 emulsified with Freund's incomplete adjuvant. The second,

rabbit B, was immunized intradermally with SDS–polyacrylamide gel slices containing 5 μg of immunopurified gC-3 emulsified with Freund's complete adjuvant followed by boosting 5 weeks later with similar gC-3-containing gel slices emulsified with Freund's incomplete adjuvant. Antisera were collected 1 week after boosting.

Immunoelectroblot (Western Blot) Analysis. A modification of the procedure described by Towbin et al. (1979) was used. An NP-40 lysate of unlabeled KOS-infected cells (50 μL) was boiled for 2 min in SDS–PAGE sample buffer and was run on SDS–PAGE as described above. Proteins were electrophoretically transferred to nitrocellulose (Schleicher & Schuell BA85) in a buffer of 25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20% methanol (v/v) using a Bio-Rad TransBlot apparatus at 0.25 A for 2 h. The blot was then reacted with gC-specific monoclonal antibodies or with rabbit antisera specific for gC diluted 1:100 in TBS–BSA, with protein A–horseradish peroxidase conjugate, and with 4-chloro-1-naphthol by the method used for immunoslot blots.

Enzyme Immunoassay. An enzyme immunoassay was used to test the reactivity of rabbit antisera with HSV-1 (KOS) infected HEL cells as described previously (Marlin et al., 1985).

Deglycosylation of gC-3 by Treatment with Trifluoromethanesulfonic Acid (TFMS). Samples were deglycosylated with TFMS by a modification of the method of Kalyan and Bahl (1983). Lyophilized samples containing 5–10 μg of gC were treated with 50 μL of TFMS in the presence of 25 μL of anisole as a scavenger. After digestion at room temperature, the reaction was stopped by adding 250 μL of pyridine diluted 1:4 in water and by cooling samples on ice for 15 min. Samples were then dialyzed overnight against water and recovered by lyophilization.

Raman Spectroscopic Analysis of gC. Raman spectra were measured by using a computer-controlled Spex 1403 double monochromator with 1800 grooves/mm holographic gratings. A sample of 50 μL was added to a solid copper capillary cell maintained at 23 °C by circulating fluid. The 4880-Å line was used as the excitation source, the polarization was randomized, and the scattered light was collected at 90°. The average power at the sample was 120 mW. Monochromator slits were set to achieve a frequency resolution of ±1.3 cm^{-1}. The frequency-shifted light was detected by a low dark count (<7 photons/s) RCA 31034 photomultiplier tube cooled to −22 °C and operated at 1600 V. Scanning speed was 0.5 cm^{-1}/s. The spectra shown were simple arithmetic averages of 65 runs, each obtained by point by point subtraction of the buffer spectrum from the spectrum of the sample in buffer. The spectra thus obtained had an average signal to noise ratio of 30. Deconvolution of Raman bands was carried out by using Gaussian profiles, and the areas were calculated by numerical integration using a simple triangulation algorithm (Merajver et al., 1985).

Computer Analysis of the Primary Sequence of gC-3. The computer program "SEQ", written by Drs. Shawn Black and Joseph Glorioso (available from Dr. J. C. Glorioso), was used to calculate the secondary structure of gC-3 by the method of Chou and Fasman (1974) using the primary amino acid sequence predicted from the DNA sequence of HSV-1 gC determined by Homa et al. (1986).

RESULTS

Purification of gC. To purify microgram quantities of gC-3 protein, the culture medium from HEL cells infected with $gC^{-}3$ virus was used as starting material. Contaminating proteins, which were present even though serum-free medium was used, were removed after concentration of the cell culture medium by separation on a gel filtration column of Sephacryl S-200. Fractions 80–90 eluted from the column (Figure 1) were enriched in gC-3 as determined by binding of gC-specific monoclonal antibodies in immunoslot blot analyses. This gC-3-enriched material was well separated from most of the nonreactive protein, detected by the absorbance at 280 nm, which eluted in fractions 105–120 (Figure 1). SDS–PAGE analyses (Figure 2) showed that the starting material applied to the S-200 column (lane A) contains predominantly proteins of low molecular weight. Material eluting from the column in fractions 84–92 (lanes D–G) was enriched in protein of the known molecular weight for gC. Taken together, these data demonstrate that separation on the S-200 column provided an

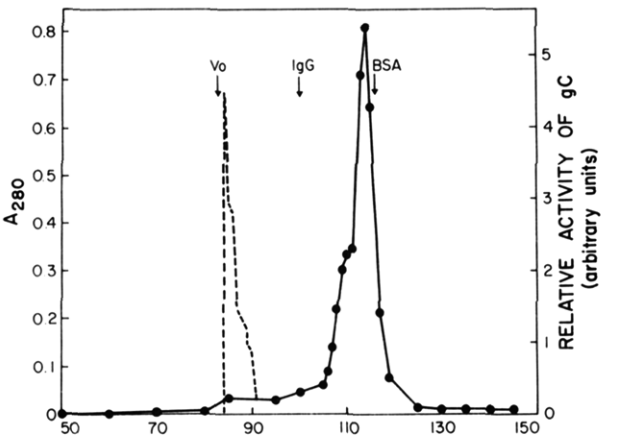

FIGURE 1: Chromatography on a column of Sephacryl S-200 of an Amicon concentrate of the cell culture medium from HEL cells infected with $gC^{-}3$ virus. The column size was 2.5 × 180 cm; the running buffer was 0.15 M NaCl–0.05 M Tris-HCl, pH 7.4, containing 0.02% NaN_3; the fraction size was 4.0 mL, and the flow rate was 10 mL/h. The absorbance at 280 nm (solid line) and the relative activity of gC (in arbitrary units) as determined by immunoslot blot analysis followed by densitometric analysis of the blot (dashed line) are shown for fractions eluted from the column. The migration positions of marker proteins IgG (M_r 150000) and BSA (M_r 69000) are indicated. V_0 indicates the void volume of the column.

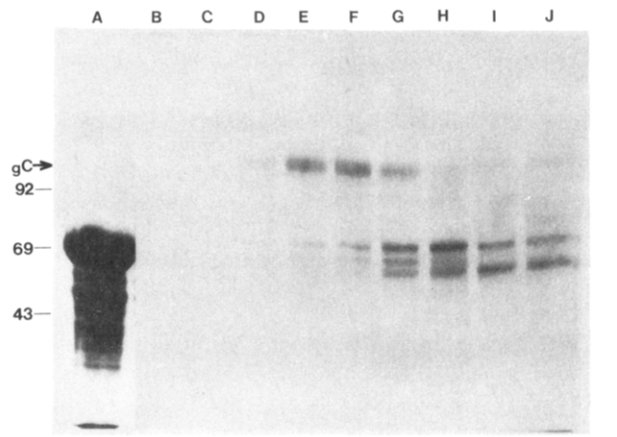

FIGURE 2: SDS–PAGE analysis of fractions from the Sephacryl S-200 column. Fifty microliters of the Amicon concentrate applied to the column or 100 μL of fractions eluted from the Sephacryl S-200 column described in the legend to Figure 1 was boiled in SDS–PAGE sample buffer (0.5 M Tris-HCl, pH 6.9, 1% SDS, and 5% 2-mercaptoethanol) and electrophoresed in an SDS gel consisting of 8.5% acrylamide cross-linked with 0.47% *N,N'*-diallyltartardiamide for approximately 2 h at 70 mA until the bromophenol blue marker ran near the end of the gel. The gel was then silver stained by using the Bio-Rad (Richmond, CA) silver stain kit 161-0443. Molecular weight markers and the migration position of native gC are indicated on the left. (A) Amicon concentrate; (B) fraction 80; (C) fraction 82; (D) fraction 84; (E) fraction 86; (F) fraction 88; (G) fraction 90; (H) fraction 92; (I) fraction 94; (J) fraction 96.

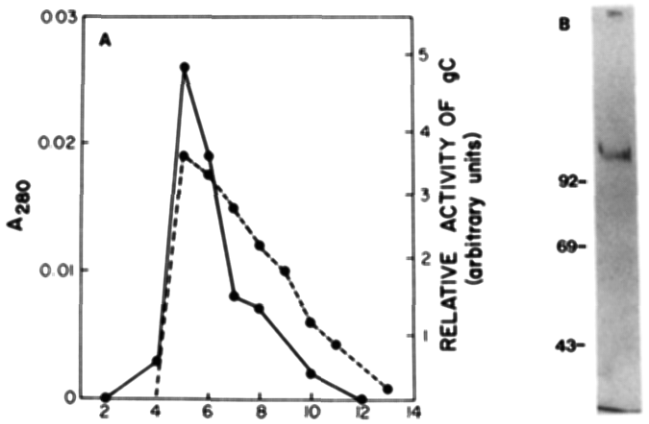

FIGURE 3: Immunoaffinity column chromatography. The immunoaffinity matrix, consisting of gC-specific monoclonal antibodies cross-linked with dimethyl pimelimidate to protein A–Sepharose CL-4B, was mixed with carrier Sepharose CL-4B at a 1:1 ratio of specific to carrier beads and used to form a column 1 × 7 cm. Fractions enriched in gC-3 from Sephacryl S-200 chromatography were applied to the column, and the column was washed with 60 mL of 0.1 M borate, pH 8.2. gC-3 was eluted in 1.9-mL fractions with 30 mL of 0.05 M diethylamine, pH 11.0. (A) Absorbance at 280 nm (solid line) and the relative activity of gC, in arbitrary units, as determined by immunoslot blot analysis followed by densitometric analysis of the blot (dashed line) are shown for fractions eluted from the immunoaffinity column. (B) SDS–PAGE analysis of immunopurified gC-3. Fifty microliters of fraction 5 eluted from the immunoaffinity column was boiled in SDS–PAGE sample buffer, electrophoresed in an 8.5% acrylamide gel, and silver stained as described in the legend to Figure 2. The migration positions of molecular weight markers are indicated on the left.

efficient partial purification of gC-3.

An immunoaffinity column was used in the next step of purification of gC-3. The column was prepared by cross-linking gC-specific monoclonal antibodies to protein A–Sepharose CL-4B beads with dimethyl pimelimidate, according to the method of Schneider et al. (1982). This method ensures that the antigen binding sites of the monoclonal antibodies are optimally oriented for binding gC-3 in the applied sample. The peak of gC activity, as determined by immunoslot blot analysis, coeluted from the immunoaffinity column with a protein peak detected by the absorbance at 280 nm (Figure 3A). SDS–PAGE analyses of the peak fraction eluted from the immunoaffinity column (Figure 3B) showed a single protein band with a molecular weight of 130000, the known molecular weight for gC-3. Approximately 300 μg of gC-3 was purified from the supernatant of 3×10^8 HEL cells infected with gC⁻3 virus.

Characterization of Purified gC. Immunoaffinity-purified gC-3 was analyzed by radiochemical microsequence methods. No sequence of the native immunoaffinity-purified molecule could be obtained, for unknown reasons. Consequently, cyanogen bromide fragments of immunopurified gC-3 labeled with ^{3}H-amino acids were prepared. The chromatogram obtained after separation of cyanogen bromide fragments in 6 M guanidine hydrochloride (data not shown) was similar to chromatograms obtained previously for gC (Kikuchi et al., 1984). Because of the reproducibility of the chromatographic analyses, fractions 99–103 were predicted to contain a cyanogen bromide fragment with a sequence starting after the methionine residue at position 138 of gC. Alignment of the amino acid sequence of this fragment with the amino acid sequence predicted from the DNA sequence (Figure 4) demonstrates the identity of immunopurified gC-3. Since only one unique sequence was obtained, this is additional evidence for the homogeneity of immunopurified gC-3.

To further establish its identity, immunopurified gC-3 was used to elicit polyclonal rabbit antisera. Two rabbits were used, one immunized with native gC-3 and the other with polyacrylamide gel slices containing gC-3. Antisera were characterized by immunoprecipitation, immunoelectroblot (western blot) analyses, and enzyme immunoassays. Antiserum from the rabbit immunized with native gC-3 immunoprecipitated wild-type (KOS) gC, as did gC-specific monoclonal antibodies (Figure 5A, compare lanes 3 and 1). Antiserum from the rabbit immunized with polyacrylamide gel slices containing gC-3 weakly immunoprecipitated gC (lane 5), whereas preimmune antisera (lanes 2 and 4) did not immunoprecipitate gC. Minor bands that are seen in all lanes of the autoradiogram may be due to nonspecific precipitation of viral proteins. In immunoelectroblot analyses (Figure 5B), only serum from the rabbit immunized with native gC-3 reacted positively with a KOS-infected HEL cell lysate (lane 3). Monoclonal antibodies (lane 1), serum from the rabbit immunized with polyacrylamide gel slices containing gC-3 (lane 5), and preimmune antisera (lanes 2 and 4) all failed to react in immunoelectroblots. In enzyme immunoassays, serum from the rabbit immunized with native gC-3 reacted with KOS-infected HEL cells at a titer of 320. By comparison, a typical gC-specific monoclonal antibody has a titer in enzyme immunoassays of about 10000. These results demonstrate that antiserum from a rabbit immunized with native gC-3 reacts with wild-type gC. However, unlike monoclonal antibodies, polyclonal rabbit antiserum reacts with denatured gC in immunoelectroblot analyses.

Deglycosylation of gC. To examine the effect of removing carbohydrate from gC-3 on its secondary structure, gC-3 was treated with trifluoromethanesulfonic acid (TFMS). This method, described by Edge et al. (1981) and Kalyan and Bahl (1983), was selected because it can completely remove both N-linked and O-linked oligosaccharides from glycoproteins. gC-3 was treated with TFMS for 30, 60, and 120 min, and the reduction in molecular weight was measured by SDS–PAGE analysis (Figure 6). Treatment with TFMS for 30 min (lane A) reduced the molecular weight of gC-3 to 87000, treatment for 60 min (lane B) reduced the molecular weight of gC-3 to 84000, and treatment for 120 min (lane C) reduced the molecular weight of gC-3 to 81000. Further TFMS treatment did not reduce the apparent molecular weight on

138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154
Met	*Gly*	*Pro*	*Ser*	*Pro*	*Pro*	*Ile*	*Ala*	*Pro*	*Ala*	*Pro*	*Asp*	*Leu*	*Glu*	*Glu*	*Val*	*Leu*
---	**Pro**	---	**Pro**	**Pro**	---	---	**Pro**	---	**Pro**	---	**Leu**	---	---	---	**Leu**	

FIGURE 4: Comparison of the amino acid sequence determined for gC-3 with the predicted amino acid sequence. HEL cells were biosynthetically labeled with 5 mCi each of [^{3}H]arginine, -proline, and -leucine and infected with gC⁻3. gC-3 was purified from the cell supernatant by gel filtration and immunoaffinity chromatography as described in Figures 1 and 3. Cyanogen bromide fragments of gC were prepared and separated on a column of Sephacryl S-200 in 6 M guanidine hydrochloride as described in the text. Fractions 99–103 were pooled, and the amino acid sequence of this pool was determined by methods described in the text. The numbers above the sequence indicate the residue positions in gC. The leader, or signal sequence, is not included in the sequence, so that residue 1 is the amino terminus of the fully processed gC molecule. The first line is the amino acid sequence predicted from the DNA sequence (Frink et al., 1983; Homa et al., 1986). The second line is the amino acid sequence obtained for the radiolabeled cyanogen bromide fragment.

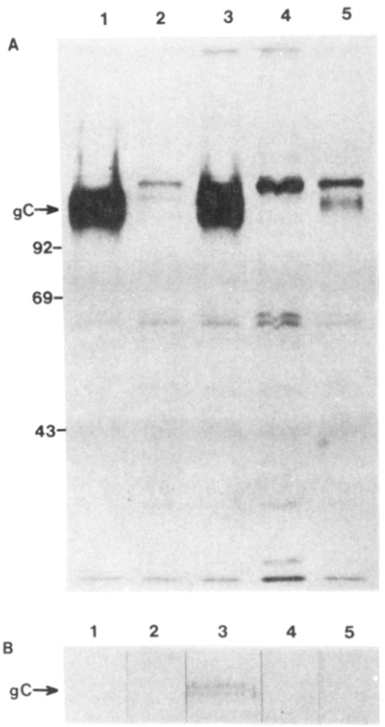

FIGURE 5: Analysis of anti-gC reactivity of rabbit antisera. (A) Immunoprecipitation. HEL cells were infected with wild-type (KOS) HSV and were biosynthetically labeled with [^{3}H]arginine, and an NP-40 lysate of infected cells was prepared. This lysate was immunoprecipitated as described previously (Kikuchi et al., 1984) with (lane 1) gC-specific monoclonal antibodies, (lane 2) preimmune serum from rabbit A, (lane 3) immune serum from rabbit A (immunized with native gC-3), (lane 4) preimmune serum from rabbit B, and (lane 5) immune serum from rabbit B (immunized with polyacrylamide gel slices containing gC-3). gC was eluted by boiling the precipitates in SDS–PAGE sample buffer and was electrophoresed in an 8.5% acrylamide gel as described in the legend to Figure 2. The gel was treated with 0.1 M sodium salicylate in 0.1 M Tris to provide fluorographic enhancement, then dried, and autoradiographed at −70 °C using XR-5 film (Kodak, Rochester, NY). The migration positions of molecular weight markers and of native gC are indicated on the left. (B) Immunoelectroblot analysis. HEL cells were infected with wild-type (KOS) HSV, and an NP-40 lysate of the infected cells was prepared. One hundred microliters of this lysate was mixed with SDS–PAGE sample buffer, boiled, and electrophoresed in an 8.5% acrylamide gel as described in the legend to figure 2. Proteins in this gel were electrophoretically transferred to nitrocellulose as described in the text. The blot was then reacted with a pool of nine monoclonal antibodies specific for gC or with rabbit antisera specific for gC, followed by protein A–horseradish peroxidase conjugate (Bio-Rad), and developed with 4-chloro-1-naphthol. The reagents used were as follows: lane 1, monoclonal antibodies; lane 2, preimmune serum from rabbit A; lane 3, immune serum from rabbit A (immunized with native gC-3); lane 4, preimmune serum from rabbit B; lane 5, immune serum from rabbit B (immunized with polyacrylamide gel slices containing gC-3).

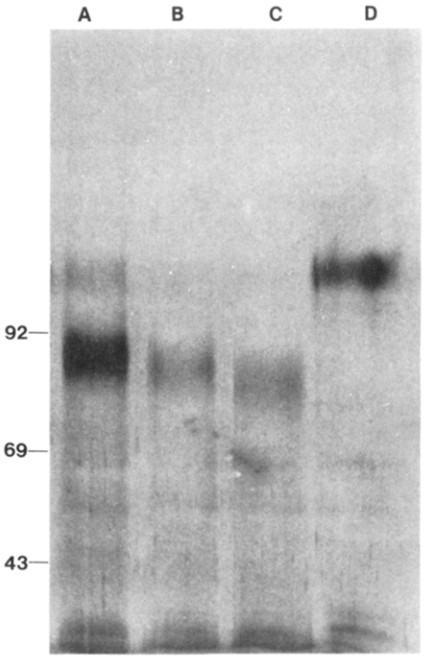

FIGURE 6: SDS–PAGE analysis of deglycosylated gC-3. Samples of gC-3 eluted from the immunoaffinity column were dialyzed overnight against water, then lyophilized, and deglycosylated by treatment with TFMS for 30, 60, and 120 min. Samples were boiled in SDS–PAGE sample buffer, electrophoresed in an 8.5% acrylamide gel, and silver stained as described in the legend to Figure 2. Migration of molecular weight markers is indicated on the left. (A) 30-min treatment with TFMS; (B) 60-min treatment with TFMS; (C) 120-min treatment with TFMS; (D) no treatment with TFMS.

SDS–PAGE of gC-3 to less than 81 000, which is evidence that all the carbohydrate has been removed. The discrepancy between the observed molecular weight and the molecular weight predicted from the DNA sequence (55 000) may reflect anomalous migration of gC-3 on SDS–PAGE, perhaps due to the high content of proline residues. gC-3 treated with TFMS for 60 min was used for Raman spectroscopic analyses. It is likely that this treatment has completely removed all the carbohydrate from gC-3 but this has not been independently verified.

Raman Spectroscopic Analyses of Native and Deglycosylated gC. Purification of gC-3 in microgram quantities allowed Raman spectroscopic analysis, a sensitive method for obtaining information about the secondary structure of proteins. The Raman spectra of both native gC-3 (Figure 7A,B) and deglycosylated gC-3 (Figure 7C,D) were measured. The most useful regions for analysis of secondary structure are the amide III band complex (1200–1300 cm^{-1}) and amide I band complex (1600–1700 cm^{-1}). Both regions contain peptide bond vibrations which have frequencies that vary according to the type of secondary structure in which the bonds participate. The bands between 1200 and 1400 cm^{-1} correspond to vibrations from disordered (1235 and 1245 cm^{-1}) (Yu et al., 1973) and α-helical segments (frequency greater than 1270 cm^{-1}) (Yu et al., 1973; Small et al., 1970) and to ring vibrations of tryptophan (1332 and 1356 cm^{-1}) (Yu, 1974).

Qualitative comparisons of the Raman spectra established that deglycosylated gC-3 is more disordered than native gC-3. For example, the band at 928 cm^{-1}, due to α-helical secondary structure, was reduced in the spectrum of deglycosylated gC-3 compared to native gC-3, whereas bands due to the vibrations of amino acids such as the tyrosine band at 827–837 cm^{-1} remained largely unchanged in deglycosylated gC-3 (compare panels A and C of Figure 7). The intensity of the band between 1190 and 1195 cm^{-1} (in the amide III complex) due to random-coil structures was increased in deglycosylated gC-3. In the region 1400–1800 cm^{-1}, the increase in amplitude of the disordered band in deglycosylated gC-3 at 1672–1714 cm^{-1} (in the amide I complex) was particularly informative (compare panels B and D of Figure 7). The bands at 1604–1607 cm^{-1}, due to phenylalanine and tyrosine residues, remained unchanged in amplitude in both samples. The band at 1129 cm^{-1} was an artifact, due to incomplete filtering of a plasma excitation line.

To quantitate the secondary structures that produce characteristic Raman spectra of both native and deglycosylated gC-3, the band profiles in the amide I and amide III regions were deconvoluted by using Gaussian band shapes. The total integrated intensity was calculated from individual bands in

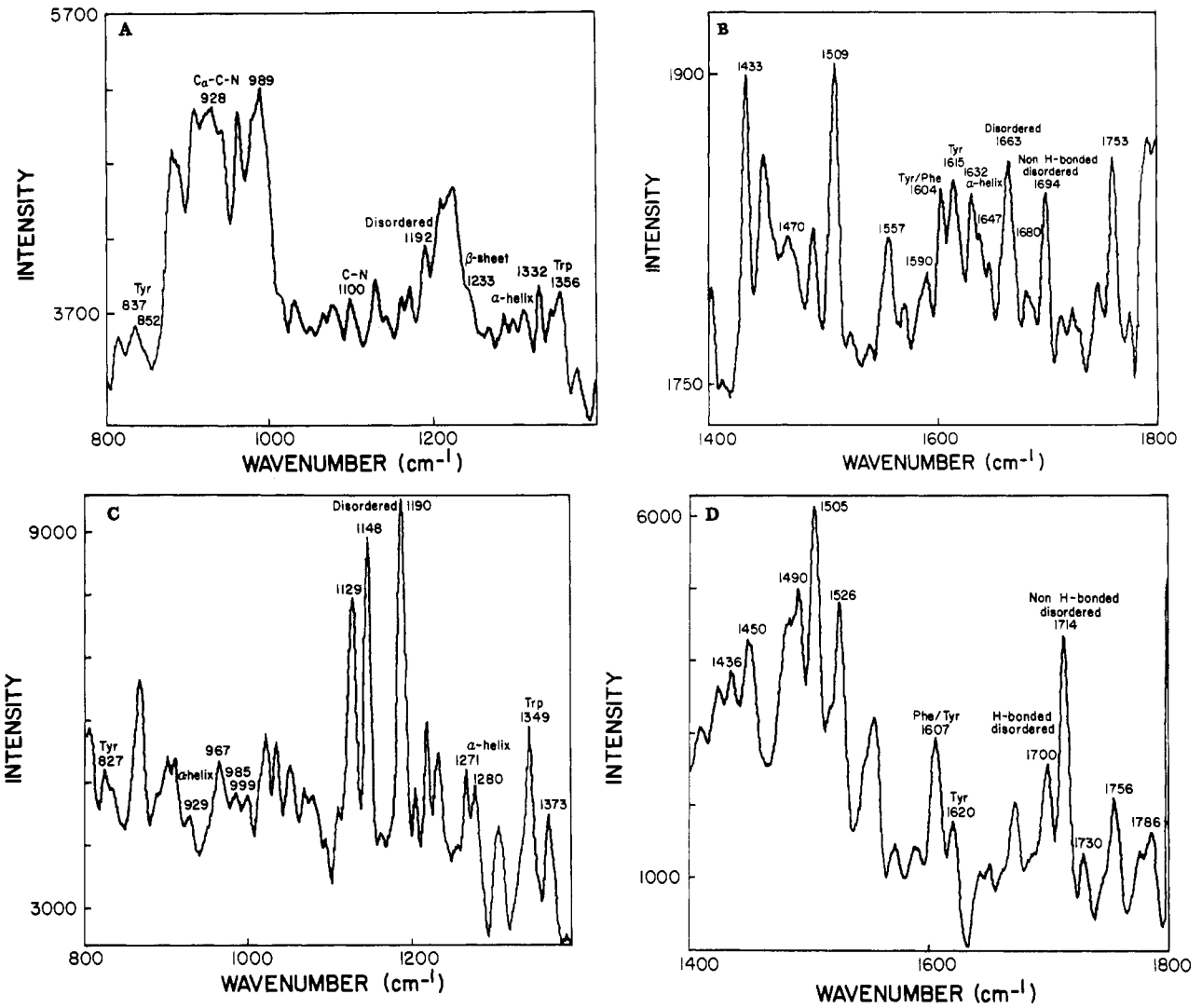

FIGURE 7: Raman spectra of native and deglycosylated gC-3. Fifty-microliter samples of gC-3 eluted from the immunoaffinity column or gC-3 that was deglycosylated by treatment with TFMS were analyzed by using conditions described fully in the text. Panel A is the Raman spectrum of native gC from 800 to 1400 cm^{-1}; panel B is the Raman spectrum of native gC from 1400 to 1800 cm^{-1}; panel C is the Raman spectrum of deglycosylated gC from 800 to 1400 cm^{-1}; panel D is the Raman spectrum of deglycosylated gC from 1400 to 1800 cm^{-1}.

Table I: Ratios of Relative Abundance of Secondary Structures Obtained from Raman Spectroscopic Analysis[a]

sample	ratio	amide I	amide II
native gC	α-helix:disordered	1:4.0	1:4.4
	α-helix:β-sheet	1:1.6	1:1.4
deglycosylated gC	α-helix:disordered	1:10	1:9
	α-helix:β-sheet	1:1.2	1:1.5

[a] These ratios are calculated for band profiles in the amide I (1600–1700 cm^{-1}) and amide III (1200–1300 cm^{-1}) regions by deconvolution using Gaussian band shapes and integration of the total intensity.

Table II: Secondary Structural Features of gC As Obtained from Raman Spectroscopy or Chou–Fasman Calculations[a]

	% α-helix	% β-sheet	% disordered
Raman data			
native gC	17 ± 2	24 ± 1	60 ± 2
deglycosylated gC	8 ± 2	10 ± 2	81 ± 2
Chou–Fasman calculations	11	18	β-turn 61 disordered 9

[a] The percentages of α-helix, β-sheet, and disordered structures were calculated from the ratios given in Table I that were derived from Raman spectroscopic analysis. These are compared to percentages of α-helix, β-sheet, β-turn, and random secondary structures calculated from analysis by the method of Chou and Fasman (1974) from the amino acid sequence of gC predicted from the DNA sequence of Homa et al. (1986).

the envelope, and the ratios of relevant band intensities were obtained (Table I). The accuracy of the band profile deconvolution scheme was tested by calculating the ratio of intensities, $I(624\ cm^{-1})/I(664\ cm^{-1})$, which gives an estimate of the phenylalanine:tyrosine ratio of the protein sample. For gC-3, this ratio was 0.952, which is in good agreement with the value of 0.926 determined from the amino acid sequence. According to the additional assumptions that only the peptide backbone vibrations contribute to the intensity of the deconvoluted regions and that α-helix, β-pleated sheet, and random-coil are the only structures present, the percent distribution of each secondary structure in native and deglycosylated gC-3 was calculated (Table II).

Further analysis of the Raman spectra provides information about the environment near tyrosine residues of gC-3. The bands at 852 and 837 cm^{-1} correspond to Fermi resonance interactions between the tyrosine ring fundamental and its first overtone. The ratio $R = I(852\ cm^{-1})/I(837\ cm^{-1})$ such that $0.9 < R < 1.43$ indicates exposed hydrogen bonds, while a value of R in the interval $1.43 < R < 3.33$ indicates buried tyrosine residues (Siamwiza et al., 1975). The value of R for gC-3 is 2.0 ± 0.3, indicating a predominance of buried tyrosines. The tyrosine bands exhibited very little frequency shift

when the spectrum of deglycosylated gC-3 was compared to that of native gC-3. This is evidence that deglycosylation of gC-3 did not greatly affect the electrostatic environment near tyrosine residues. The amino acid sequence of gC-3 contains nine tyrosine residues at widely scattered locations, so complex folding of the molecule may be involved in maintaining these tyrosine residues in an internal location. None of the tyrosines are located in the N-terminal 100 or so amino acids, which is where the majority of the carbohydrate is presumably attached (Frink et al., 1983).

Discussion

The secondary structure of gC was predicted by the empirical method of Chou and Fasman (1974) (see Table II) using the primary amino acid sequence predicted from the DNA sequence (Frink et al., 1983; Homa et al., 1986). However, the results of empirical Chou–Fasman predictions of the secondary structure of gC were subject to question for several reasons. First, the amino acid sequence of gC predicted from the nucleotide sequence (Frink et al., 1983; Homa et al., 1986) suggests that gC contains an unusually high percentage of proline residues (23%), residues that tend to break α-helices and β-sheets and introduce β-turns into the secondary structure of proteins. Chou–Fasman calculations therefore predict an unusually high percentage of β-turn secondary structure for gC, and the accuracy of this prediction was not known. Furthermore, gC is extensively glycosylated, and the influence of oligosaccharides, which are not allowed for in Chou–Fasman calculations, was not known. For these reasons, an independent measurement of secondary structure by spectroscopic methods was undertaken.

To perform direct spectroscopic measurement of the secondary structure, it was necessary to purify gC in microgram quantities. gC-3 was selected for structural analysis because it is soluble in the absence of detergents, which simplifies isolation and purification. On the basis of its nucleotide sequence, gC-3 is predicted to be only 19 amino acids shorter at the carboxy terminus than wild-type gC (Homa et al., 1986). gC-3 also reacts with 10 gC-specific monclonal antibodies (Marlin et al., 1985). Because of these features, the structure of gC-3 analyzed in this paper should closely resemble that of wild-type gC, even though the molecules are not completely identical. A two-step protocol using gel filtration and immunoaffinity chromatography allowed preparation of highly purified gC-3 with an overall yield of about 1 μg per 10^6 cells.

The identity of immunopurified HSV gC–3 was confirmed by using a variety of criteria. First, purified gC-3 has the correct molecular weight as determined by SDS–PAGE analyses. Second, purified gC-3 is reactive with monoclonal antibodies specific for gC in immunoslot blot analyses, demonstrating that it has the correct antigenic properties. Third, radiochemical amino acid microsequence analyses were performed on purified gC-3. Although an N-terminal sequence of gC-3 could not be obtained, an internal amino acid sequence characteristic of gC was obtained from a cyanogen bromide fragment of purified gC-3. Whether or not the N-terminus is blocked or if there are other reasons why an N-terminal sequence could not be obtained is not known.

Polyclonal rabbit antisera were prepared to further confirm the identity of immunoaffinity-purified gC-3. Antiserum from a rabbit immunized with native gC-3 reacted with wild-type gC in immunoprecipitation and enzyme immunoassays. The polyclonal rabbit antiserum also reacted with gC in immunoelectroblots, whereas gC-specific virus-neutralizing monoclonal antibodies did not react in immunoelectroblots. This result suggests that epitopes of gC that are important for recognition by neutralizing monoclonal antibodies may be sensitive to denaturation by SDS–PAGE.

Because gC is extensively glycosylated, carbohydrate may be important in stabilizing the secondary structure of gC and maintaining the integrity of its antigenic epitopes. To determine the effect of removing the carbohydrate residues, gC was treated with TFMS (Edge et al., 1981; Kalyan & Bahl, 1983). Treatment with TFMS for 60 min reduced the molecular weight of gC-3 by 35%. Treatment with TFMS for this length of time probably removes all the carbohydrate from gC-3, but this point has not been independently verified. In a control experiment, radiolabeled wild-type gC that was treated for 60 min with TFMS could be immunoprecipitated with polyclonal rabbit antiserum (data not shown), which indicates that TFMS treatment did not denature gC to the extent that major antigenic properties were altered. These results agree with the finding that TFMS treatment of human chorionic gonadotropin did not alter its antigenic or receptor recognition properties (Kalyan & Bahl, 1983) and that TFMS treatment of human myelin-associated protein did not alter its reactivity with rabbit antisera (Frail et al., 1984; Shy et al., 1984).

Because of its sensitivity, Raman spectroscopy was used to analyze the secondary structure of both native gC-3 and gC-3 that was deglycosylated by treatment with TFMS. Information about the secondary structure of both proteins was determined from the intensity and frequency of specific bands in the Raman spectra, especially those in the amide III complex (1200–1300 cm^{-1}) and the amide I complex (1600–1700 cm^{-1}), which are due to peptide bond vibrations that vary with secondary structure. A deconvolution procedure, which allowed quantitation of the relative peak intensity, was performed on bands in the amide III and amide I complexes. From the relative intensity of characteristic Raman bands, the relative percentages of α-helix, β-sheet, and disordered secondary structures were calculated (Table II). In native gC, there is 17% α-helix, 24% β-sheet, and 60% disordered secondary structures. By comparison, influenza hemagglutinin, another virus membrane glycoprotein, has approximately 25% α-helix, 26% β-sheet, and 16% β-turns, as determined from X-ray crystallographic analysis (Wilson et al., 1981).

Several conclusions can be drawn from this Raman spectroscopic analysis of gC-3. First, gC-3 consists of a low percentage of α-helix and β-sheet secondary structures and a relatively high percentage of disordered secondary structures. Second, deglycosylation of gC-3 with TFMS induced a small decrease in the percentage of α-helix and β-sheet secondary structures and a correspondingly small increase in the percentage of disordered secondary structures. Finally, the percentages of α-helix and β-sheet measured by Raman spectroscopic analysis were in surprisingly good agreement with percentages predicted by empirical Chou–Fasman calculations. This agreement is remarkable because gC has an unusual secondary structure containing a high percentage of β-turns, and because Chou–Fasman calculations do not take into account carbohydrate moieties. β-Turns have been shown to be particularly antigenic in other molecules such as lysozyme (Teicher et al., 1973), so the high percentage of β-turns in HSV gC may indicate several epitopes at distinct locations that are antigenic.

In this study, data on the purification of microgram amounts of HSV-1 gC and analyses of its secondary structure by Raman spectroscopy are presented. The purification methods presented could be used to obtain sufficient quantities of gC-3

to prepare crystals for X-ray crystallographic analysis. It will be necessary to obtain such detailed structural information about gC before we can fully understand the structure–function relationships of this molecule.

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